Characterization of PmHS2 glycosyltransferases for the controlled synthesis of heparosan

- a precursor of heparin and heparan sulfate -

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Characterization of PmHS2 glycosyltransferases for the controlled synthesis of heparosan

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Chapter 1

General Introduction

A part of this Chapter is in preparation for publication as a Review article.

Heparin and Heparan sulfate

The glycosaminoglycans (GAGs) are important bioactive polysaccharides due to their role in cell adhesion, chemokine signaling, biochemical cascades, signal transduction and even pathogen recognition (Linhardt and Toida 2004; Bishop, Schuksz et al. 2007). They are un-branched and negatively charged polysaccharides, composed of a repetition of disaccharide units (Table 1). GAGs are present in most of the vertebrate cells, where they are synthesized by glycosyltransferases (GTs) from activated monosaccharides (UDP-sugars) (DeAngelis 2002). Some of these GAGs such as hyaluronic acid, chondroitin sulfate and heparin are commonly used in health care.

Table 1. Disaccharide composition of GAG polysaccharide. The monosaccharides present in GAGs are: glucuronic acid (GlcUA); *N*-acetylglucosamine (GlcNAc), galactose (Gal), *N*-acetylgalactosamine (GalNAc), and iduronic acid (IdoUA).

Glycosaminoglycans	Disaccharide units
Hyaluronan	-4GlcUAβ1-3GlcNAcβ1-
Keratan	-3Galβ1-4GlcNAcβ1-
Chondroitin	-4GlcUAβ1-3GalNAcβ1-
Dermatan	-4IdoUAβ1-3GalNAcβ1-
Heparosan *(precursor of heparin/heparan sulfate)*	-4GlcUAβ1-4GlcNAcα1-

Heparin is used since the middle of the 1930's as an anticoagulant compound to prevent blood clotting during surgery. Anticoagulant heparin is also administrated in other therapeutic applications including kidney dialysis and acute coronary syndromes (Rabenstein 2002), and it is used as adjuvant and coating compound in medical devices to avoid blood coagulation.
Worldwide, about 40 tons of pharmaceutically grade heparin product is annually produced and used (Peterson, Frick et al. 2009). Most of this heparin product is isolated from animal derivatives (Liu, Zhang et al. 2009) (Fig. 1). Due to the harsh process conditions to isolate the heparin, the amount of waste produced, the shortage of raw materials of animal origin (Petitou and van Boeckel 2004), and the potential safety risk that represents the use of animal derivatives (Guerrini, Beccati et al. 2008; Liu, Zhang et al. 2009), the trend is to progressively replace this traditional production system. A small fraction of the anticoagulant heparin product is obtained by chemical synthesis (Choay, Petitou et al. 1983; Petitou and van Boeckel 2004). The synthetic heparin anticoagulant products are expensive, and despite the fact that these products are well defined and homogenous, they increase the risk of complications due to their long half time in the body. For these reasons, the chemical

synthesis is not expected to take over the market of the traditionally produced heparin (Liu, Zhang et al. 2009).

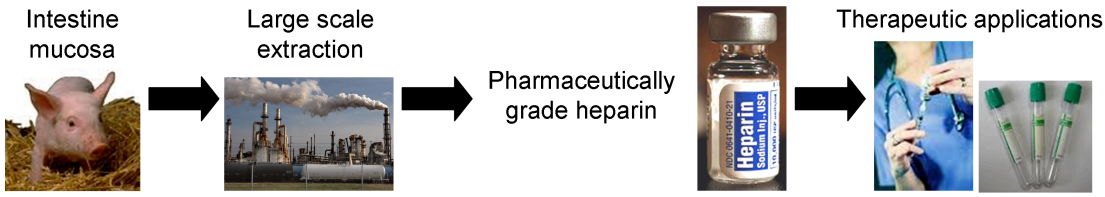

Figure 1. Chart flow of the production of pharmaceutically grade anticoagulant heparin: from animal derivatives to medical applications.

In addition to its anticoagulant effect, heparin has a therapeutic potential for the treatment of cancer (Yip, Smollich et al. 2006) and the prevention of virus infections (Rusnati, Vicenzi et al. 2009). Heparan sulfate (HS), an analog of heparin which is not used yet in medical applications, has the same therapeutic potential as heparin (Bishop, Schuksz et al. 2007). Heparin and heparan sulfate biological activity is influenced by the polymer chain length, the sugar unit composition, and the sulfation patterns. Thus, the utilization of Hep/HS drugs in new therapeutic settings requires the availability of well defined heparin and heparan sulfate-like molecules. Since the traditional production system using animal derivates does not yield homogenous and well defined products, and the chemical synthesis of heparin oligomers is laborious and not economically feasible for the synthesis of heparin longer than hexasaccharides, there is a general interest in developing alternative systems that control each of synthesis step of Hep/HS in order to produce well defined structures.

Hep/HS biosynthesis in mammalian cells

In mammalian cells, the biosynthesis of heparin and heparan sulfate takes place in the Golgi apparatus and involves many enzymatic steps (Rabenstein 2002; Gorsi and Stringer 2007). As shown in figure 2, Hep/HS chains are initiated by the synthesis of a tetrasaccharide linker composed of one glucuronic acid (GlcUA), two galactoses (Gal), and one xylose (Xyl). The xylose residue is covalently bound to a serine of a core protein (Xylβ1-*O*-Ser). The linker, GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser, serves as template for the synthesis of the unsulfated precursor of both the Hep- and HS-proteoglycans (Hep-PGs and HS-PGs). However, the core proteins differ for Hep- and HS-PGs; heparin polysaccharide is attached to a serglycin, while heparan sulfate can be linked to distinct proteins such as for example

syndecans, perlecans, and glypicans depending on its cellular location (Salmivirta, Lidholt et al. 1996).

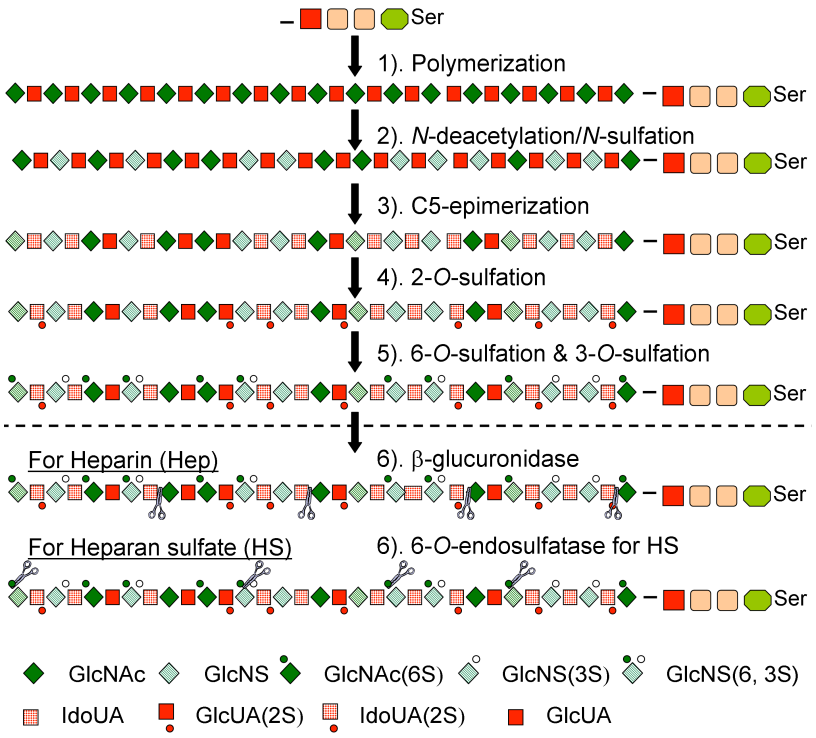

Figure 2. Multi-steps synthesis of heparin (Hep) and heparan sulfate (HS). The synthesis of Hep/HS is initiated by the polymerization of a tetrasaccharide linker: GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser. Then, heparin and heparan sulfate are synthesized by a cascade of catalytic steps.

The unsulfated precursor of Hep/HS is known as heparosan (Table 1 and Fig. 3). Heparosan is polymerized by glycosyltransferases belonging to the EXT (hereditary multiple exostosin) and EXT-like families (EXTL) (McCormick, Duncan et al. 2000; Gorsi and Stringer 2007). The glycosyltransferases EXT1 and EXT2 catalyze alternatingly the transfer of the *N*-acetylglucosamine (GlcNAc) and GlcUA residues from UDP-sugars to the growing polymer chain.

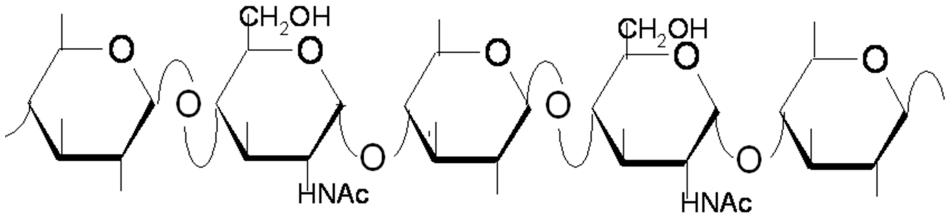

Figure 3. Heparosan structure. Heparosan is the unsulfated precursor of heparin (Hep) and heparan sulfate (HS) and it is constituted of a repetition of glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc) disaccharide unit (-4GlcUAβ1-4GlcNAcα1-).

Once heparosan elongation is terminated, the GlcNAc residues are randomly *N*-deacetylated into *N*-glucosamine (GlcN), prior to be *N*-sulfated into GlcNS by the dual action of the *N*-deacetylase/*N*-sulfatase (NDST) enzyme (Bame, Lidholt et al. 1991; Bame, Reddy et al. 1991). Following the *N*-deacetylation/*N*-sulfation step, some of the GlcUA residues are converted into iduronic acid (IdoUA) residues by the action of the glucuronyl C5-epimerase (Hepsi). The C5-epimerase converts GlcUA into IdoUA when the GlcUA residue is attached to the reducing end of a GlcNS residue (marked with *). Thus, only the GlcUA residues present in GlcNS*-GlcUA-GlcNS and GlcNS*-GlcUA-GlcNAc can be converted into IdoUA (Rabenstein 2002). Then, the polysaccharide chain is *O*-sulfated by three *O*-sulfotransferases (*O*ST): the 2-*O*ST, the 6-*O*ST, and the 3-*O*ST. These enzymes transfer a sulfate group to the oxygen molecules of distinct saccharide residues. The uronic acid residues (GlcUA and IdoUA) are sulfated in C2 position by the 2-*O*ST. The 6-*O*ST and the 3-*O*ST catalyze the *O*-sulfation of the glucosamine units (GlcNAc, GlcN, and GlcNS) on the C6 and C3 position, respectively (Rabenstein 2002).

At the end of the synthesis, endo-β-D-glucuronidase cleaves randomly the heparin chain (60000 - 100000 Da) at the GlcUA residues (Rabenstein 2002). Due to the uneven repartition of GlcUA residues, it results in a polydisperse mixture of smaller heparin chains (5000 - 25000 Da) (Lindahl, Feingold et al. 1986; Rabenstein 2002). Unlike heparin, heparan sulfate is not extensively cleaved by the β-glucuronidase and thus mostly HS-PGs are found in the mammalian cells (Rabenstein 2002).

The sulfation pattern of the HS-PG chain is critical for the biological activity and the interaction with specific ligands such as for example the fibroblast growth factor (FGF) family (Lamanna, Baldwin et al. 2006; Gorsi and Stringer 2007). For a long time it was assumed that the sulfation patterns of HS resulted from the sulfotransferase catalytic activity only. However, it was found that endosulfatase (SULF) participate also in the heparan sulfate sulfation pattern by reducing the amount of *O*-sulfate groups, mainly in position C6 (Lamanna, Baldwin et al. 2006; Gorsi and Stringer 2007). It is not fully understood yet which domains of the heparan sulfate are considered as substrate for SULF, but it was observed that the modification of the sulfation pattern by SULF is essential to confer HS-PG its biological activity (Lai, Sandhu et al. 2008).

Hep/HS structure and biological activity

During each of these Hep/HS synthesis steps, the heparosan polysaccharide is modified randomly and partially (Rabenstein 2002). Many enzymes and isoforms are involved in the

synthesis of heparin and heparan sulfate, which result in distinct complex saccharide structures (Table 2). The structural diversity of heparin and heparan sulfate polymers is large due to the amount of disaccharide unit and sulfation pattern combinations. In addition to the primary structure, the flexibility of the heparin and heparan sulfate chains and the van der Waals interactions with proteins add an additional complexity to these GAGs (Coombe and Kett 2005).

Table 2. Disaccharide units present in heparin and heparan sulfate.

Disaccharide composition	Catalyzed by
GlcUA-GlcNAc	EXT and EXTL
GlcUA-GlcNS	NDST
IdoUA-GlcNAc IdoUA-GlcNS	Hepsi
GlcUA(2S)-GlcNAc IdoUA(2S)-GlcNAc GlcUA(2S)-GlcNS IdoUA(2S)-GlcNS	2-*O* ST
GlcUA-GlcNAc(6S) IdoUA-GlcNAc(6S) GlcUA-GlcNS(6S) IdoUA-GlcNS(6S) GlcUA(2S)-GlcNAc(6S) IdoUA(2S)-GlcNAc(6S) GlcUA(2S)-GlcNS(6S) IdoUA(2S)-GlcNS(6S)	6-*O* ST
GlcUA-GlcNS(3S) IdoUA-GlcNS(3S) GlcUA-GlcNS(3, 6S) IdoUA-GlcNS(3, 6S) GlcUA(2S)-GlcNS(3S) IdoUA(2S)-GlcNS(3S) GlcUA(2S)-GlcNS(3, 6S) IdoUA(2S)-GlcNS(3, 6S)	3-*O* ST

Heparin and heparan sulfate polysaccharides differ in their structural organization, disaccharide composition, and also degree of sulfation. Heparan sulfate is composed of three domains: the non-sulfated block (NA) made of GlcUA-GlcNAc repeats, the intermediate block (NA/NS) more sulfated than NA and composed of GlcNAc and GlcNS in combination with GlcUA, and the highly sulfated block (NS) (Rabenstein 2002) (Fig. 4). In heparan sulfate the NA domain is the most abundant, the number of GlcNAc and GlcNS are about the same, and the number of IdoUA residue is lower than the GlcUA residues (Coombe and Kett 2005). Heparin is only composed of the NS-like domain and is therefore highly sulfated. More than 80% of the Heparin polysaccharide is *N*-sulfated, and the *O*-sulfated groups are even more present than the *N*-sulfated groups (Gallagher and Walker 1985; Gallagher, Lyon et al. 1986).

Heparin is mainly composed of GlcNS and IdoUA; 70% of the heparin composition found in porcine intestinal mucosa is composed of the disaccharide units (IdoUA(2S)-GlcNS(6S)).

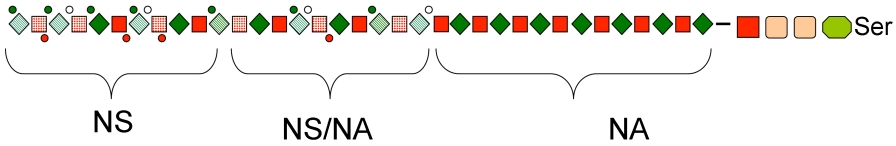

Figure 4. Heparan sulfate polysaccharide organization. The legend of the monosaccharide units is presented in Fig. 2. NA, NS/NA and NS stand for the non-sulfated block, the intermediate block, and the highly sulfated block, respectively.

For both heparin and heparan sulfate, the *O* sulfation pattern ensure biological activity (Gorsi and Stringer 2007; Peterson, Frick et al. 2009). The IdoUA residue is also very important because it interferes in the binding affinity of the polysaccharide with proteins. Indeed, unlike the GlcN and GlcUA residues present in the "chair" conformation, the IdoUA residues oscillate rapidly between the "chair" and the "skew-boat" conformations (Coombe and Kett 2005). This ability to adopt conformational changes enhances the binding of the Hep/HS polymers to protein. In the cell matrix and on the cell surface, the interactions between protein and Hep/HS-PG depend on the specific composition of these latter. Thus, the polymer chain length, the disaccharide unit combinations and the sulfation patterns dramatically influence the Hep/HS biological activity.

Hep/HS polymers are involved in many physiological process (Linhardt and Toida 2004). A mouse animal model in which the enzymes involved in the heparan sulfate synthetic pathway had been knockout, revealed that each steps in synthesis of HS-PG, from the polymerization to the post-polymerization modifications, are critical to ensure a good embryo development (Coombe and Kett 2005). Since, heparin-like structures bind to a multitude of proteins involved in a variety of biological functions, these molecules have a large therapeutic potential. In addition to their well known anticoagulant activity, these molecules appear to be also promising in the treatment of cancer and virus infection (Coombe and Kett 2005; Yip, Smollich et al. 2006; Rusnati, Vicenzi et al. 2009).

Pharmaceutical applications of Hep/HS

Anticoagulant activity

Endogenous heparin and HS-PG participate in the inhibition of the blood coagulation cascade. While their basal anticoagulation activity in the body is rather low, when heparin is administrated by injection it exhibits a high anticoagulant activity (Rabenstein 2002). Heparin

anticoagulant activity is mainly due to its interaction with antithrombin, known as a proteinase inhibitor involved in the blood coagulation cascade. When antithrombin comes in contact with the unique heparin pentasaccharide sequence (-GlcNAc(6S)-GlcUA-GlcNS(3S,6S)-IdoUA(2S)-GlcNS(6S)-), it changes its conformation into an active form which results in the inhibition of the blood coagulation cascade (Lindahl, Feingold et al. 1986). Only 1/3 of the heparin extracted from animal derivates has this specific structure. The 6-*O* and 3-*O* sulfation pattern is essential for the binding of heparin to antithrombin (Peterson, Frick et al. 2009). The IdoUA does not bind with antithrombin, but it facilitates the binding of heparin polymer by increasing the flexibility of the chain (Casu, Petitou et al. 1988). The requirement for IdoUA in order to facilitate the binding to antithrombin is size dependent. Heparin-like chains of about 10 monosaccharide residues do not need IdoUA or IdoUA(2S) to exhibit anticoagulant activity (Chen, Jones et al. 2007).

Potential applications in cancer treatment

Heparan sulfate-proteoglycans are involved in many aspects of cancer development from cell growth to metastasis. It was reported that the deregulated synthesis of HS-PGs, as well as the deregulated expression of the enzymes involved in the HS-PG post-polymerization modifications and degradation, contribute to the different steps of tumor progression (Sanderson, Yang et al. 2004; Yip, Smollich et al. 2006).

In normal physiological conditions, the HS-PGs present on the cell surface serve as co-receptor for several growth factors and thus induce cell proliferation. In cancer cells, it was observed that the changes in the expression of the HS-PGs present on the cell-surface lead to the reduction of cell adhesion (Yip, Smollich et al. 2006) which promote cell invasion, cancer progression, and angiogenesis (Götte, Joussen et al. 2002). In addition, deregulation of the heparanase (β-endoglucuronidase) activity on the cell surface and in the cell matrix, results in the degradation of HS-PGs that favors cell invasion and metastasis (Yip, Smollich et al. 2006). The silencing of the heparanases reduced metastasis and tumor angiogenesis (Cohen, Pappo et al. 2006).

In cancer, the anticoagulant activity of heparin affects tumor progression by decreasing the angiogenesis and by having an antimetastasic effect (Yip, Smollich et al. 2006). Several animal studies suggest that heparin antimetastasic activity is based on the anticoagulant activity, the inhibition of heparanase (Bar-Ner, Eldor et al. 1987), the interference with HS-PG interactions (Ludwig, Boehme et al. 2004). Yet, although it is not well understood how heparin interferes in the angiogenesis process, it was observed that heparin modulates the expression and the function of the angiogenic growth factors and inhibitors (Casu, Vlodavsky et al. 2007). Depending on the heparin molecular weight, the biological activity differs

(Bishop, Schuksz et al. 2007). High molecular weight heparin enhances the binding of the growth factors to their receptors and thus promotes angiogenesis, while low molecular weight heparin has the opposite effect by inhibiting their binding. The use of low molecular weight heparin during the treatment of patients suffering from cancer, improved their life time (Yip, Smollich et al. 2006; Lazo-Langner, Goss et al. 2007). This result confirms the beneficial effect of heparin molecules during cancer treatment. The use of anticoagulant heparin in therapy against cancer is not suitable due to the high risk of hemorrhagic complications. To circumvent these complications, non-anticoagulant Hep/HS molecules but still exhibiting anti-tumoral activity have been produced (Casu, Vlodavsky et al. 2007). Yip *et al*, reviewed different therapeutic strategies using GAGs against cancer progression (Yip, Smollich et al. 2006). Currently, inhibitors of the enzymes involved in the Hep/HS synthesis and degradation, and also competitive inhibitors of angiogenic factors such as heparin-like molecules are being investigated for their therapeutic effect in cancer treatment.

Potential applications to prevent virus infection

In addition to the anticoagulant and antitumoral activities, Hep/HS are of interest for the prevention of virus infections. Viral infections are often initiated by the binding of viruses to proteoglycans such as HS-PGs localized on the cell surface (Saphire, Bobardt et al. 2001; Rusnati, Vicenzi et al. 2009). It was observed that the basic amino acid residues of viral proteins interact with the negatively charged sulfated/carboxyl groups of the HS-PG chains, prior to the cell entry (Lee, Pavy et al. 2006). Thus, the aim of antiviral compounds is to hinder the interaction between the viral proteins and the components of the cell surface. A powerful antiviral drug should have a high affinity for the virus and binds to it before the virus binds to the proteoglycan localized on the cell surface (Rusnati, Vicenzi et al. 2009). The antiviral activity of heparin and heparan sulfate against DNA and RNA viruses such as human immunodeficiency virus (HIV) (Baba, Snoeck et al. 1988) and as well flaviviruses (Chen, Maguire et al. 1997; Lee, Pavy et al. 2006) has been reported already since many years. Since HS-PG are implicated in virus cell entry, heparin-based antiviral agents disrupting the interaction between the HS-PG and the viral proteins (Saphire, Bobardt et al. 2001; Lee, Pavy et al. 2006) are promising compounds. Preclinical *in vitro* studies showed that heparin-like compounds can inhibit HIV, herpes simplex virus (HSV) and human papilloma virus (HPV) infections (Rusnati, Vicenzi et al. 2009) and also are active against dengue and encephalitic flaviviruses (Baba, Snoeck et al. 1988; Chen, Maguire et al. 1997; Lee, Pavy et al. 2006). It was observed that the antiviral activity of Hep/HS can also be modulated by varying the degree of epimerization, sulfation or/and the chain length (Chen, Maguire et al. 1997; Rusnati, Vicenzi et al. 2009). Depending on the virus, the interaction

with the HS-PG is different (Coombe and Kett 2005) and thus the structure of potential antiviral compounds should be investigated against each viral species.

Production of Hep/HS pharmaceutical compounds

Recovery of active compounds from animal derivatives

In 2008, the market of anticoagulant heparin products represented a turn over of about 4.3 billion euros and showed an annual growth rate of 5 to 10% from 2003 to 2008. The major players involved in this worldwide market are Sanofi-Aventis –France– (68%), GlaxoSmithKline –UK– (8%), Pfizer Inc –USA– (7%), Leo Pharma (4%), Novartis, Rovi, Boehringer Ingelheim, Abbot, and others. (personal communication, MSD-Oss). MSD-Oss; formally known as Organon, is also an important player by producing 8 to 10% (in heparin units) of the heparin market. About 80% of the unfractionated heparin produced by MSD-Oss is sold to GlaxoSmithKline and Sanofi-Aventis in order to be used as starting material for low molecular weight heparin.

Most of the commercialized anticoagulant heparin products are obtained from pig intestine mucosa as starting material (Liu, Zhang et al. 2009). The first step of this industrial process is the pre-hydrolysis of the raw material using proteolytic enzymes at room temperature (Liu, Zhang et al. 2009). The pre-hydrolysis is followed by a hydrolysis at 50-75°C for about 6 h. The temperature is raised by the addition of 1 to 3 volumes of hot aqueous solvent (80-100°C) to the reaction mixture. The digested mixture is then cooled down to ambient temperature before proceeding to the recovery of heparin. Heparin is a polyanionic molecule and it is therefore extracted from the hydrolysate by using an anion exchange resin. The adsorbed heparin is eluted with a high salt solution (Houdenhoven. van 1999), and recovered from the eluant by ethanol precipitation.
From pig mucosa, a mixture of polydisperse heparin polysaccharides of 5000 to 30000 Da is recovered (Rabenstein 2002). In order to suit the medical requirements, low molecular weight heparin 4000 to 6000 Da is produced from the native unfractionated heparin. Heparin fractionation can be done by chemical cleavage using nitrous acid or by enzymatic cleavage using heparinase. The low molecular weight heparin products represent the largest part of the heparin product sells. In the US market, the low molecular weight heparin product Lovenox (Adventis) corresponds to 70% of the heparin product sells (Liu, Zhang et al. 2009).

Chemical synthesis of heparin products

Chemical synthesis of Hep/HS tetra- and pentasaccharide was introduced for the first time by Choay and co-workers (Choay, Petitou et al. 1983). In the 80's, the collaboration between Sanofi-Synth/labo and Organon (currently MSD-Oss) resulted in the synthesis of a heparin synthetic analog pentasaccharide (Choay, Petitou et al. 1983; Petitou and van Boeckel 2004). From this work, and after about ten years of a successful clinical development, Sanofi commercialized in 2002 a specific heparin anticoagulant pentasaccharide motif known as fondaparinux (brand name Arixtra, Sanofi-Synthelabo). Despite the fact that the production of synthetic heparin requires many steps, multi-kilogram synthesis of highly pure compound is performed in industry (Petitou and van Boeckel 2004).

Alternative method for the production of Hep/HS polymers

As an alternative to the extraction from animal derivates and the chemical synthesis of heparin anticoagulants, (chemo)enzymatic catalysis could be used to produce defined Hep/HS chains (Lindahl, Li et al. 2005). The enzymes involved at each step of the Hep/HS synthesis (Fig. 2) have been isolated from mammalians cells or microorganisms, expressed and characterized as recombinant proteins in *E. coli* (Peterson, Frick et al. 2009) in order to be used for the production of well defined Hep/HS polymers. During the biosynthesis of Hep/HS, the synthesis of heparosan determines the chain length and the size distribution of the Hep/HS polymers.

Heparosan production strategies

Extraction of heparosan from microorganisms

GAGs are present in the polysaccharide capsule of some microorganisms in order to mimic the host polysaccharides and to attenuate the immune response during infection (Roberts 1996). Until now, heparosan polymer has been found in the capsule of the pathogenic bacteria *Escherichia coli* K5 (Vann, Schmidt et al. 1981), *Pasteurella multocida* Type D (Pandit and Smith 1993; Rimler 1994), and *Avibacterium paragallinarum* (Wu, Chen et al. 2010). Heparosan production has only been reported for *E. coli* K5, and large scale fermentation enabled the recovery of 15 g of heparosan per liter of culture (Wang, Ly et al. 2010) (Fig. 5A). It was found that during the fermentation and the purification processes, lyases cleave heparosan K5 polymers. This lyase activity results in a heterogeneous heparosan polymer mixture of about 1.5 kDa and 16 kDa (about 8 and 80 monosaccharides) (Manzoni 1996;

Manzoni, Rollini et al. 2004). Despite the fact that this production strategy is cost effective, neither the heparosan chain length nor the incorporation of analog sugar residues can be controlled.

Synthesis of heparosan using recombinant enzymes

Recombinant heparosan synthases from mammalians and drosophila

In mammalians, the synthesis of heparosan is catalyzed by glycosyltransferases belonging to the EXT (hereditary multiple exostosin) and EXT-like (EXTL) family. EXT1 and EXT2 catalyze the elongation of heparosan chains. Together EXT1 and EXT2 form an active hetero-complex, and their simultaneous expression in recombinant cells resulted in their full catalytic activity (Senay, Lind et al. 2000). The complex EXT1/2, as well as only EXT1 were able to elongate *in vitro* K5 heparosan acceptors by adding some extra sugar units (10 to 20 sugar units) (Busse and Kusche-Gullberg 2003). Unlike EXT1, no significant transferase activity was observed when EXT2 was incubated in the absence of EXT1 (McCormick, Duncan et al. 2000). Kim et al. (2003), observed that the purified EXT1/2 complex synthesized heparosan polymers of about 170 kDa in the presence of GlcUA-Gal-O-C_2H_4NH-benzyloxycarbonyl, and of about 200 kDa in the presence of glypican-I core protein or α-trombomodulin proteoglycan as template molecules (Kim, Kitagawa et al. 2003).

In drosophila, a family of homolog proteins to the mammalians EXT is involved in the synthesis of heparosan: TTV, SOTV and BOTV (Bellaiche, The et al. 1998; Izumikawa, Egusa et al. 2006).

Recombinant heparosan synthases from Escherichia coli K5

In *E. coli* K5, the synthesis of heparosan is mainly regulated by the glucosaminyl transferase KfiA (Hodson, Griffiths et al. 2000; Chen, Bridges et al. 2006) and the glucuronyl transferase KfiC (Griffiths, Cook et al. 1998), which transfer monosaccharide units to the non-reducing end of the heparosan growing chain. Sugiura *et al*, (2010) expressed KfiC and KfiA in *E. coli* BL21 (DE3) and showed that KfiC does not exhibit a transferase activity when incubated without KfiA in the presence of substrate and template molecules (Sugiura, Baba et al. 2010). In contrast, KfiA exhibited acetylglucosaminyl transferase activity when incubated in the absence of KfiC. In addition, they observed that the presence of an excess of KfiA increased the polymerization activity, while an excess KfiC had no effect on the GlcNAc transferase activity. Heparosan chains of about 10 kDa and 20 kDa were synthesized by the complex

KfiA/KfiC after 8 h and 18 h of incubation in the presence of heparosan oligosaccharides (7-mer), respectively.

Recombinant heparosan synthases from Pasteurella multocida Type D

Unlike in *E. coli* K5, in *P. multocida* Type D the synthesis of heparosan is controlled by a bifunctional glycosyltransferase; the heparosan synthase PmHS1. PmHS1 and PmHS2, which is a PmHS1 cryptic homolog, were expressed as recombinant proteins in *E. coli* (May, Zhang et al. 2001; DeAngelis and White 2002). Despite their high amino acid level homology (73 %), the recombinant PmHS1 and PmHS2 exhibit different polymerization properties (DeAngelis and White 2004; Sismey-Ragatz, Green et al. 2007). PmHS1 synthesized heparosan polymers with an average molecular weight of 800 kDa, while heparosan chains of 28 kDa were polymerized by PmHS2. In the presence of heparosan oligosaccharide templates PmHS1 synthesized heparosan with a lower molecular weight and a lower size distribution, while the addition of templates had almost no effect on the PmHS2 polymerization process (Sismey-Ragatz, Green et al. 2007).

Site directed mutagenesis was applied on the two conserved D*X*D amino acid domains of PmHS1 (Kane, White et al. 2006), and two PmHS1 single action transferase mutants were obtained. It was found that both the PmHS1 glucuronyl transferase and PmHS1 acetylglucosaminyl transferase, incubated separately, were capable to transfer GlcUA and GlcNAc residue to a heparosan template molecule, respectively. This finding might be of interest to control heparosan polymer synthesis. Indeed, a tight control of the hyaluronan (-4GlcUAβ1-3GlcNAcβ1-) (DeAngelis, Oatman et al. 2003), and chondroitin (-4GlcUAβ1-3GalNAcβ1-) (Sugiura, Shimokata et al. 2008) oligosaccharide synthesis was achieved by immobilizing each of the respective single action transferase on distinct columns, and by re-circulating the reaction mixtures (Fig. 5B).

In addition, PmHS2 exhibits interesting polymerization characteristic since it was observed to transfer modified sugars such as *N*-acetylglucosamine residues having different acyl chain length in the C2 position (Sismey-Ragatz, Green et al. 2007). Heparosan analogs might confer new biological properties to heparin and heparan sulfate.

Production of heparosan using recombinant bacteria

The production of heparosan by recombinant bacteria has not been reported yet, but it was shown to be a successful method to produce hyaluronan (Fig. 5C). Hyaluronan, also known as hyaluronic acid, is a GAG composed of the same sugar units as heparosan but with different glycoside linkages (-4GlcUAβ1-3GlcNAcβ1-).

The expression of the *Streptococcus equisimilis* hyaluronan synthase *has*A gene and the UDP-glucose dehydrogenase gene, in *Bacillus subtilis* permitted to recover from the supernatant multi-grams of hyaluronan (1.1 to 1.2 MDa) per liter culture (Widner, Behr et al. 2005). This production yield was comparable to the yield observed with *Streptococcus equisimilis* strains (7 g/L) a natural producer of hyaluronan (Kim, Yoo et al. 1996). The fact that hyaluronan synthase from *Pasteurella multocida* has been expressed into recombinant *E. coli* (Yu and Stephanopoulos 2008; Mao, Shin et al. 2009) and succeeded in the production of hyaluronan, is promising for the production of heparosan using *P. multocida* heparosan synthase into recombinant *E. coli*.

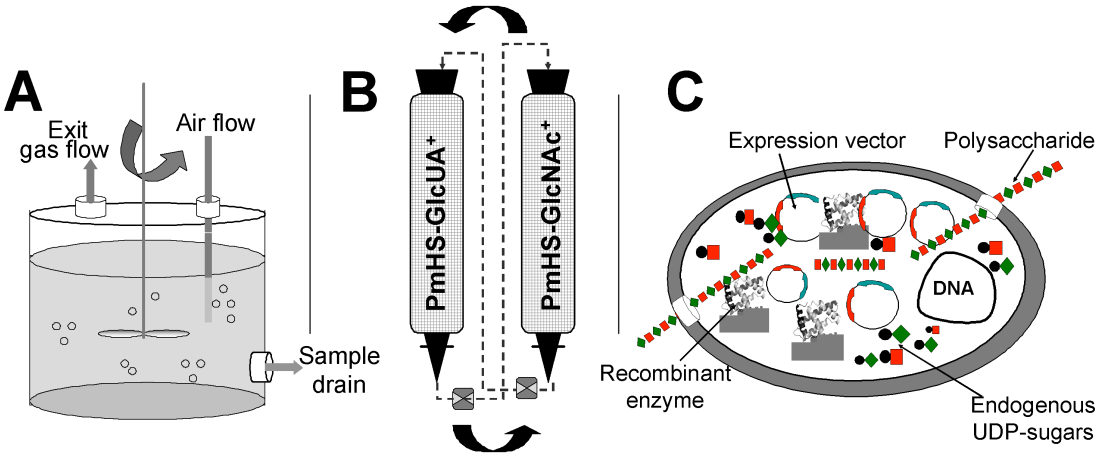

Figure 5. Overview of heparosan production strategies. A). *E. coli* fermentation for the extraction of capsular heparosan. B). Synthesis of heparosan by recombinant heparosan synthases (controlled synthesis of heparosan oligosaccharides using immobilized single action transferases). C). Production of heparosan polysaccharides using recombinant bacteria

Conclusion

In the preceding paragraphs, we described different methods used to synthesize heparosan polymers. Table 3 gives an overview of the advantages and disadvantages of each strategy. Despite the fact that the isolation of heparosan from bacterial capsule is economically advantageous, *E. coli* K5 is a human pathogen. Thus, rounds of random mutagenesis should be applied to decrease its virulence (Kim, Yoo et al. 1996) in order to use it for the production of pharmaceutical compounds. In addition, the production of heparosan from bacterial capsule does not enable to control the chain elongation and disaccharide unit composition (Roman, Roberts et al. 2003). The use of recombinant bacteria is interesting since non-pathogenic microorganisms can be used, and regulated expression of engineered heparosan synthases could enable a partial control of the polymer synthesis. However, with the current knowledge the polymer chain length cannot be controlled yet (Chen, Marcellin et al. 2009).

Despite the requirements for the expensive UDP-sugars, the use of biocatalysts to synthesize heparosan appears to be the best strategy to control the chain length and to incorporate unnatural sugar units.

Table 3. Advantages and disadvantages of heparosan production strategies.

Methods	**A** Extraction from *E. coli* K5	**B** Recombinant heparosan synthase	**C** Recombinant bacteria
Advantages	- UDP-sugars produced by *E. coli* - Cost effective	- Control polymer length (immobilized enzyme: disaccharide to 20-mers/ non-immobilized enzyme: 20 to 800 kDa) - Process free of contamination	- UDP-sugars produced by recombinant bacteria - non pathogenic bacteria - Cost effective
Disadvantages	- Polymer length not controlled - *E. coli* K5 pathogen	- Need UDP-sugar - Not cost effective	- Polymer length not controlled
Next focus	- Strain improvement (virulence, yield, lyase activity, etc.)	- UDP-sugar production system	- To be investigated for the production of heparosan

Thesis outline

Due to their involvements in many physiological processes and their large potential for medical applications, Hep/HS are interesting polysaccharides. Since their biological activity depends on their structure and composition, the utilization of Hep/HS based drugs in new therapeutic settings will require the synthesis of well defined heparin and heparan sulfate-like molecules. Here, the polymerization of heparosan was studied in detail in order to be able to control the polymer elongation and thus regulate the chain length and size distribution of Hep/HS polymers. PmHS2 was considered as a promising candidate since it exhibits both an acetylglucosaminyl and a glucuronyl transferase activity (DeAngelis and White 2004; Kane, White et al. 2006) and it can use modified UDP-sugars to elongate heparosan (Sismey-Ragatz, Green et al. 2007). Therefore, in order to control enzymatically the synthesis of heparosan, the polymerization mechanism of the *Pasteurella multocida* heparosan synthase 2 -PmHS2- was studied in detail.

In Chapter 2, the incubation parameters influencing the PmHS2 polymerization activity are described. It was observed that the UDP-sugar concentration influences the PmHS2 polymerization activity with respect to the polymer chain length and size distribution.

Using site-directed mutagenesis techniques, two functional and active PmHS2 single action transferases (PmHS2-GlcUA^{+} and PmHS2-GlcNAc^{+}) were obtained from PmHS2. In Chapter

3, these two single action transferases were used to investigate the PmHS2 polymerization process in more detail. Not only the overall UDP-sugar concentration influences the polymer molecular weight but also the amount of each UDP-sugar. In addition, PmHS2 was found to exhibit glycoside hydrolase activity.

In Chapter 4, the influence of the each transferase activity and each UDP-sugar concentration on the polymerization process was studied. For this purpose, the polymerization process was investigated in the presence of non-equimolar PmHS2 single action transferase concentrations, and a fractional factorial design (4 variables and 3 levels) was also included. In addition, the heparosan chain elongation was controlled by re-circulating the reaction mixture from one column with immobilized PmHS2-GlcUA^{+} to another column with immobilized PmHS2-GlcNAc^{+}, and vise versa.

A general assay using agarose gel electrophoresis analysis to screen a library of PmHS2 mutants for their ability to synthesize polysaccharides is described in Chapter 5. The isolation of thermostable PmHS2 mutants validated this assay. In addition, the effect of a *C*-terminus tag on PmHS2 stability was investigated.

In Chapter 6, the obtained results are discussed with respect to the control of heparosan elongation and size distribution. In addition, (chemo)enzymatic alternative production systems to synthesize defined heparin and heparan sulfate molecules are discussed.

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Chapter 2

In vitro synthesis of heparosan using recombinant *Pasteurella multocida* heparosan synthase PmHS2

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Abstract

In vertebrates and bacteria, heparosan the precursor of heparin is synthesized by glycosyltransferases via the stepwise addition of UDP-*N*-acetylglucosamine and UDP-glucuronic acid. As heparin like molecules represent a great interest in the pharmaceutical area, the cryptic *Pasteurella multocida* heparosan synthase PmHS2 found to catalyze heparosan synthesis using substrate analogs has been studied. Here, we report an efficient way to purify PmHS2 and to maintain its activity stable during 6 months storage at -80°C using His-tag purification and a desalting step. In the presence of 1 mM of each nucleotide sugar, purified PmHS2 synthesized polymers up to an average molecular mass of 130 kDa. With 5 mM of UDP-GlcUA and 5 mM UDP-GlcNAc an optimal specific activity, from 3 to 6 h of incubation, was found to be about 0.145 nmol/μg/min and polymers up to an average of 102 kDa were synthesized in 24 h. In this study we show that the chain length distribution of heparosan polymers can be controlled by change of the initial nucleotide sugar concentration. It was observed that low substrate concentrations favor the formation of high molecular weight heparosan polymer with a low polydispersity while high substrate concentration did the opposite. Similarities in the polymerization mechanism between PmHS2, PmHS1 and PmHAS are discussed.

Introduction

Bioactive carbohydrates play an important role in many organisms. In vertebrates they are implicated in many physiological processes and biological functions at the molecular level from gene expression to protein regulation and interaction (Jackson, Busch et al. 1991). Therefore some of these carbohydrates, especially glycosaminoglycans (GAGs), are of great interest in medicine. GAGs are linear polysaccharides, sulfated or not, made of repeated disaccharides units of hexose or hexuronic acid and *N*-acetyl-hexosamine. The GAG heparosan (-4GlcUAβ1-4GlcNAcα1-) is the unsulfated precursor of heparin. Heparin acts as an anticoagulant and is known for its use in surgery to prevent e.g. vein thrombosis (Rabenstein 2002). Recent studies have evidenced that the molecular weight of heparin greatly influences its biological activity (Rabenstein 2002). Low molecular weight heparin ranging from 2.4 kDa to 5.4 kDa (8 to 18 monosaccharides units) may have an anti-tumor effect by reducing or suppressing tumor growth and metastasis in some cases (Castelli, Porro et al. 2004; Norrby 2006; Lee 2007). This finding implies that a tight control of the molecular weight of heparin and heparin-like molecules during production seems to be the clue to enlarge its therapeutic use. However the current ways to produce heparin and low molecular weight heparin are far from being optimal with respect to the control of the chain length distribution. Monodisperse heparin fractions with low molecular weight are difficult to obtain from animal tissue and the chemical synthesis of polymers longer than 6 units is not economically feasible (Chen, Bridges et al. 2006).

In vivo GAGs are polymerized by glycosyltransferase (GT) enzymes that catalyze the polymer elongation through stepwise addition of α-linked uridine nucleotide sugars. Depending on the polysaccharide structures, the polymerization involves GT able to perform an inverting (α→β) or a retaining (α→α) mechanism (Coutinho, Deleury et al. 2003). The polymerization step involving the (α→β) mechanism is well known, however, the mechanism (α→α) is less described and still remains not fully understood. Due to the nature of the heparosan polymer, the heparosan synthase exhibits both of these mechanisms to elongate heparosan chains.

Bacterial capsules composed of heparosan have been reported in *Escherichia coli* K5 (Vann, Schmidt et al. 1981) and *Pasteurella multocida* Type D (Pandit and Smith 1993; Rimler 1994; DeAngelis, Gunay et al. 2002). In *E. coli* K5, the synthesis of heparosan is regulated by 4 genes, KfiA, KfiB, KfiC, and KfiD, located on the same operon. However only two of them, KfiA and KfiC, encoding respectively for the *N*-acetylglucosaminyl transferase and for the D-glucuronyl transferase seem to be involved in the elongation of heparosan polymers

(Griffiths, Cook et al. 1998; Hodson, Griffiths et al. 2000; Chen, Bridges et al. 2006). Different from what is observed in *E. coli*, in *P. multocida* Type D the synthesis of heparosan is performed by only one enzyme with two glycosyltransferase activities; the heparosan synthase PmHS1. The *P. multocida* Type D heparosan synthase gene *pm*hssA also known as *pm*hs1 located on the putative capsule locus was cloned and PmHS1 active proteins were expressed (DeAngelis and White 2002). Based on homology with *pm*hssA a cryptic gene *pm*hssB (described also as *pgl*A) encoding for an active recombinant heparosan synthase PmHS2 was discovered in *P. multocida* Type A, D and F (May, Zhang et al. 2001; DeAngelis and White 2002). Characterization of recombinant PmHS1 and PmHS2 enzymes showed that despite their high amino acid level homology (73%), the enzymes differ considerably with respect to their kinetic properties and the molecular weight distribution of the synthesized heparosan polymers (DeAngelis and White 2004; Sismey-Ragatz, Green et al. 2007).

Kinetic studies on both *P. multocida* heparosan synthase enzymes report an initiation rate and an elongation rate of 2.6 and 76 pmol/μg/min respectively for PmHS1 while 5.2 and 28 pmol/μg/min were observed for PmHS2 (Sismey-Ragatz, Green et al. 2007). When incubated without acceptor the polymerization properties of PmHS1 and PmHS2 were largely different with respect to molecular weight of the synthesized polymer. PmHS1 synthesized heparosan polymers with an average molecular mass of 800 kDa while heparosan of 28 kDa were observed when PmHS2 was used as a catalyst. The addition of heparosan oligosaccharide acceptors lowered the molecular weight of heparosan polymers synthesized by PmHS1 while it had almost no effect on the PmHS2 polymerization process. Sismey-Ragatz et al., 2007, also showed that contrary to PmHS1, PmHS2 is able to synthesize glycosaminoglycan polymers with new biological properties using unnatural donor sugar analogs. These heparin analog molecules with potentially new biological activities represent a great interest to enlarge therapeutic uses. It was indeed observed that depending on the sugar substitution, digestion of the polymer by heparinase III could be avoided (Sismey-Ragatz, Green et al. 2007).

Because of the above-mentioned polymerization properties PmHS2 was investigated in detail in this study. An efficient way to express, purify and store the PmHS2 enzyme is reported. The PmHS2 polymerization activity in time, as well as the heparosan polymer elongation process have been characterized by use of a coupled enzyme assay, agarose gel electrophoresis and HPSEC analysis. The influence of parameters such as the nucleotide sugar, the cofactor and the UDP concentrations on the enzyme activity and the product molecular weight are described. In this report we show that PmHS2 is capable of synthesizing heparosan polymers with a high Mw and that heparosan polymer average Mw and size

distribution can be controlled by the initial nucleotide sugar concentration present in the polymerization reaction.

Experimental Procedures

Cloning, expression and purification of recombinant *P. multocida* heparosan synthase 2

Based on the sequence of the *Pasteurella multocida* Type D heparosan synthase cryptic gene *pm*hssB (GenBank acc. No. AY292200), the *pm*hssB forward primer 5′-CGCCATGAAGGGAAAAAAAGAGATG-3′ and reverse primer 5′-GGATCCTTATAAAAAATAAAAAGGTAAAC-3′ were designed. Using a High Fidelity PCR mix (Roche) on genomic DNA isolated from a *Pasteurella multocida* Type D (Strain number: 40456 from the Wageningen-UR Central Veterinary Institute collection) the *pm*hssB open reading frame (ORF) was amplified by 30 cycles of PCR (94°C, 30 sec; 60°C, 30 sec; 72°C, 1 min). The PCR fragment was ligated into pCR-BluntII vector and transformed into *E. coli* Top10 cells (Invitrogen). The identity of the *pm*hssB gene was confirmed by sequencing (Base Clear B.V). Using the forward primer 5′-CACCATGAAGGGAAAAAAAGAGATG-3′ and the reverse primer 5′-TAAAAAATAAAAAGGTAAACAGGGGATA-3′, *pm*hssB fragments were obtained by applying 30 cycles of PCR (94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min) which were cloned in the pET101-D-TOPO expression vector (Invitrogen).

The pET101-D-TOPO vector allowing the fusion of a V5 epitope and a C terminal His-tag to the *pm*hssB fragment was first transferred into the *E. coli* Top10. Subsequently positive plasmids were isolated and transformed into the *E. coli* BL21*(DE3) expression strain (Invitrogen). Recombinant protein was expressed according to the manufacturer's instruction. Briefly, a culture inoculated with an overnight pre-culture was grown in LB ampicillin (50 μg/ml) media at 37°C under shaking condition (250 rpm). When the OD_{600} was around 0.6 the protein expression was induced by addition of 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) and growth was continued for 2 h at 30°C/250 rpm. Cells were harvested by centrifugation (Allegra 6R centrifuge. Beckman Coulter. Rotor: GG-3.8A.) at 5°C/3000 g/20 min and the pellet was stored at -80°C. For enzyme purification the pellet (from 75 ml culture) was thawed on ice and resuspended in 4 ml phosphate buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8) before being sonicated 4x30 sec on ice with a sonication tip (Vibra cell. Sonic and materials INC, output control set on 40). Prior to centrifugation at 5°C/18000 g/15 min (Eppendorf centrifuge 5417R), $MgCl_2$ was added to the lysate to a final concentration of 6 mM (Kane, White et al. 2006). After centrifugation, the supernatant was kept on ice for

further purification and the pellet was discarded. In order to perform optimal His-tag purification, imidazole was added to the supernatant to a final concentration of 10 mM. To 4 ml of supernatant, 1 ml of 50% Ni-NTA super-flow resin slurry (Qiagen) was added and gently mixed for 1 h at 4°C on a rotating shaker. The resin slurry and supernatant mix was washed with increasing imidazole concentrations respectively 10 mM and 20 mM. To allow the elution of PmHS2, 250 mM of imidazole was present in the elution buffer. The first 3 eluted fractions of 1 ml known to have the highest PmHS2 concentrations were pooled and then desalted by gel filtration using a PD10 column with Sephadex G-25 Medium resin (GEA Healthcare) with 50 mM Tris, pH 7.2 as eluent. Ethylene glycol to a final concentration of 1 M was added to the desalted PmHS2 fraction before storage at -80°C.

Purity of the PmHS2 desalted fraction was monitored by 10% SDS-PAGE stained with SimplyBlue Safe Stain (Invitrogen) and the protein content was quantified by the BCA method (Pierce) using BSA as reference.

Characterization of the PmHS2 polymerization process

Standard polymerization conditions

Unless noted, polymerization assays with a final volume between 30 to 200 µl contained 40 mM Tris pH 7.2, 4 mM $MnCl_2$, 4 mM $MgCl_2$, 5 mM UDP-glucuronic acid (UDP-GlcUA), 5 mM UDP-*N*-acetylglucosamine (UDP-GFlcNAc) and 55-60 µg/ml of 95% pure PmHS2 enzyme. The reaction occurred in the dark at 30-32°C under mild shaking conditions for time ranging from 1.5 to 48 h depending on the experiment. In order to study the effect of different parameters the composition of the polymerization mix was changed. Modifications in the standard incubation conditions are stated in the legend to the figures.

Note that in this study no oligosaccharide acceptor was added to the polymerization reaction.

Determination of the heparosan polymer chain length and polydispersity

After synthesis, the reactions were quenched at 99°C for 10 min and then cooled on ice. The polymer chain length and the size distribution of the synthesized products were assessed by agarose gel electrophoresis and by high performance size exclusion chromatography (HPSEC) analysis. For gel analysis the samples were mixed with glycerol to a final concentration of 12%, loaded on a 2% (w/v) agarose gel and run for 2 h at 50 V in Tris-acetate EDTA buffer (TAE buffer) (Lee and Cowman 1994). The gels were stained overnight in the dark in 50% ethanol / 0.005% Stains-All (Sigma). The destaining of the gels was carried out in pure water for 30 min in the dark followed by an exposure to the light till

background discoloration. Images from the gel were acquired with a BioRad GS-800 calibrated densitometer and analyzed by the Quantity One program. Selected hyaluronan molecular mass markers ranging from 27 kDa to 495 kDa (Select-HALoLadder, Hyalose, Oklahoma, USA) were used to estimate the heparosan molecular weight. Since Stains-All tends to over-stain the polysaccharides on an agarose gel it is was difficult to estimate the average molecular weight of the polymers in an accurate way by this type of analysis.

Alternatively the size distribution and the molecular weight of the polymers were analyzed by High Performance Size Exclusion Chromatography (HPSEC) on a SpectraSystem HPLC (Thermo Separation Products, USA) using three TosoHaas TSK-gel columns in series (4000, 3000, and 2500 PWx1, 300x7.5 mm; TosoHaas, Japan) preceded by a TSK PWx1 guard column (TosoH, Japan). Samples (20 µL) were injected and eluted at 30°C using 0.8 ml/min $NaNO_2$ (0.2 M). Detection was performed using a Shodex RI 71 refractive index detector (Showa Denko K.K., Japan). Hyaluronan molecular mass standards of respectively 30 kDa, 160 kDa, and 262 kDa were used for calibration. From the logarithmic model fitting the elution time of the hyaluronan molecular weight standards we established the following equation: Mw = Mi = exponential [(elution time - 27.811)/(-1.4765)]. By assuming that heparosan and hyaluronan elution patterns are comparable, the molecular weight of the eluted samples (Mi) was determined by applying the equation mentioned above. The molecular weight distribution (polydispersity index PDI) was determined using the following formula: $PDI = M_w / M_n$

$$Mn = \frac{\sum_i (Ni \times Mi)}{\sum_i (Ni)} \qquad Mw = \frac{\sum_i (Ni \times Mi^2)}{\sum_i (Ni \times Mi)}$$

The RI signal, known to be proportional to the relative amount of eluted product, was considered as being equivalent to the amount of polymer chains (Ni) of molecular weight Mi.

Determination of PmHS2 specific activity

The polymerization reaction was performed with 95% pure PmHS2 and in presence of nucleotide sugars. Both nucleotide sugars (UDP-glucuronic acid and UDP-*N*-acetylglucosamine) were added in equimolar ratio and in sufficient amount in order to initiate the polymerization and enable us to follow the elongation process in time by means of the coupled enzyme assay and agarose gel analysis. The activity of PmHS2 was assessed by measuring the UDP formation with a non continuous spectrophotometric assay at 340 nm (Gosselin, Alhussaini et al. 1994; Krupa, Shaya et al. 2007). In this assay the UDP released

from the conversion of the nucleotide sugars during the polymerization is coupled via pyruvate kinase (PK) and lactate dehydrogenase (LDH) to the oxidation of NADH into NAD^+. With 0.3 mM NADH as initial concentration and in presence of UDP, the NADH conversion into NAD^+ was established to be linear. According to the stoichiometric equation 1 mM UDP will convert 1 mM NADH. This was confirmed with preliminary experiments in which the NADH/UDP conversion ratio was found to be 1.016.

Due to the degradation of nucleotide sugars at high temperature, heat shock was not used to quench the reactions. Polymerization reactions were stopped by immersion in liquid nitrogen and stored at -20°C. Prior to the UDP measurement, the samples were thawed on ice and diluted to contain less than 0.3 mM of UDP. No residual PmHS2 activity was detected using this procedure. The following compounds were added to the diluted samples: 0.3 mM NADH, 2 mM PEP (phospho(enol) pyruvic acid trisodium salt heptahydrate 98%. Across organic, Belgium), 112.5 mM KCl, and 25 U/ml LDH (L-lactate dehydrogenase from Rabbit muscle. Fluka). The mix with a final volume of 140 µl was pre-incubated in a Tecan Safire thermostated spectrophotometer during 10 min at 30°C and its absorbance was measured at 340 nm. The assay was performed in microtiterplate wells (UV-star plate 96 wells, flat bottom, Greiner bio-one). After the pre-incubation, PK (pyruvate kinase from rabbit muscle. Sigma) was added to a final concentration of 10 U/ml. The final reaction volume was 150 µl. The OD was measured at 340 nm for 30 min at 30°C. A stable absorbance indicated full conversion of the UDP. The pre-incubation and incubation absorbance data were combined to calculate the amount of UDP released in the polymerization reactions and thus, estimate the enzymatic activity using the conversion ratio of 1.016. All experiments were performed in independent duplicate; the average is presented in the "Results" section.

Identification of the polymer chains synthesized by PmHS2

The identification of the polymer obtained by enzymatic activity of the recombinant PmHS2 was carried out using Heparinase III from *Flavobacterium heparinum* (Sigma). Heparinase III is known to specifically cleave at the α1-4 linkage between the hexosamine and glucuronic acid residues of heparosan and heparan sulfate. First the polymerization assay was quenched at 99°C for 30 min then Heparinase III (0.5 U/µl in 0.05 M sodium phosphate buffer pH 7.6) was added to it and the mixture was incubated for 24 h at 30°C (DeAngelis, Gunay et al. 2002). The samples obtained after polymerization and incubated with and without Heparinase III were analyzed on a 2% (w/v) agarose gel. Full degradation of the polymer confirmed the identity of PmHS2 synthesized product as being heparosan polymers (data not shown).

Results

Cloning, expression and purification of the recombinant P. multocida heparosan synthase PmHS2

The *Pasteurella multocida pm*hssB gene was cloned and PmHS2 recombinant proteins expressed using the vector pET101 in *E. coli* BL21*(DE3) strain. After sonication of the cell suspension, 15-20% of PmHS2 protein was found in the soluble fraction (Fig. 1). Most of the PmHS2 remained in the membrane fraction. After Ni-NTA purification and PD10 desalting steps we estimated the purity of PmHS2 enzyme to be about 95% based on SDS-PAGE gel analysis. BCA assay showed that 5 to 10 % of pure PmHS2 enzyme was lost during the PD10 desalting step. We estimated that about 7 ± 1 mg of 95% purified PmHS2 was obtained from a 1 L of *E. coli* culture after 2 h of induction at 30°C.

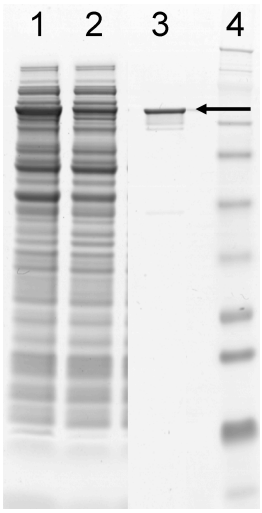

Figure 1. SDS-PAGE analysis of recombinant PmHS2 protein. E. coli protein fractions were separated on a 10% polyacrylamide gel and stained with SimplyBlue Safe Stain (Invitrogen). The expressed PmHS2 is present in the total E. coli cell fraction (dilution x4) (lane1), in the soluble fraction after cold sonication and centrifugation (dilution x2) (lane2) and in the His-tag purified and PD10 desalted fraction (dilution x1) (lane3). The approximate protein molecular mass (around 77 kDa) was estimated by comparison with the SeeBlue Plus2 Prestain (Invitrogen) molecular mass standard (lane 4).

Characterization of the PmHS2 polymerization process

Storage stability of PmHS2

The storage stability of PmHS2 was determined by comparing the catalytic activity of the newly extracted and purified PmHS2 enzyme with the PmHS2 enzyme stored at -80°C. The catalytic activity of PmHS2 was estimated by gel electrophoresis analysis of the polymerization products obtained under standard conditions. Addition of $MnCl_2$ and $MgCl_2$ to the storage buffer increased enzyme instability at -80°C. Under these storage conditions a significant loss of activity was observed after 3 weeks at -80°C. Addition of ethylene glycol to a final concentration of 1 M in the storage buffer was crucial to ensure the enzyme stability. With ethylene glycol, both the activity of the enzyme expressed as the amount of nucleotide

sugars converted by PmHS2 after 24 h of incubation at 30°C and the chain length of the synthesized heparosan were the same before and after 6 months at -80°C (Fig. 2). After 1 year of storage a decrease of about 10 to 20% of the enzymatic activity was observed. SDS-PAGE analysis showed that PmHS2 was not degraded during the storage at -80°C (data not shown).

Process stability during the polymerization

In the presence of 55-60 μg/ml of 95% pure PmHS2 and 20 mM of each UDP-sugar we observed that PmHS2 maintained at least 80% of its initial activity during the incubation time from 24 to 48 h. After 24 h of incubation in the presence of 5 mM or 20 mM of both UDP-sugar PmHS2 converted comparable amount of nucleotide sugars. According to these results we assumed that PmHS2 process stability was constant for 24 h under the assay conditions used. Thus, we considered 24 h of incubation as being the reference time point to study the influence of different parameters such as the divalent metal ion, the nucleotide sugar and the UDP concentrations on the polymerization activity.

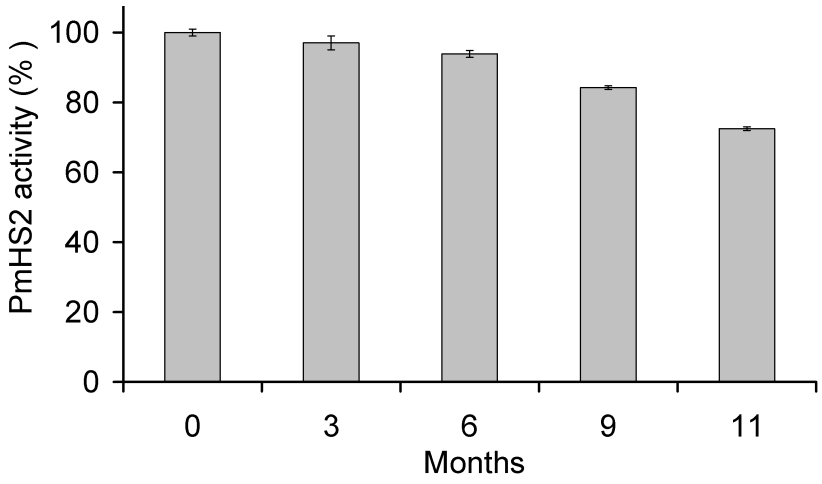

Figure 2. Influence of the time of storage at -80°C on PmHS2 polymerization activity. After several months of storage at -80°C, purified PmHS2 (55-60 μg/ml) was thawed on ice and its activity was assayed in the standard polymerization buffer described in the Materials and Methods section. Enzyme activity expressed as the amount of substrate converted after 24 h of incubation at 30°C was determined using the coupled enzyme assay. 100% activity corresponds to the amount of substrate converted by freshly extracted enzyme.

Influence of the divalent metal ions Mn^{2+} and Mg^{2+}

Divalent metal ions such Mn^{2+} and Mg^{2+} have been described as being cofactors of some glycosyltransferases (Markovitz, Cifonelli et al. 1959; Stoolmiller and Dorfman 1969). DeAngelis and White, 2004, showed that in the case of PmHS2, Mn^{2+} is the optimal divalent

metal ion to support the polymerization but Mg^{2+} also contributes to the substrate conversion (DeAngelis and White 2004). To study the influence of these divalent metal ions on the PmHS2 catalytic activity, an equimolar mixture of $MnCl_2$ and $MgCl_2$ ranging from 0 to 16 mM was investigated (Fig. 3). The polymerization was not initiated in absence of metal cofactor. PmHS2 activity after 24 h of reaction time was optimal with 4 mM of both $MgCl_2$ and $MnCl_2$. With 0.5 mM of $MgCl_2$ and 0.5 mM of $MnCl_2$, a 40% decrease of the activity was found. In the range from 2 mM to 6 mM of each divalent metal ion, a decrease of activity of not more than 10% was observed. In presence of 8 mM to 16 mM of each divalent metal ion a decrease of activity, respectively, about 20 to 50% was observed. Not only the amount of substrate converted in 24 h was affected by the cofactor concentrations but also the average polymer molecular mass. Based on agarose gel electrophoresis analysis, PmHS2 incubated in presence of 4 mM of Mg^{2+} and 4 mM of Mn^{2+} synthesized heparosan polymers with an average of 102 kDa while 30-40 kDa heparosan polymers were obtained in presence of 10 mM of each ion.

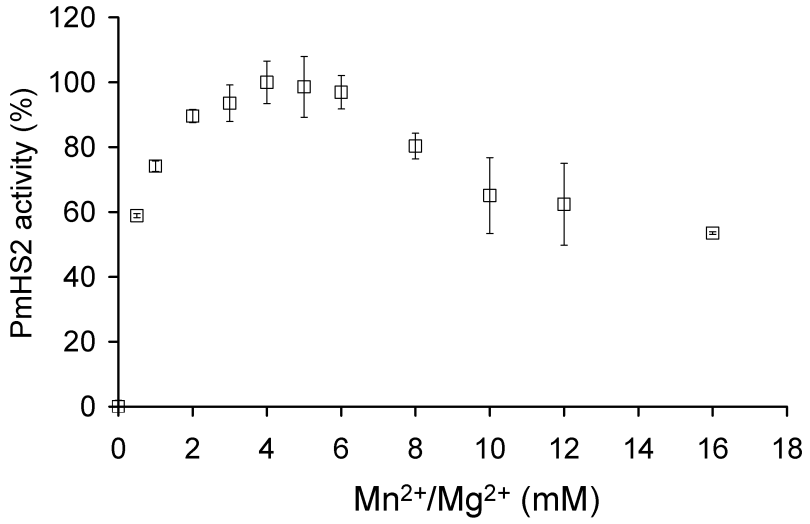

Figure 3. Effect of the $MnCl_2$ and $MgCl_2$ concentration on PmHS2 activity. Enzymatic activity of purified PmHS2 (55-60 μg/ml) was assayed in the standard polymerization buffer described in the Materials and Methods section with an equimolar mixture of divalent metal ions ($MnCl_2$ and $MgCl_2$) ranging from 0 to 16 mM. 100% activity corresponds to the amount of substrate converted after 24 h of incubation at 30°C in presence of 4 mM of $MnCl_2$ and 4 mM of $MgCl_2$. The data represent the average of three independent experiments ± SD.

Influence of the nucleotide sugar concentrations

Since for each mole of nucleotide sugar converted 1 mole of UDP is released, the free UDP generated during the polymerization reaction was used to estimate the substrate conversion by PmHS2. The molecular mass and the size distribution of heparosan polymer were determined

by agarose gel electrophoresis and HPSEC using hyaluronan polymers standards (27 kDa to 495 kDa).

Nucleotide sugars, UDP-GlcUA and UDP-GlcNAc, mixed in an equimolar ratio, were added to the polymerization buffer in a sufficient amount to initiate the polymerization and enable us to follow the elongation process in time by means of the coupled enzyme assay and agarose gel analysis. In order to study the influence of the nucleotide sugar concentration on the polymerization process, substrate concentrations of 1 mM, 5 mM, and 20 mM of both UDP-GlcUA and UDP-GlcNAc were added to the polymerization reaction with purified PmHS2.

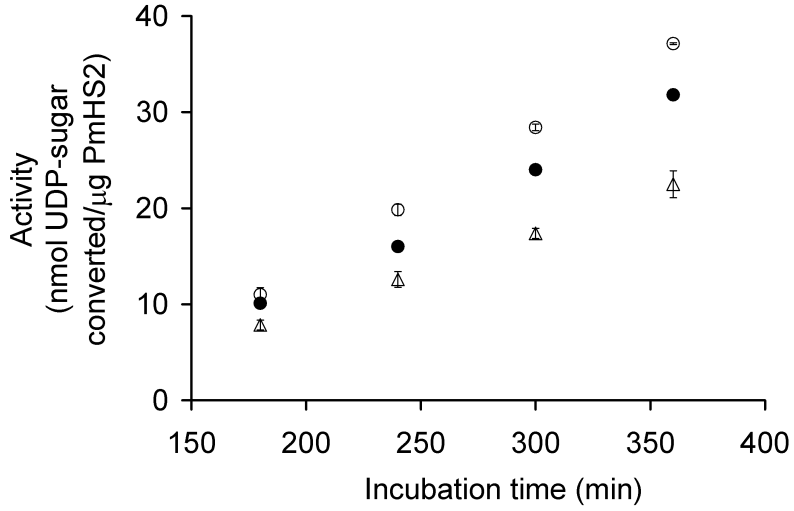

Figure 4. Influence of the substrate concentration on PmHS2 activity during 180 min to 360 min of incubation. Purified PmHS2 (55-60 µg/ml) was assayed in the standard polymerization buffer described in the Materials and Methods section with increasing substrate concentration ranging from 1 mM up to 20 mM of both nucleotide sugars. The specific activity observed during 180 min to 360 min of incubation for the following substrate concentrations 1 mM (Δ), 5 mM (○), and 20 mM (•) of both UDP-GlcUA and UDP-GlcNAc was respectively about 0.080, 0.145, 0.128 nmol/µg/min.

It is important to notice that for the 3 equimolar nucleotide sugar concentrations chosen in this study the specific activity of PmHS2 remained constant from 180 min to 360 min (3 h to 6 h) of incubation despite the decrease of available nucleotide sugars and the subsequent increase in UDP a possible inhibitor of the polymerization reaction (Fig. 4) (Tlapak-Simmons, Baron et al. 2004; Baggenstoss and Weigel 2006).

We observed that the substrate concentration influenced PmHS2 polymerization process in two distinct manners. First, as shown in figure 4, the substrate concentration influenced the specific activity of the enzyme. The highest activity of 0.145 nmol/µg/min was observed between 180 min to 360 min of incubation when 5 mM of each UDP-sugar were added. Adding 20 mM of each nucleotide sugar lowered PmHS2 specific activity to 0.128 nmol/µg/min. At a lower substrate concentration, 1mM of UDP-GlcUA and 1 mM of UDP-

GlcNAc, the specific activity was about 0.080 nmol/μg/min. In this last case, based on the amount of substrate converted per unit of time, we calculated that the nucleotide sugars were totally converted after about 8 h of incubation.

Secondly the substrate concentration greatly influenced the molecular weight and the size distribution of the heparosan polymers synthesized by PmHS2. Both agarose gel electrophoresis and HPSEC analysis showed that PmHS2 elongated high molecular weight heparosan polymers when incubated in presence of 1 mM of each nucleotide sugar while lower molecular weight products were obtained when incubated with 5 mM or 20 mM of each UDP-sugar (Fig. 5A and 5B). Also the polydispersity of the formed heparosan was influenced by the UDP-sugar concentrations, a low substrate concentration resulted in a lower polydispersity. HPSEC analysis showed that in presence of 1 mM, 5 mM, and 20 mM of both UDP-GlcUA and UDP-GlcNAc the average molecular mass of the heparosan formed was respectively 130 kDa; 102 kDa, and 45 kDa. These data showed an inverse correlation between the nucleotide sugar concentration and the polymer chain length. Depending on the initial conditions PmHS2 lengthened heparosan chains to a different polymerization degree. In presence of 1 mM of each nucleotide sugar, polymers with an average of 650 monosaccharide units were synthesized, in comparison to 225 monosaccharide units per polymer when incubated in the presence of 20 mM of each UDP-sugar.

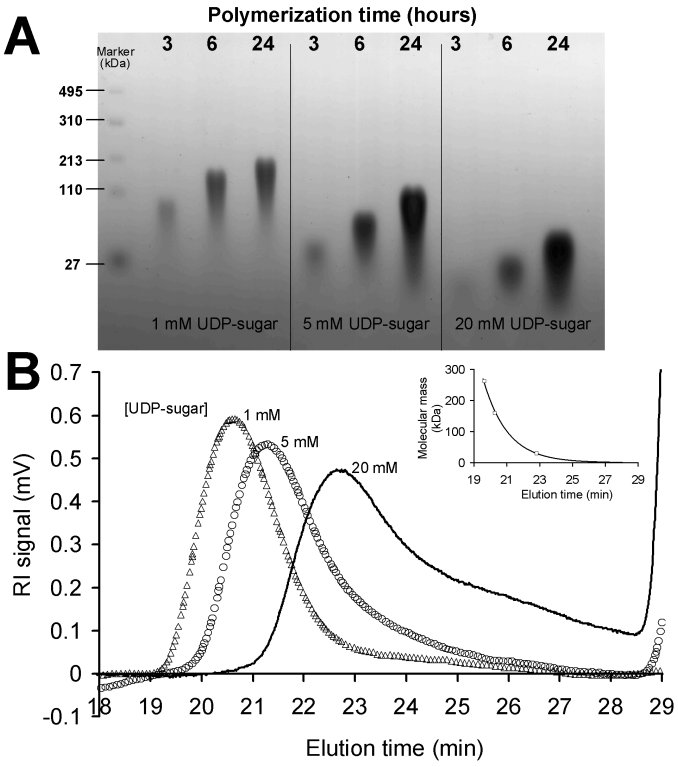

Figure 5. Influence of the substrate concentration on PmHS2 polymerization process. Purified PmHS2 (55-60 μg/ml) was incubated at 30°C in the standard polymerization buffer described in the Materials and Methods section with increasing substrate concentration (UDP-GlcUA and UDP-GlcNAc). (A) Depending on the substrate concentration and incubation time, heparosan polymer chain length and size distribution was analyzed on 2% (w/v) agarose gels stained with Stains-All. (B) HPSEC analysis of the synthesized heparosan after 24 h of incubation in presence of 1 mM, 5 mM and 20 mM of both UDP-GlcUA and UDP-GlcNAc. The average molecular mass and polydispersity (PDI) of heparosan polymers were respectively: (Δ) 130 kDa (PDI =1.04), (○) 102 kDa (PDI =1.36), and (•) 45 kDa (PDI =1.52).

Table 1 shows for each studied substrate concentration, the relation between the amount of nucleotide sugars converted after 3 h of incubation and the corresponding average polymer molecular weight observed on agarose gel electrophoresis. From these data we have calculated the amount of heparosan polymer chains present in each sample and their elongation rate.

The following equation was used to determine the amount of heparosan polymer chains (nmole P_{Tn}) initiated during the first 3 h of incubation in a 100 µl reaction volume per µg of PmHS2.

$$\text{nmole } P_{Tn} = \frac{m_{\text{nucleotide sugar}} \text{ at } T_n}{Mw_{\text{average}} \text{ at } T_n}$$

During the first 3 h of polymerization we calculated that about 9 times less heparosan polymer chains were initiated and elongated with 1mM of UDP-GlcUA and 1 mM of UDP-GlcNAc compared to 20 mM of both UDP-sugars.

Assuming that only the oligosaccharides present after 3 h of polymerization reaction will be elongated, in other words by neglecting the initiation of new polymers, we calculated the elongation rate per polymer chain. For this calculation the PmHS2 specific activity values previously determined for a period of incubation between 3 to 6 h were used (Fig. 4).

At an incubation time T_n we estimated the total amount of nucleotide sugar converted (nmole T_{Tn}).

$$\text{nmole } T_{Tn} = \left(T_n - T_{(n-x)}\right) \times \text{specific activity} + \text{nmole } T_{T(n-x)}$$

For each incubation time T_n, the average polymerization degree for each polymer chain could be determined (DP_{Tn}).

$$DP_{Tn} = \text{nmole } T_{Tn} / \text{nmole } P_{Tn}$$

From the DP_{Tn}, the average Mw of the heparosan polymer chains at T_n could be assessed.

$$Mw_{Tn} = DP_{Tn} \times Mw_{\text{monosaccharide}}$$

The average number of monosaccharide units transferred per min and per heparosan polymer chain (U_{MS}, equal to elongation rate) has been estimated for the elongation phase using the equation:

$$U_{MS} = \frac{\left(Mw_{Tn} - Mw_{T(n-x)}\right) / Mw_{\text{monosaccharide}}}{T_n - T_{(n-x)}}$$

The calculated elongation rate was about 3.6 monosaccharide unit/min/heparosan chain versus 0.6 monosaccharide unit/min/heparosan chain respectively with 1 mM versus 20 mM

of each UDP-sugar. Thus, at low substrate concentration both a decrease in number of polymer chains and an increase in elongation rate per heparosan chain result in longer polysaccharides.

Finally based on our assumptions and the calculations we determined the average heparosan polymer Mw (kDa) that should be synthesized after 6 h of polymerization.

$$Mw_{\text{average Tn}} = DP_{Tn} \times Mw_{\text{monosaccharide}}$$

We were able to describe PmHS2 polymerization behavior in presence of different substrate concentrations. However, the calculated average heparosan Mw after 6 h of incubation are higher than what is observed on agarose gel electrophoresis (Table. 1). For example, in presence of 1 mM of each UDP-sugar, we calculated that after 6 h of incubation heparosan polymers should have an average molecular mass of 198 kDa, while on agarose gel heparosan polymers of about 120 ± 10 kDa were observed. The difference between the calculated and the observed data is even more significant if we consider 24 h of incubation. The increased tailoring of the spots (Fig. 6B) indicated enhanced polydispersity due to initiation of new heparosan chains at the same time as the elongation of heparosan polymers.

To conclude, the effect of the nucleotide sugar concentration on PmHS2 polymerization process can be described thanks to equations estimating the amount of polymer chains initiated and their approximate molecular weight. However, the results suggest that the estimation can be further improved when taking into account that during the elongation, the initiation of new polymer chain occurs.

Table 1. Influence of the nucleotide sugar concentration on the PmHS2 polymerization process. The polymerization reaction was performed in 100 µl reaction volume containing 55-60 µg/ml purified PmHS2. The detail of the calculation is presented in the Result section.

UDP-GlcUA UDP-GlcNAc mM	UDP-sugar converted per microgram PmHS2 (nmol, 3 h incubation)	Observed average heparosan molecular mass (kDa, 3 h incubation)[a]	Heparosan chain initiated per microgram PmHS2 (nmol, 3 h incubation)	Calculated U_{MS}[b]	Calculated average heparosan molecular mass (kDa, 6 h incubation)[a]	Observed average heparosan molecular mass (kDa, 6 h incubation)[a]
1	8 ± 0.20	70 ± 5	0.022	3.6	198	120 ± 10
5	11 ± 0.70	28 ± 2	0.082	1.8	84	60 ± 5
20	10 ± 0.50	10 ± 2	0.200	0.6	33	25 ± 2

a. The polymer average molecular mass (± estimated error) was determined using agarose gel electrophoresis analysis in comparison with hyaluronan molecular weight marker as a reference.

b. Refers to the calculated average number of monosaccharide units transferred per min and per heparosan polymer chain.

PmHS2 polymerization process in time

The polymerization process in time was investigated with 55-60 μg/ml of 95% pure PmHS2 using the optimal nucleotide sugar concentrations to get the highest specific activity (5 mM of each UDP-sugar). The quantification of free UDP was used to determine the amount of nucleotide sugar converted in time. We observed that the PmHS2 polymerization process could be divided into phases depending on the specific activity observed in time (Fig. 6A). These polymerization phases have been studied and characterized for the enzyme specific activity, the product molecular weight synthesized and the polymer size distribution. The polymerization starts with the synthesis of short oligosaccharide from nucleotide sugars. This phase called initiation is quite short and in the case of PmHS2 "the level of *de novo* initiation" is low (Sismey-Ragatz, Green et al. 2007). As shown in Table. 1, we observed a correlation between the UDP-sugar concentrations and the amount of heparosan polymer chain initiated.
Following the initiation, the short heparosan chains synthesized will be elongated. This phase is usually referred as the elongation phase and is characterized by a constant ratio of nucleotide sugar conversion over the time ($\Delta P/\Delta T$). Between 2 h and 12 h of incubation the enzyme specific activity was about 0.100 ± 0.01 nmol/μg/min (Fig. 6A.). This corresponds to a transfer of 1.2 monosaccharide unit/min/heparosan chain. The average molecular mass of representative polymer samples increased significantly in time from 61 kDa (PDI= 1.25) after 4 h to 102 kDa (PDI= 1.36) after 24 h of incubation meaning that about 500 monosaccharide units in average have been incorporated into each polymer chain. However, as noticed before the increase in polydispersity in time showed that initiation of new chains and elongation proceed simultaneously (Fig. 6B). The last phase observed is characterized by a sudden level off of the enzyme activity. After about 12 to 13 h of incubation, the specific activity of PmHS2 slowed down to about 0.020 ± 0.005 nmol/μg/min (Fig. 6A). Between 12 h and 24 h the polymer size only slightly increased in contrast with an increase in polydispersity (Fig. 6B).

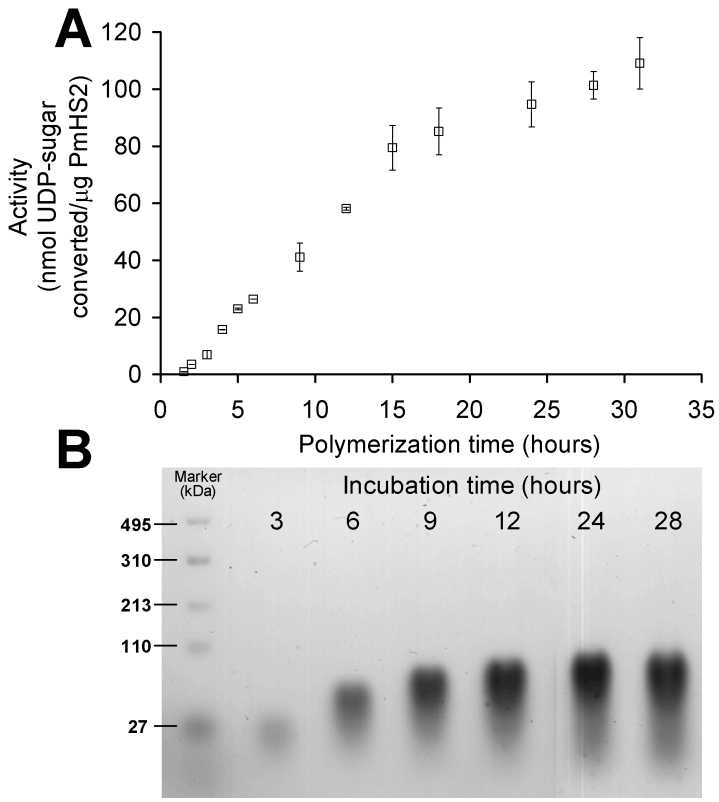

Figure 6. Analysis of PmHS2 polymerization process in time. The PmHS2 (55-60 μg/ml) enzymatic activity, incubated during 28 h at 30°C, was analyzed with standard assay conditions as described in the Materials and Methods section. At many time points the amount of substrate converted was determined using the coupled enzyme assay. (A) The specific activity for the elongation phase (2-12 h) and the level off were respectively 0.100 ± 0.010 nmol/μg/min and 0.020 ± 0.005 nmol/μg/min. Five independent experiments were completed for each data set, their average ± SD is presented. (B) The molecular weight distribution of heparosan polymer in the samples was analyzed by 2% (w/v) agarose gels stained with Stains-All.

Inhibition of the PmHS2 polymerization process by UDP

UDP has been reported to inhibit GT enzymes activity (Markovitz, Cifonelli et al. 1959; Stoolmiller and Dorfman 1969; Tlapak-Simmons, Baron et al. 2004; Baggenstoss and Weigel 2006). When added to the polymerization buffer prior to the synthesis, UDP showed severe inhibition of the PmHS2 activity. The inhibition level of 5 mM of UDP on the polymerization activity was around 50% with 5 mM or 20 mM of each nucleotide sugar (Fig. 7A). In the range of 8 to 10 mM of UDP the level of inhibition was dependent on the nucleotide sugar concentration (Fig. 7B).

With 5 mM of each nucleotide sugar the complete inhibition of the PmHS2 polymerization activity was observed in presence of 8 mM UDP, while with 20 mM of each nucleotide sugar the full inhibition was obtained with 10 mM of UDP. These results suggest that UDP

inhibition is somehow related to the concentration of nucleotide sugars present in the reaction assay. The inhibitory UDP concentration does not seem to be reached during the first hours of incubation. We indeed observed that from 2 to 12 h of incubation, the UDP rise and the decrease of substrate availability did not affect PmHS2 specific activity (Fig. 4 and Fig. 6A). To conclude, the inhibition by UDP does not seem to occur when the UDP concentration is below or equal to 4 mM.

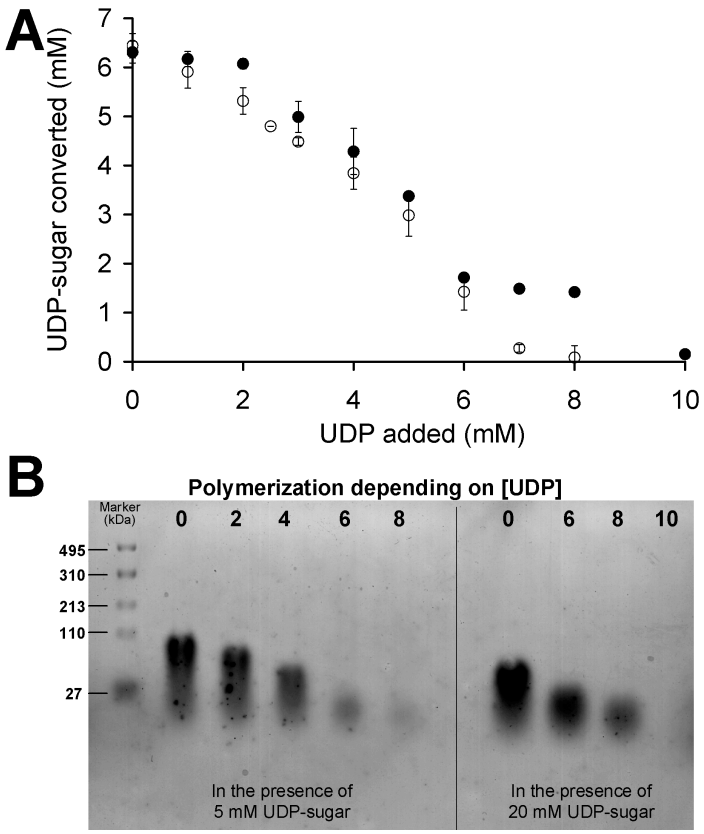

Figure 7. Effect of initial UDP concentration on PmHS2 activity. Purified PmHS2 (55-60 μg/ml) was assayed in the standard polymerization buffer described in the Materials and Methods section with increasing initial UDP concentrations from 0 mM to 10 mM. (A) The nucleotide sugar concentration of both UDP-GlcUA and UDP-GlcNAc was respectively 5 mM (○) and 20 mM (•). After 24 h of incubation at 30°C the amount of substrate converted by PmHS2 in presence of UDP was determined using the coupled enzyme assay. (B) Samples from both series were analyzed on a 2% (w/v) agarose gel stained with Stains-All.

Discussion

In this report we describe the successful cloning of the *P. multocida* Type D heparosan synthase-2 gene (*pm*hssb) and the expression of the corresponding PmHS2 protein into *E.coli* BL21*(DE3) using pET101 as an expression vector. Protein expression and purification allowed the recovery of 7 ± 1 mg of 95% pure and active PmHS2 enzyme per liter of culture. This level of PmHS2 enzyme recovery after purification, which is stable for at least 6 months when stored at –80°C, has not been described so far.

We found that, in absence of divalent metal ions, PmHS2 did not exhibit activity and that cofactor concentration added in excess inhibit the enzymatic activity. The optimal cofactor concentration for PmHS2 polymerization activity was determined to be 4 mM of both Mn^{2+} and Mg^{2+}. This finding support the classification reviewed by Coutinho et al. 2003 that PmHS2 belongs to the GlycosylTransferase-A (GT-A) superfamily due to the requirement for divalent metal ions in order to be active and its D*X*D amino acid conserved domains.

Polymerization reactions catalyzed by the recombinant PmHS2 showed 3 distinct polymerization phases. PmHS2 initiates the polymerization process by the synthesis of short oligosaccharides using nucleotide sugars. For PmHS2, the initiation phase has been described to have a slow “de novo” initiation rate (Sismey-Ragatz, Green et al. 2007). We demonstrated that for PmHS2 the nucleotide sugar concentrations during the initiation phase play an important role in the average Mw and the polydispersity of the resulting heparosan polymers. Low substrate concentrations favor the formation of higher molecular weight polymers with a lower polydispersity while high substrate concentrations do the opposite.

On one hand we assume that in an early stage of the polymerization the PmHS2 enzyme has more affinity for oligomers than for nucleotide sugars. On the other hand we also hypothesize that in presence of a low substrate concentration the probability for PmHS2 to encounter a synthesized heparosan oligomers instead of a nucleotide sugar is relatively high. A lower substrate concentration causes less heparosan chains to be initiated. Consequently lesser heparosan polymers to be elongated and thus, it will result in the synthesis of longer heparosan polymer chains. This polymerization phenomenon is similar to what has been observed with PmHAS and PmHS1 in presence of oligosaccharide acceptors. For both enzymes, the affinity for the oligosaccharide acceptor was higher than for activated monosaccharides. In addition, a decrease in oligosaccharide acceptor concentration resulted in polymer chains with a higher Mw, while an increase in acceptor lowered the average Mw of the polymer formed (Jing and DeAngelis 2004; Sismey-Ragatz, Green et al. 2007). We demonstrated that for PmHS2 the initiation is a determinant step in the polymer synthesis. During this phase the amount of heparosan chains initiated is regulated by the nucleotide

sugar concentration. Another conclusion from this experiment is that PmHS2 is a non-processive enzyme. With a processive enzyme the dissociation of the polymer and the enzyme implicates the end of the chain elongation. The amount of chains initiated will not differ depending on the nucleotide sugar concentrations and thus, low substrate concentrations will result in shorter polysaccharides. At the opposite, with a non-processive enzyme such PmHS2 the addition of a single monosaccharide to the growing chain is followed by the dissociation of the polymer from the enzyme. The polymer is re-capture before proceeding to the transfer of new monosaccharide unit. As in presence of low substrate concentration PmHS2 initiates less chains it leads to higher molecular weight products.

The substrate concentration also influenced the specific activity of the PmHS2 enzyme during the elongation phase. The highest activity was observed by adding 5 mM of each UDP-sugar, from 3 to 6 h of incubation the specific activity was found to be about 0.145 ± 0.010 nmol/µg/min. In this research, the specific activity observed for PmHS2 is considerable higher than the elongation rate of 28 pmol/µg/min that has been reported so far for PmHS2 (Sismey-Ragatz, Green et al. 2007). When using the optimal nucleotide sugar concentration PmHS2 showed an elongation phase of about 10 h and under these conditions the polymers chains were elongated with average up to 500 to 550 monosaccharides units in 24 h.

Although we have observed that at the first stage of the polymerization that the enzyme has more affinity towards products with a higher molecular weigh both the elongation and the initiation of new heparosan chain occur simultaneously. Weigel, 2002, stipulated that despite the fact that in theory non-processive enzyme could elongate the polymer chains till infinite, it has not been observed. Due to the increase of the polymer chain length and the polymer concentration, interaction between the non reducing end of the polymer chains and the enzyme become more difficult in time. As the system enzyme/polymer is becoming more rigid it will favor the initiation of new polymer chains from nucleotide sugars or short oligosaccharides that are more mobile than longer chains (Weigel 2002). This phenomenon supports the fact that new heparosan chains are also initiated while the elongation of polymer occurs. As newly elongated heparosan oligomers cannot obtain the same molecular weight as the previously synthesized heparosan polymers during the incubation time it results in a broader molecular weight distribution and therefore, a higher polydispersity.

In many studies UDP was found to be an inhibitor of glycosyltransferase activity (Markovitz, Cifonelli et al. 1959; Stoolmiller and Dorfman 1969; Tlapak-Simmons, Baron et al. 2004; Baggenstoss and Weigel 2006). PmHS2 also is inhibited by UDP. The UDP inhibition is influenced by the nucleotide sugar concentration; high substrate concentrations reduce the UDP inhibition. We observed that the specific activity of PmHS2 remained constant during the incubation period from 2 to 12 h despite the decrease of available nucleotide sugars and the subsequent increase in UDP. This showed that the UDP concentration present in the

polymerization assay has to reach a certain concentration limit which seems to be above 4 mM in order to be an inhibitor of PmHS2 activity.

To summarize, we observed that purified PmHS2 is able to synthesize relatively long heparosan polymers with a molecular mass up to 130 kDa. In the presence of 5 mM of each UDP-sugar a maximal specific activity of 0.145 nmol/µg/min was found. We showed evidence that a change in the initial nucleotide sugar concentrations influenced the final product molecular weight and polydispersity and confirmed that PmHS2 is a non-processive enzyme. Future studies on heparosan synthase will allow us to develop an *in-vitro* production system in which heparosan polymer chain length can be controlled.

Abbreviations

GAG, glycosaminoglycan; UDP, uridine diphosphate; GlcUA, glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; GT, glycosyltransferase; HPSEC, high performance size exclusion chromatography; OD, optical density; PmHS2, *Pasteurella multocida* heparosan synthase 2; HS1, heparosan synthase 1; HAS, hyaluronan synthase

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Chapter 3

Analysis of the polymerization initiation and activity of *Pasteurella multocida* heparosan synthase PmHS2, an enzyme with glycosyltransferase and UDP-sugar hydrolase activity

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Abstract

Heparosan synthase catalyzes the polymerization of heparosan (-4GlcUAβ1-4GlcNAcα1-) 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.

Introduction

Due to its extensive use in the medical area, the world market of heparin is yearly increasing with about 15%. In 2009, it represented a turnover of approximately 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 (Rabenstein 2002). 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 patient suffering from cancer (Lazo-Langner, Goss et al. 2007). However, due to the risk of hemorrhagic complications heparin cannot be used as an anticancer therapeutic agent, and thus analog 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 (Casu, Vlodavsky et al. 2007). 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 un-epimerized precursor of heparin, is constituted of the repeating disaccharide unit (-4GlcUAβ1-4GlcNAcα1-). In nature, apart from vertebrates, heparosan 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 and PmHS2 (the cryptic homolog of PmHS1), have been characterized and both recombinant enzymes exhibit different polymerization properties (DeAngelis and White 2002; DeAngelis and White 2004; Sismey-Ragatz, Green et al. 2007). PmHS2 is a non-processive glycosyltransferase containing a glucuronyl transferase ($GlcUA^{+}$) and a *N*-acetylglucosaminyl transferase ($GlcNAc^{+}$) activity. Each of the catalytic transferase domain contains a D*X*D amino acid motif, considered to be the key residue involved in the substrate binding and the catalysis (Charnock and Davies 1999; Pedersen, Tsuchida et al. 2000). The inactivation of the catalytic domain by substitution of both aspartic acids (D) by asparagines (N) has been described for *P. multocida* hyaluronan synthase PmHAS (Jing and DeAngelis 2000; Jing and DeAngelis 2003) and for PmHS1 (Kane, White et al. 2006). Here we describe, based on amino acid sequence homology with PmHS1 and with *Escherichia coli* heparosan

synthase KfiC and KfiA (Griffiths, Cook et al. 1998; Hodson, Griffiths et al. 2000; Chen, Bridges et al. 2006), 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 on 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 D*X*D amino acid motif of each PmHS2 transferase domain. The positions of the point mutations in *pm*hssB (GenBank acc. No. AY292200) were based on amino acid homology with PmHS1 (Kane, White et al. 2006) and *E. coli* KfiC and KfiA (Griffiths, Cook et al. 1998; Hodson, Griffiths et al. 2000; Chen, Bridges et al. 2006). The primers forward FW_{GlcUA} 5′-CTTTCAAAATAGTAATGATGTATGTCATCATG-3′ and reverse RV_{GlcUA} 5′-CATGATGACATACATCATTACTATTTTGAAAG-3′ were designed to allow the substitution D215N/D217N in the glucuronyl transferase domain leading to PmHS2-GlcNAc$^+$. To obtain the PmHS2-GlcUA$^+$, the primers forward FW_{GlcNAc} 5′-CCTGTAATGATAACATTATCTATCCAAGCG-3′ and reverse RV_{GlcNAc} 5′-CGCTTGGATAGATAATGTTATCATTACAGG-3′ enabled the substitution D479N/D481N in the *N*-acetylglucosaminyl transferase domain. The double transferase knock out (PmHS2-nul) was obtained by using as DNA template *pm*hs2-GlcUA$^+$ and the primer set FW_{GlcUA}/RV_{GlcUA}.

To introduce the nucleotide modification into the *pm*hssB gene, two PCR reactions of 30 cycles (94°C/30 sec, 60°C/30 sec, 72°C/1 min) amplified *pm*hssB 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/30 sec, 65°C/30 sec, 72°C/2 min). Finally the modified gene was amplified by 30 cycles of PCR (94°C/30 sec, 60°C/30 sec, 72°C/2 min) and for each gene: *pm*hs2-GlcUA$^+$, *pm*hs2-GlcNAc$^+$ and *pm*hs2-nul, the full length was obtained using the primer set FW_{HS2} 5′-CGTAGGATCCATGAAGGGAAAAAAAGAGATG-3′ and RV_{HS2} 5′-GCATGAGCTCTAAAAAATAAAAAGGTAAACAGG-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 D*X*D motifs.

Table 1. Primer combinations for the site directed mutagenesis of *P. multocida pm*hssB (*pm*hs2). The amino acid modifications occurred at the D*X*D motif; (-) and (+) stand for unmodified and modified D*X*D motif, respectively.

Gene/Protein	D215N/ D217N	D479N/ D481N	Primer combinations
***pm*hs2/PmHS2**	-	-	FW_{HS2}-RV_{HS2}
***pm*hs2-GlcUA$^+$/PmHS2-GlcUA$^+$**	-	+	FW_{HS2}-RV_{GlcNAc} / FW_{GlcNAc}-RV_{HS2}
***pm*hs2-GlcNAc$^+$/PmHS2-GlcNAc$^+$**	+	-	FW_{HS2}-RV_{GlcUA} / FW_{GlcUA}-RV_{HS2}
***pm*hs2-nul/PmHS2-nul**	+	+	[a] FW_{HS2}-RV_{GlcUA} / FW_{GlcUA}-RV_{HS2}

a. DNA template = *pm*hs2-GlcUA$^+$

Expression and purification of the PmHS2 recombinant proteins

The expression and the purification of the recombinant proteins: PmHS2-GlcUA$^+$, PmHS2-GlcNAc$^+$, PmHS2-nul, was done in the same way as previously described for PmHS2 (Chapter 2).

Determination of PmHS2 enzyme activities

For the standard polymerization conditions, the polymerization reaction with a final volume between 30 µl and 600 µl contained 40 mM Tris-HCl pH 7.15, 4 mM $MnCl_2$, 4 mM $MgCl_2$, UDP-GlcUA, UDP-GlcNAc and freshly purified recombinant PmHS2 enzymes (PmHS2, PmHS2-nul, PmHS2-GlcUA$^+$, PmHS2-GlcNAc$^+$). The amount of UDP-sugar and enzymes varied with the experiments; details are mentioned in the legend to the figures. 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; PDI = 1.17) were synthesized using the standard polymerization condition. The polymerization reaction was quenched by heating (99°C/30 min), and subsequently was centrifuged (18000x*g*/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-ionisation 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 (Gosselin, Alhussaini et al. 1994; Krupa, Shaya et al. 2007). The assay was performed in the same way as previously described (Chapter 2).

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% (w/v) agarose gel and the gels were run for 2 h at 50 V in Tris-acetate-EDTA (TAE) buffer, and then were stained overnight in the dark in a ethanol/Stains-All buffer. The destaining was carried out in pure water as we previously described.

In addition, Novex 20% Tris Borate-EDTA (TBE) polyacrylamide gels (Invitrogen) were used depending on the experiment. The gels were run for 45 min at 200 V in Tris-Boric acid-EDTA (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 (Tris 45 mM pH 9.2, formamide 7.5% (v/v), isopropanol 25% (v/v)). The TBE gels were destained in pure water for 30 min in the dark.

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

High performance anion exchange chromatography (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 x 250 mm) in combination with a CarboPac PA guard column (2 x 25 mm)

and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA). The standards were purchased from Sigma, unless indicated, UMP (uridine monophosphate disodium salt Mw 368.15), UDP-GlcUA (UDP-glucuronic acid trisodium salt Mw 646.2), UDP-GlcNAc (UDP-*N*-acetylglucosamine sodium salt Mw 651.3), GlcUA (D-glucuronic acid Mw 194.14), GlcNAc (*N*-acetyl-D-glucosamine Mw 221.21. Merck), and UDP (uridine diphosphate disodium salt hydrate Mw 448.18 + aq. 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 min, 44 min 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, Sunnyvale, USA) 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 hereafter 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 min, 21 min, 24 min, 27 min, and 34 min, respectively.

High performance size exclusion chromatography (HPSEC) analysis

The size distribution and the molecular weight of the polymers were analyzed by HPSEC on an Ultimate 3000 (Dionex, Sunnyvale, USA) using three TosoH Bioscience TSK-gel columns in series (4000, 3000, and 2500 Super AW, 150 x 6.0 mm) preceded by a TSK AW-L guard column (35 x 4.6 mm;TosoH Bioscience, Japan). Samples (20 μL) were injected and eluted at 40^{o}C using 0.6 ml/min 0.2 M $NaNO_3$. All samples were injected and analyzed in duplicate. Detection was performed using a Shodex RI 101 refractive index detector (Showa Denko K.K., Japan). Selected hyaluronan molecular mass markers (Hyalose) of respectively 30 kDa, 160 kDa and 262 kDa were used for calibration. For each HPSEC analysis, the molecular weight (M_i) and the size distribution (polydispersity index, PDI) of the samples were determined by fitting the elution time of the hyaluronan molecular weight markers to a logarithmic model (Chapter 2).

Matrix assisted laser desorption-tie-of-flight mass spectrometry (MALDI-TOF MS) analysis

An Ultraflex workstation (Bruker Daltonics, Germany) 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 mixure was transferred to a MALDI-sample plate and dried under a stream of warm air.

Results

The D*X*D 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 (Charnock and Davies 1999; Pedersen, Tsuchida et al. 2000). The substitution of both aspartic acids (D) by asparagines (N) in the D*X*D 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 heparosan 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 enable us to understand the initiation of the heparosan 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 (Chapter 2), while 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 heparosan 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 D*X*D motif inactivated only the modified transferase domain but maintained the function of the other transferase.

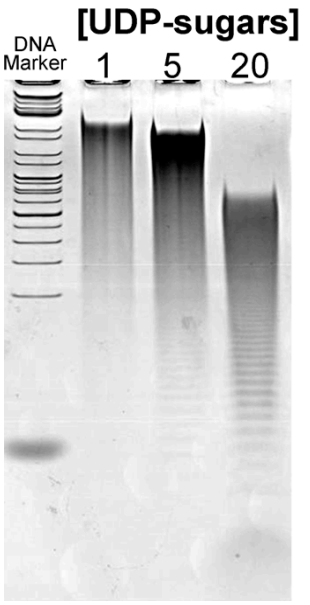

Figure 1. Influence of the UDP-sugar concentration on the polymerization process of the combined PmHS2 single action transferases. PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ (1/1) (45 μg/ml of each) were incubated together for 24 h in the presence of 1 mM, 5 mM and 20 mM of each UDP-sugar, respectively. The chain length and size distribution of heparosan polymer is assessed with a Novex 20% TBE gel.

Just as previously observed with PmHS2 (Chapter 2), PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ favored the formation of high molecular weight heparosan polymers with a low polydispersity in the presence of low UDP-sugar concentrations. The opposite was observed in the presence of high UDP-sugar concentrations. The specific activity of a 1/1 mixture of PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ 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-GlcUA$^+$/PmHS2-GlcNAc$^+$, measured in the period from 100 to 500 min, was in average 0.06 nmol/μg/min (nmol UDP-sugar converted/μg protein/min) and was 2-fold lower than the PmHS2 specific activity which was about 0.12 nmol/μg/min.

Influence of the incubation temperature on the polymerization activity

A rise in the incubation temperature from 30°C to 38-40°C resulted, after 4 h of incubation, in a 5-fold increase of the PmHS2 polymerization activity based on the UDP-sugar conversion (Fig. 2). Agarose gel analysis of the polymerization product showed that the incubation temperature did not influence the overall molecular weight distribution of heparosan polymers. Independently of the incubation temperature, within the range from 30°C to 39°C, the polymerization activity leveled off when about 450 nmol of UDP-sugar had been converted. The absence of polymerization activity at incubation temperatures above 47°C suggests the inactivation of PmHS2.

The polymerization activity of the single action transferases (PmHS2-GlcUA^{+}/PmHS2-GlcNAc^{+}) increased by 1.6-fold when the incubation temperature was raised from 30°C to 35°C. Complete inactivation of the enzymes was observed at 40°C and at higher temperatures.

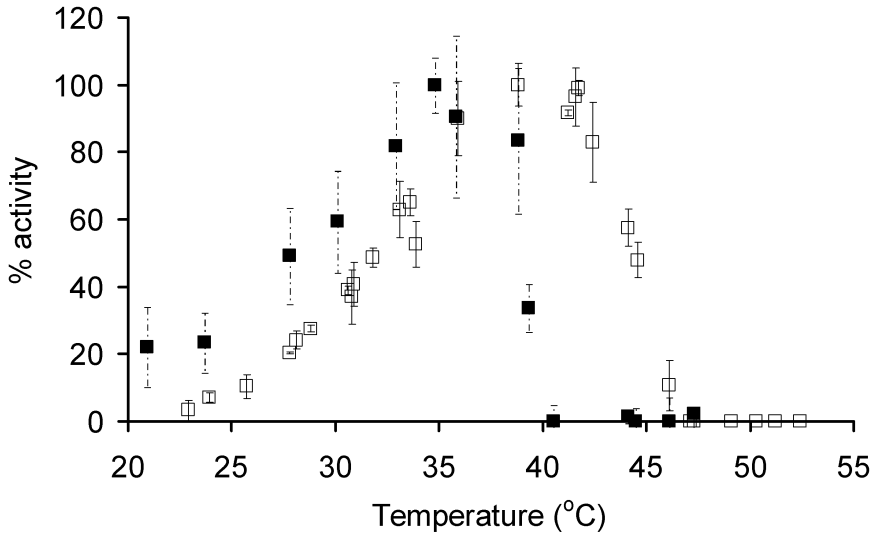

Figure 2. Influence of the incubation temperature on the polymerization activity of PmHS2 and of the combined PmHS2-GlcUA^{+}/PmHS2-GlcNAc^{+}. The activity of PmHS2 (□) or the single action transferases incubated together (■) was determined in the presence of 5 mM of each UDP-sugar. The UDP-sugar conversion after 4 h of incubation at temperatures from 22°C to 49°C was assayed by the coupled enzyme assay, 100% activity corresponds to the highest UDP-sugar conversion observed on the temperature range. The maximal conversion was 54.0 ±3.5 nmol/ μg (□) for PmHS2 and 8.0 ±0.7 nmol/ μg (■) for PmHS2-GlcUA^{+}/PmHS2-GlcNAc^{+}.

Hydrolysis activity of PmHS2

The reaction mixtures of the PmHS2 enzymes (PmHS2, PmHS2-nul, PmHS2-GlcUA$^+$ or PmHS2-GlcNAc$^+$) incubated for 24 h in the presence of both UDP-sugars or in the presence of only one UDP-sugar were analyzed by HPAEC.

The HPAEC analysis (condition 2) of PmHS2 incubated with UDP-GlcUA, showed the presence of free glucuronic acid (electrochemical detection) and the presence of free UDP (UV detection). When PmHS2 was incubated in the presence of UDP-GlcNAc, free *N*-acetylglucosamine and free UDP were also observed. Analysis of the reaction mixtures by the coupled enzyme assay also revealed the presence of UDP. Reaction mixtures incubated 24 h in the presence of inactivated enzymes (99°C/15 min) and UDP-sugars were also analyzed by HPAEC analysis (condition 2) and by the coupled enzyme assay. The UDP-sugars (UDP-GlcUA and UDP-GlcNAc) degradation was 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 mM to 0.05 mM of UDP-GlcNAc were converted into *N*-acetylglucosamine and UDP. The conversion represents about 12 to 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 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, 0.15 mM to 0.20 mM UDP-GlcUA was converted which represents about 60 to 80% of the initial concentration (0.25 mM). 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 h and 24 h of incubation and represented around 5% of the initial concentration.

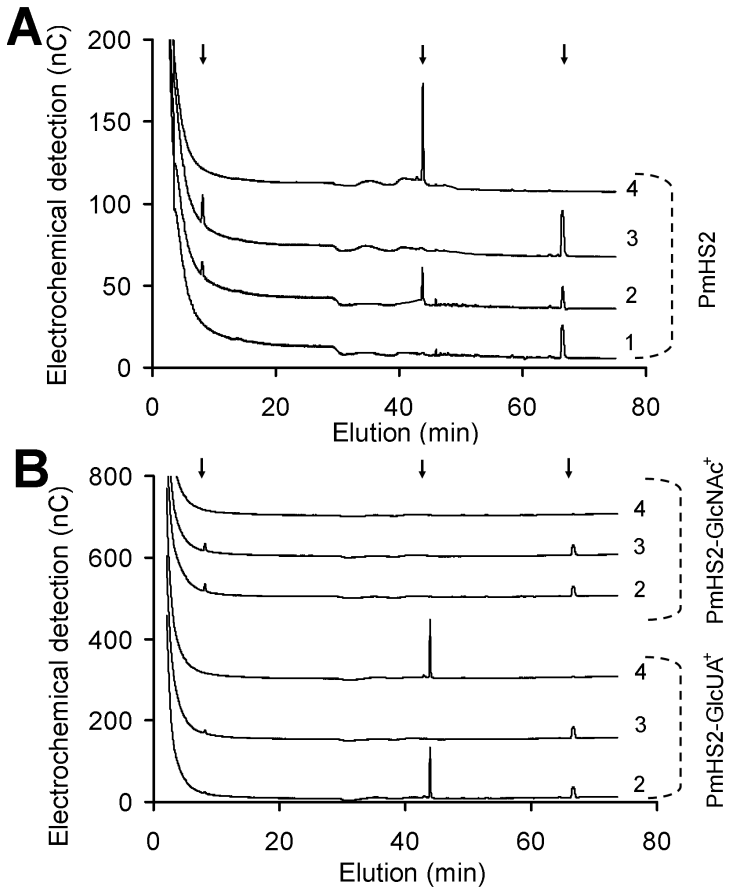

Figure 3. High performance anion exchange chromatography to assess PmHS2-enzymes hydrolysis activity. A). Purified PmHS2 (55-60 μg/ml) and B). Purified single action transferases (80-90 μg/ml) (PmHS2-GlcUA$^+$ or PmHS2-GlcNAc$^+$) were incubated in the presence of both UDP-sugars or with only one UDP-sugar (0.25 mM). Each reaction mixture was analyzed by HPAEC (condition 1) after 24 h of incubation. Sample [1] is the inactivated PmHS2 (99°C/15 min) incubated in the presence of UDP-GlcNAc/UDP-GlcUA. The samples [2, 3, 4] are the reaction mixtures of active PmHS2 enzymes (PmHS2, PmHS2-GlcUA$^+$, or PmHS2-GlcNAc$^+$) incubated with: [2] UDP-GlcNAc/UDP-GlcUA, [3] UDP-GlcNAc, and [4] UDP-GlcUA, respectively. The monosaccharides GlcNAc, GlcUA, and the UDP-GlcNAc eluted after 8 min, 44 min and 67 min, respectively (arrows).

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$^+$ and PmHS2-GlcNAc$^+$ were incubated together. After 24 h of incubation in the presence of 0.5 mM of each UDP-sugar, the reaction mixtures of PmHS2-GlcUA$^+$ (glucuronyl transferase) incubated alone, and as well PmHS2-GlcNAc$^+$ (N-acetylglucosaminyl transferase) 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).

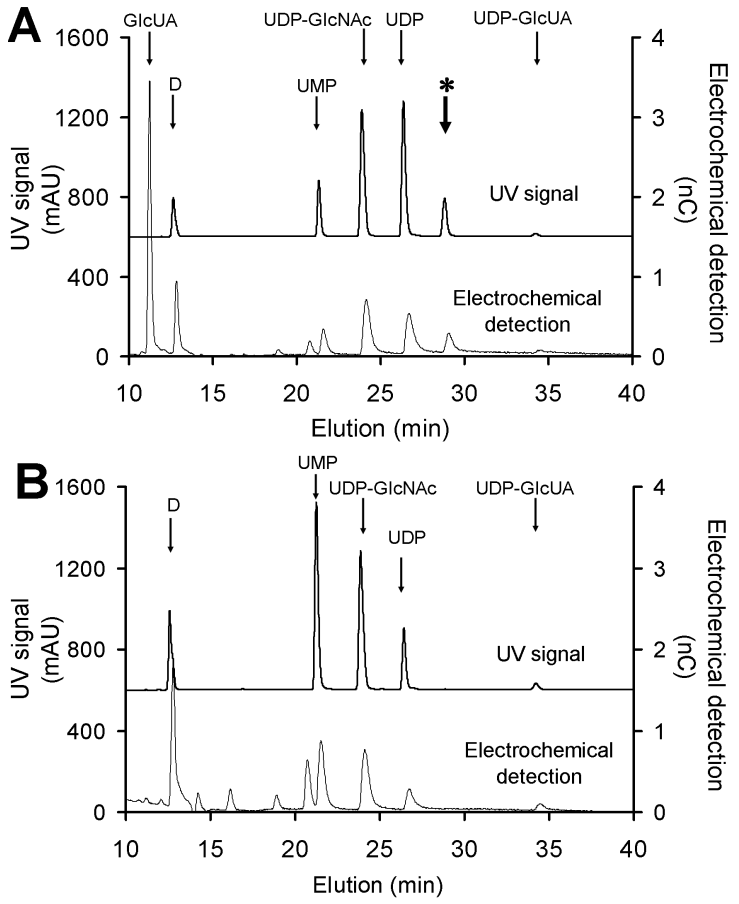

Figure 4. High performance anion exchange chromatography analysis of the reaction mixture of each single action transferase (PmHS2-GlcUA$^+$ or PmHS2-GlcNAc$^+$) using electrochemical and UV detection (HPAEC condition 2). A). PmHS2-GlcUA$^+$ and B). PmHS2-GlcNAc$^+$ (80-90 μg/ml) were incubated for 24 h in the presence of 1 mM UDP-GlcUA and 1 mM UDP-GlcNAc. In the presence of PmHS2-GlcUA$^+$ (A), an additional product [*] eluted around 29 min; the product was detected by both electrochemical and UV detection. Using HPAEC condition 2, the degradation of UDP-GlcUA was observed and it resulted in the formation of UMP and a degradation product [D].

In addition, the reaction mixtures of both the PmHS2-GlcUA$^+$ and the PmHS2-GlcNAc$^+$ were analyzed by normal phase thin layer chromatography (TLC) (silica gel 60 F_{254}. Merck) with butyl alcohol/acetic acid/water (1.5:1:1) (Sismey-Ragatz, Green et al. 2007) 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 R_f 0.24 and UDP-GlcNAc R_f 0.30). This additional product (R_f 0.18) was extracted from the TLC, resuspended in pure water and analyzed by MALDI-TOF MS (Fig. 5). It showed a *m/z* of 782 which corresponds to a UDP-disaccharide containing a glucuronic

acid sugar unit and a *N*-acetylglucosamine sugar unit. In addition, the presence of mono- and di- sodium UDP-disaccharides was also observed by mass spectrometry (*m/z* 804 and 826) in the unpurified reaction mixture. Subsequently, the product was re-run 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).

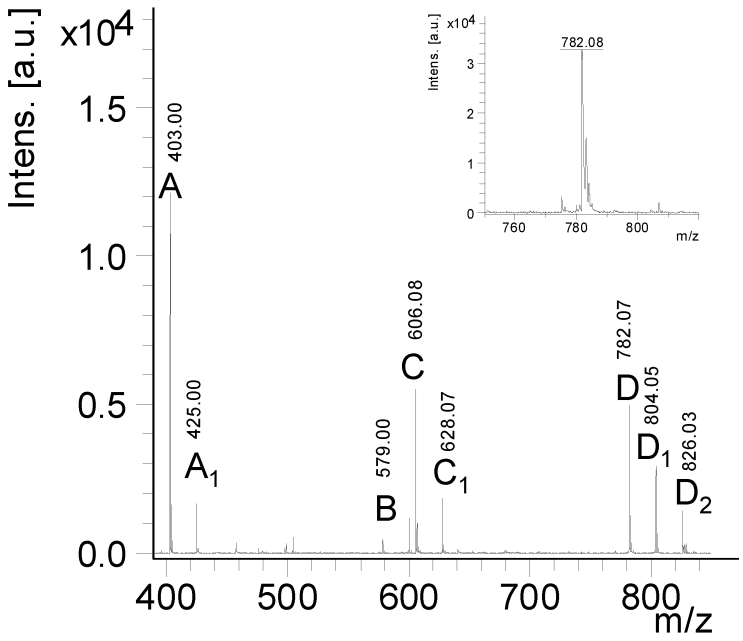

Figure 5. Matrix assisted laser desorption/ionization time-of-flight mass spectrum of the PmHS2-GlcUA$^+$ unpurified reaction mixture with a zoom view from 390-850 *m/z*. PmHS2-GlcUA$^+$ reaction mixture was incubated for 24 h in the presence of both UDP-sugars. The reaction mixture is composed of UDP [A], UDP-GlcUA [B], UDP-GlcNAc [C] and the synthesized UDP-disaccharide [D]. For each of the compound: [X] is [compound X - H], [X1] is [sodium compound X - H] and [X2] is [disodium compound X - H]. In the insert is presented the MALDI-TOF mass spectrum of the UDP-disaccharide isolated from the TLC analysis, with a zoom view from 750-850 *m/z*.

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-sugars concentration. The influence of non-equimolar UDP-sugar concentrations on the heparosan chain length elongation and its size distribution was investigated (Table. 2).

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). Estimated polydispersity index (PDI) is calculated as (Mw/Mn in which Mw, average molecular mass (kDa) and Mn, number average molecular weight (kDa).

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

In agreement with what has been previously observed in the absence of heparosan template (Chapter 2), PmHS2 polymerized longer heparosan chains when incubated with low UDP-sugar concentrations. Heparosan chain with an average molecular mass of 135 kDa and 65 kDa were synthesized in the presence of 0.25 mM of each UDP-sugar or 5 mM, respectively after 24 h of incubation. Differences in heparosan polymers 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 while 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 from 0.25 mM to 20 mM of 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 mM 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. 6). In contrast, with an excess of UDP-GlcUA (5 mM) and a limiting concentration of

UDP-GlcNAc (0.1 mM to 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.

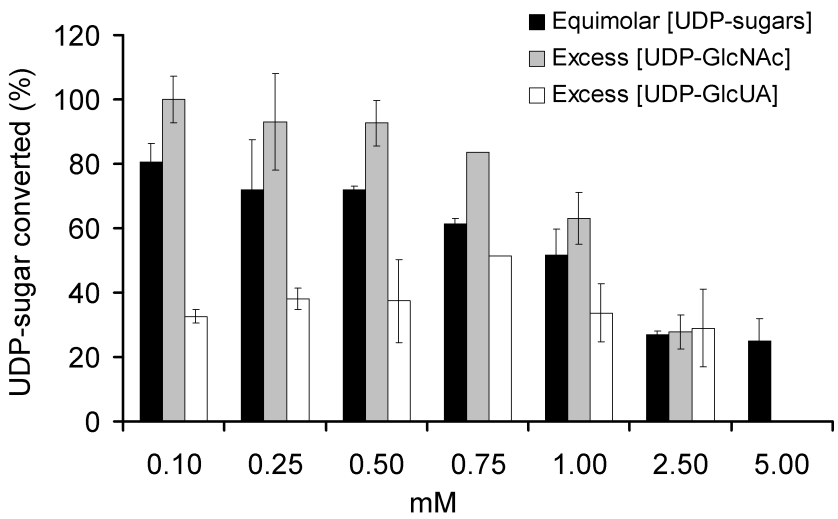

Figure 6. Conversion of UDP-sugars by PmHS2 in the presence of equimolar and non-equimolar UDP-sugar concentrations. The polymerization activity after 3 h of incubation was assayed by the coupled enzyme assay and expressed in % of UDP-sugar converted (100% corresponds to the limiting UDP-sugar concentration present at time zero). The UDP-sugar in excess was added to a 5 mM concentration and the limiting UDP-sugar to a concentration from 0.10 to 2.50 mM.

To confirm the fact that UDP-GlcNAc favors the heparosan chain initiation, PmHS2 polymerization process was investigated in the presence of heparosan template (35 kDa) (Fig. 7). The HPSEC analysis of the PmHS2 reaction mixture incubated for 3 h in the presence of heparosan template and 5 mM of each UDP-sugar revealed the presence of two products (25 kDa and 65 kDa). It was previously reported that PmHS2 initiates and elongates simultaneously heparosan polymers (Chapter 2), 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 mass of the two polymer groups was less visible; it resulted in an increase of the overall polydispersity. In the reaction mixture of PmHS2 incubated during 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 kDa 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 chains simultaneously. When using a limiting concentration of UDP-GlcNAc, PmHS2 cannot initiate new heparosan chains but can only elongate the templates.

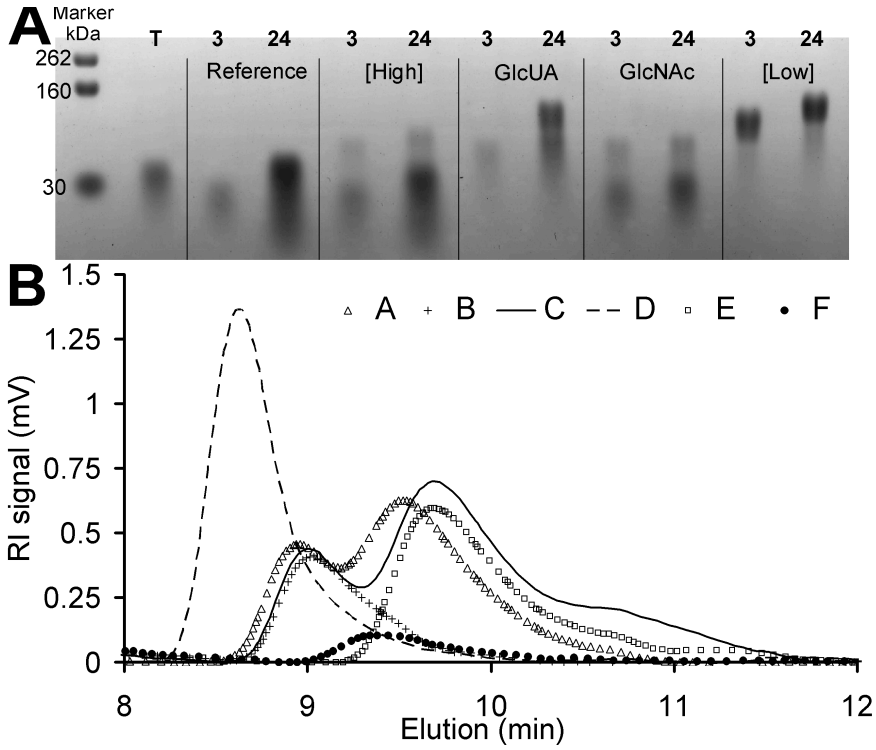

Figure 7. PmHS2 polymerization process in the presence of "heparosan template". Heparosan template of 35 kDa (PDI=1.17) was added to the PmHS2 (55-60 μg/ml) polymerization reaction in the presence of equimolar and non-equimolar UDP-sugar concentrations (0.25 mM and 5 mM). A). 2% agarose gel electrophoresis analysis of reaction mixtures after 3 h and 24 h of incubation. [T] heparosan template (10-fold concentrated), [Reference] polymerization reaction in the absence of template and with 5 mM of each UDP-sugar. For the polymerization reactions incubated in the presence of template, sample [GlcUA] was obtained with an excess of UDP-GlcUA, [GlcNAc] with an excess of UDP-GlcNAc, [High] was obtained with a high concentration (5 mM) and [Low] with a low concentration (0.25 mM) of each UDP-sugar, respectively. B). The corresponding reaction mixtures (3 h of incubation) were also analyzed by HPSEC. The reaction mixture incubated with template and an excess of UDP-GlcNAc [A] (30 kDa and 75 kDa), an excess of UDP-GlcUA [B] (65 kDa), and with 5 mM [C] (25 kDa and 65 kDa) or 0.25 mM [D] (130 kDa) of each UDP-sugar, respectively. In addition, the reaction mixture incubated in the absence of template [E] and 1-fold concentrated template [F] were also analyzed by HPSEC.

Discussion

By applying site directed mutagenesis on the D*X*D amino acid motifs of PmHS2, single action transferases (PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$) were successfully obtained. Just as observed with PmHS2 (Sismey-Ragatz, Green et al. 2007) (Chapter 2), PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ incubated together, in the absence of heparosan templates, are able to synthesize heparosan polymers. In addition, the PmHS2 single action transferases synthesize, in the presence of low UDP-sugar concentrations, high molecular weight heparosan polymers and thus depict the same polymerization behavior as PmHS2 (Chapter 2). During the incubation period from 2 h to 8 h, the specific activity of the single action transferases was around 0.06 nmol UDP-sugar converted/µg protein/min whereas the specific activity of PmHS2 was around 0.12 nmol UDP-sugar converted/µg protein/min. Nevertheless, despite their slower activity it cannot be concluded that PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ are less active than PmHS2. Indeed Williams *et al*., (2006) reported for PmHAS that each transferase domain might exhibit different specific activity (Williams, Halkes et al. 2006). In order to be able to compare the polymerization activity of the single action transferases with PmHS2, it is needed to estimate the activity of each transferase catalytic domain separately. Moreover, the specific activity should be expressed in nmol UDP-sugar converted/mole of active transferase/min as 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°C to 40°C resulted in a 5-fold increase of PmHS2 polymerization activity. The level-off of PmHS2 polymerization activity observed after the conversion of about 4.5 mM of UDP-sugar, is independent of the incubation temperature within the range from 30°C to 39°C. The level off is probably due to UDP inhibition as we have observed previously that PmHS2 polymerization activity was inhibited by UDP concentration above 4 mM (Chapter 2).

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.

Sugiura *et al.,* (2007) reported that in the absence of enzyme, the metallic ion Mn^{2+} participate in the hydrolysis of UDP-GlcUA at the pyrophosphate bonds resulting in the production of UMP and a monosaccharide unit (Sugiura, Shimokata et al. 2007). Here, we also observed in

the absence of PmHS2 the hydrolysis of UDP-GlcUA into UMP 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 $MnCl_2$ and therefore prevents the role of Mn^{2+} 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*-acetylglucosaminyl transferase catalytic domain. Kane *et al*., (2006) reported for PmHS1 that the transferase catalytic domains were not completely inactivated by the amino acid substitution of the D*X*D motif. They observed that PmHS1 single action transferase mutants conserved about 1 to 2% of relative specific activity for the mutated transferase domain (Kane, White et al. 2006). 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 to 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 literature and their catalytic mechanisms show similarities at the exception of the acceptor molecules; being a water molecule with the glycoside hydrolases (Perugino, Trincone et al. 2004; Hancock, Vaughan et al. 2006; Vetting, Frantom et al. 2008). 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 as a base catalyst. Based on the similarities, the glycosidic 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 (Zhang, Wang et al. 2001) and the glucosyltransferases Toxin A and Toxin B (Ciesla and Bobak 1998). 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 glucuronyl transferase catalytic domain is more sensitive to the hydrolytic attack than the *N*-acetylglucosaminyl transferase 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 glycosylsynthases were capable to synthesize new and valuable compounds for therapeutic applications (Shaikh and Withers 2008), and that versatile glycosyltransferases were found to exhibit additional

activity such being able to synthesize rare nucleotide activated sugars (Perugino, Trincone et al. 2004; Zhang, Griffith et al. 2006).

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 (Orellana and Mohnen 1999). Therefore, the presence of a larger peak with UV detection, as with electrochemical detection shows the presence of an 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 a *m/z* of 782, corresponding to a UDP moiety containing a glucuronic acid and a N-acetyl glucosamine 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 (DeAngelis and White 2004; Sismey-Ragatz, Green et al. 2007). As a consequence the UDP presents 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, Talpak *et al*., (2005) reported that PmHAS catalyzed the formation of the GlcUA-GlcNAc-UDP disaccharide, but the disaccharide GlcNAc-GlcUA-UDP was not observed (Tlapak-Simmons, Baron et al. 2005).

Therefore, it would be interesting to determine if these *P. multocida* glycosyltransferases are only capable to initiate polymer chains by using one of the two UDP-sugars 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 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. While 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 while in the absence of oligosaccharide template more polydisperse polymers were formed (Jing and DeAngelis 2004; Sismey-Ragatz, Green et al. 2007). Williams *et al*., (2006) reported that for PmHAS the binding affinity at the acceptor binding site is higher for short oligosaccharides than for monosaccharides (Williams, Halkes et al. 2006). Therefore, in the presence of oligosaccharide templates the polymerization reaction is more efficient as 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^{+}) and 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.

Abbreviations

UDP, uridine diphosphate; UDP-GlcUA, UDP-glucuronic acid; UDP-GlcNAc, UDP-N-acetylglucosamine; PmHS2, *Pasteurella multocida* heparosan synthase 2, HS1, heparosan synthase 1; HAS, hyaluronan synthase; HPAEC, High performance anion exchange chromatography; HPSEC, High performance size exclusion chromatography; MALDI-TOF MS, Matrix-assisted laser desorption-ionisation time of flight mass spectrometry; TLC, Thin layer chromatography; PmHS2-nul, PmHS2 double knock out transferase; PmHS2-GlcUA^{+}, PmHS2 glucuronyl transferase; PmHS2-GlcNAc^{+}, PmHS2 *N*-acetylglucosaminyl transferase.

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Chapter 4

Controlled synthesis of heparosan oligosaccharides by *Pasteurella multocida* PmHS2 single action transferases

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Abstract

Pasteurella multocida heparosan synthase PmHS2 is a dual action glycosyltransferase that catalyzes the polymerization of heparosan polymers in a non-processive manner. The two PmHS2 single action transferases, obtained previously by site-directed mutagenesis have been immobilized on Ni(II)-nitrilotriacetic acid (Ni-NTA) agarose during the purification step. A detailed study of the polymerization process in the presence of non-equimolar amounts of PmHS2 single action transferases revealed that the glucuronyl transferase (PmHS2-GlcUA^{+}) is the limiting catalytic step of the PmHS2 polymerization process. Using experimental design it was determined that the *N*-acetylglucosaminyl transferase (PmHS2-GlcNAc^{+}) plays an important role in the control of heparosan elongation depending on the number of heparosan chains and the UDP-sugar concentrations present in the reaction mixture.

Furthermore, for the first time, the controlled synthesis of heparosan oligosaccharides using alternately immobilized PmHS2 single action transferases is reported. It is shown that heparosan oligosaccharides are synthesized by PmHS2-GlcUA^{+} and PmHS2-GlcNAc^{+} in the absence of template molecules in the reaction mixture.

Introduction

Heparin (Hep) and heparan sulfate (HS) are analog molecules belonging to the glycosaminoglycan (GAG) family. Hep/HS polymers are ubiquitous in mammalian cells and are involved in many physiological processes (Rabenstein 2002; Linhardt and Toida 2004). Heparin is a well known pharmaceutical compound that is used in large amounts in surgery for its anticoagulant properties. Heparan sulfate, an analog of heparin, is not used yet for therapeutic purposes, but it is considered to have a large potential for medical applications (Rabenstein 2002; Lindahl 2007). Currently, the pharmaceutically grade heparin anticoagulant products are mainly obtained from animal tissues, and some are chemically synthesized. Since these systems do not enable the production of a large range of well defined Hep/HS oligomers and polymers, alternative processes to control Hep/HS synthesis are of interest (Laremore, Zhang et al. 2009).

Heparosan is the unsulfated precursor of heparin and heparan sulfate in mammalians. In the pathogenic bacteria *Escherichia coli* K5 (Vann, Schmidt et al. 1981), *Pasteurella multocida* Type D (Pandit and Smith 1993; Rimler 1994), and *Avibacterium paragallinarum* genotype II (Wu, Chen et al. 2010) heparosan is a component of the polysaccharide capsule. Heparosan can be produced either by extracting it from microorganisms or by using recombinant enzymes *in vitro*. Large scale fermentation to produce heparosan has been only reported for *E. coli* K5 and enabled the recovery of 15 g/L of heparosan (Wang, Ly et al. 2010). Despite the fact that this production strategy is cost effective, neither the heparosan chain length nor the incorporation of modified and analog sugar residues can be controlled.

Heparosan can also be synthesized *in vitro* using recombinant heparosan synthases. Heparosan synthases from mammalians (EXT1 and EXT2) (McCormick, Duncan et al. 2000; Kim, Kitagawa et al. 2003), drosophila (TTV, SOTV, and BOTV) (Bellaiche, The et al. 1998; Izumikawa, Egusa et al. 2006), *E. coli* K5 (KfiA and KfiC) (Griffiths, Cook et al. 1998; Hodson, Griffiths et al. 2000; Sugiura, Baba et al. 2010), and *P. multocida* Type D (PmHS1 or PmHS2) (Sismey-Ragatz, Green et al. 2007) (Chapter 2) have been characterized. The EXT (EXT1 and EXT2) and Kfi (KfiA and KfiC) heparosan synthases are mono-action transferases that catalyze only one transferase activity. In addition, they need to form a complex to exhibit polymerization activity. In contrast, PmHS heparosan synthases (PmHS1 and PmHS2) are both dual action glycosyltransferases exhibiting a glucuronyl transferase and a *N*-acetylglucosaminyl transferase activity. PmHS single action transferase enzymes (PmHS-GlcUA$^+$ and PmHS-GlcNAc$^+$) were obtained by applying site-directed mutagenesis on the D*X*D motif of PmHS1 (Kane, White et al. 2006) or PmHS2 (Chapter 3). The PmHS2-GlcUA$^+$

and PmHS2-GlcNAc$^+$ incubated together in the absence of templates polymerized heparosan chains (Chapter 3).

There is a close relationship between the Hep/HS structures and their biological activity. Thus, during the synthesis of heparin and heparan sulfate it is important to control the heparosan polymerization since it determines the chain length and size distribution of the Hep/HS polymers. Recently, the controlled synthesis of heparosan-like oligosaccharides has been reported by applying alternately the activity of the glycosyltransferases KfiA (*N*-acetyl-D-glucosaminyl transferase) and PmHS2 (*N*-acetylglucosaminyl transferase and glucuronyl transferase) in the presence of UDP-sugars and a disaccharide acceptor (GlcUA-AnMannose) (Liu, Xu et al. 2010). Due to the dual action of PmHS2, the synthesis had to be carefully monitored. The controlled synthesis of glycosaminoglycan hyaluronan and chondroitin oligosaccharides has also been reported. Immobilized *P. multocida* hyaluronan synthase PmHAS single action transferases (DeAngelis, Oatman et al. 2003) and immobilized *E. coli* chondroitin polymerase K4CP single action polymerases (Sugiura, Shimokata et al. 2008) synthesized monodisperse 20-mer hyaluronan chains and 16-mer chondroitin chains from short oligosaccharide templates, respectively.

Here we report the use of an immobilized-metal affinity chromatography (IMAC) technique for the immobilization of PmHS2 single action transferases (PmHS2-GlcNAc$^+$ and PmHS2-GlcUA$^+$). PmHS2 enzymes immobilized with Ni(II)-nitriloacetic acid (Ni-NTA) agarose were characterized for their polymerization activity, stability, and ability to elongate step by step heparosan oligosaccharides. We show that PmHS2 single action transferases can be used to control the synthesis of heparosan oligosaccharides.

Experimental Procedure

Protein expression, purification, and immobilization

The genes *pm*hs2-GlcUA$^+$, and *pm*hs2-GlcNAc$^+$, were ligated into pET101 and transformed in *E. coli* BL21*(DE3) (Invitrogen) (Chapter 3). The recombinant proteins PmHS2-GlcUA$^+$, and PmHS2-GlcNAc$^+$ were expressed with a V5-epitope and a polyhistidine tag (His-Tag) at their *C*-terminus.

The protein expression and the protein recovery procedures were performed as previously described (Chapter 2 and 3). The soluble fraction (4 ml) obtained from 100 ml of induced *E. coli* culture was added to 1 ml of agarose Ni-NTA slurry (50% agarose/50% buffer) (Qiagen).

In order to immobilize PmHS2-GlcUA^{+} and PmHS2-GlcNAc^{+} on Ni-NTA agarose, the His-Tag purification was slightly modified. The elution step (phosphate buffer, 250 mM of imidazole) was omitted and the subsequent desalting step (PD10 column) was removed. Instead, the column was washed and equilibrated 5 times with Tris-HCl (50 mM, pH 7.15), followed by 3 times with Tris-HCl (50 mM, pH 7.15), Mn^{2+}/Mg^{2+} (11 mM/11 mM). Prior to be stored at 4°C or to be used in polymerization assay, 0.50 ml to 1.75 ml of Tris-HCl (50 mM, pH 7.15), Mn^{2+}/Mg^{2+} (4 mM/4 mM) was added to the column.

The amount of protein immobilized onto the Ni-NTA resin was determined by a bicinchoninic acid (BCA) assay (Pierce) using bovine serum albumin (BSA) as the standard. The efficiency of the protein binding during the washing steps and the storage was evaluated by SDS-PAGE analysis (Chapter 2).

Polymerization assay

Standard polymerization conditions

The polymerization reaction with a final volume between 30 µl and 200 µl contained 40 mM Tris-HCl (pH 7.15), 4 mM $MnCl_2$, 4 mM $MgCl_2$, UDP-GlcUA, UDP-GlcNAc, and freshly PD10-purified or immobilized recombinant PmHS2 single action transferases. The amount of UDP-sugar and enzymes varied with the experiments; details are mentioned in the legend of the figures. The reactions were performed in the dark at 30-32°C under mild shaking conditions ranging from 2 h to 24 h.

In order to compare the enzyme stability when stored at 4°C, the same amount of PD10-purified and immobilized single action transferases was resuspended in 3.5 ml buffer. Immobilized PmHS2 single action transferases were stored in the presence of Tris-HCl (50 mM, pH 7.15), Mn^{2+}/Mg^{2+} (4 mM each), while the PD10-purified enzymes were stored in the presence or in the absence of ethylene glycol (1 M) and metal ions (Mn^{2+}/Mg^{2+}, 4 mM each).

Experimental design

To determine the relationship between the ratio of transferases, the UDP-sugar concentrations, and the heparosan size distribution, samples were prepared according to a fractional factorial design of 4 variables with each 3 levels (3^{4-1}). Preliminary experiments were performed to determine the lowest PmHS2 single action transferase and UDP-sugar concentrations needed to obtain activity. From these results, the concentrations for each variable were determined (Table 1).

This experimental design consisted in the preparation of 27 reaction mixtures in which different combinations of UDP-sugars and PmHS2 single action transferase concentrations were added (Table 2). The samples were incubated for 4 h to avoid the complete conversion of the UDP-sugar when low UDP-sugar concentrations were present; each reaction mixture was analyzed for UDP content (coupled enzyme assay) and polymer chain length (high performance size exclusion chromatography).

Table 1. Variables used for the fractional factorial design of 4 variables and 3 levels (3^{4-1}). The variables x_1 and x_2 are the PmHS2 single action transferase concentrations (µg/ml), and x_3 and x_4 are the UDP-sugar concentrations (mM), respectively.

		Variables			
		x_1 PmHS2-GlcNAc$^+$	x_2 PmHS2-GlcUA$^+$	x_3 UDP-GlcUA	x_4 UDP-GlcNAc
Levels	low	7	7	0.5	0.5
	medium	21	21	2.75	2.75
	high	34	34	5	5

To analyze the data it was decided to use the so-called response surface methodology described by Box and Draper (Box and Draper 1987). The analysis of the data was done by performing a linear regression in which 15 parameters (main effects, quadratic effects, and interaction effects) were taken into account. The response variable (y) was modeled using the following equation:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_1^2 + b_6 x_2^2 + b_7 x_3^2 + b_8 x_4^2 + b_9 x_1x_2 + b_{10} x_1x_3 + b_{11} x_1x_4 + b_{12} x_2x_3 + b_{13} x_2x_4 + b_{14} x_3x_4$$

In this case: y is the heparosan molecular weight, b_0 is the intercept, b_1 to b_{14} the regression coefficients and x_1, x_2, x_3, and x_4 are the variables: PmHS2-GlcNAc$^+$, PmHS2-GlcUA$^+$, UDP-GlcUA, and UDP-GlcNAc concentration, respectively. The estimation of the regression coefficients was done by least squares regression.

The calculation of the so-called Mallow's Cp criterion was used to determine the best fit for this model. The Mallow's Cp criterion is calculated by taking into account the fit of the model and giving penalties for each additional parameter used to fit a model to the data. Thus, the best model to represent the data is obtained when the Mallow's Cp is at the lowest value. The visualization of the response is given by a contour plot (Result section). In practice, the response variable can only be visualized as a function of two independent variables.

Therefore, cross-sections at different levels of the two others variables were made. The visualization is done using the mathematical software package Matlab.

Table 2. Variable combinations of the 3 levels experiment plan design. The variables x_1 and x_2 stands for PmHS2-GlcNAc$^+$ and PmHS2-GlcUA$^+$ concentrations (μg/ml) and the variables x_3 and x_4 stands for UDP-GlcUA and UDP-GlcNAc concentrations (mM), respectively. The PmHS2 single action transferases were added to a concentration of 7, 21, and 34 μg/ml; and the UDP-sugars to an end concentration of 0.5, 2.75, and 5 mM.

Sample	x_1 [PmHS2-GlcNAc$^+$]	x_2 [PmHS2-GlcUA$^+$]	x_3 [UDP-GlcUA]	x_4 [UDP-GlcNAc]
1	34	7	0.5	0.5
2	34	21	5	0.5
3	34	21	0.5	5
4	34	34	0.5	2.75
5	21	21	2.75	0.5
6	7	21	2.75	5
7	21	7	0.5	5
8	7	21	5	2.75
9	34	34	2.75	0.5
10	34	7	5	2.75
11	21	34	5	2.75
12	34	7	2.75	5
13	21	7	5	0.5
14	34	34	5	5
15	7	34	0.5	5
16	21	34	0.5	0.5
17	7	7	0.5	2.75
18	21	21	5	5
19	34	21	2.75	2.75
20	7	7	5	5
21	21	21	0.5	2.75
22	7	7	2.75	0.5
23	7	34	5	0.5
24	21	7	2.75	2.75
25	7	34	2.75	2.75
26	7	21	0.5	0.5
27	21	34	2.75	5

Controlled elongation of heparosan oligosaccharides

PmHS2 single action transferases (0.8 ± 0.05 mg/ml agarose) were immobilized, using Ni-NTA resin, on two separate columns: PmHS2-GlcUA$^+$ (column A) and PmHS2-GlcNAc$^+$ (column B) (Fig. 1). Each incubation step was performed during 3 h in the dark under shaking conditions at 32°C. The first incubation step resulting in the synthesis of UDP-heparosan dimers was conducted in column A (Chapter 3). In order to favor the chain initiation, the UDP-sugars were progressively added every 30 min into the column during the 3 h of incubation (final concentration 3.25 mM UDP-GlcUA and 6.50 mM UDP-GlcNAc). At the

end of the incubation the reaction mixture was released of column A, and was heat inactivated (15 min at 65°C) to avoid the possible presence of any residual enzyme activity, before being loaded onto column B. The reaction mixtures were transferred from column A to column B in an alternating manner to elongate heparosan oligosaccharides to a defined polymerization degree; UDP-GlcUA or UDP-GlcNAc were added to column A or B, respectively. In between each incubation cycle, the reaction mixtures were heat inactivated and the columns were washed with 2 x 1 ml of Tris-HCl (40 mM, pH 7.15), 4 mM Mn^{2+}, 4 mM Mg^{2+}.

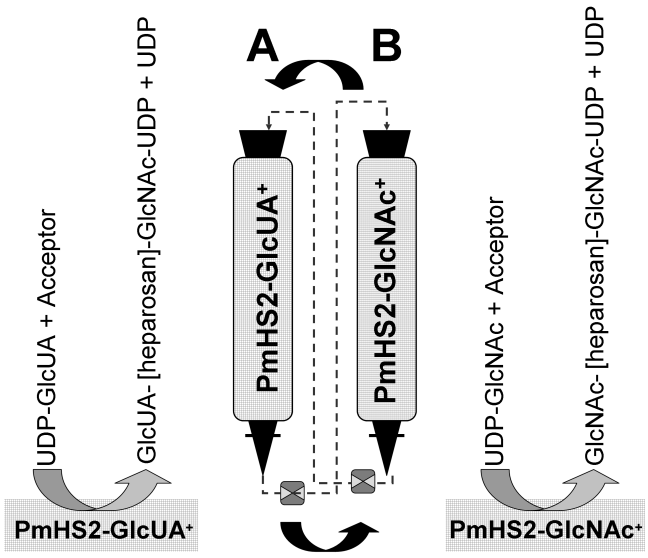

Figure 1. Schematic representation of the procedure for the step by step elongation of heparosan oligosaccharides. The first step is the initiation of the heparosan chain (GlcUA-GlcNAc-UDP) by PmHS2-$GlcUA^+$ (column A) in the presence of both UDP-GlcUA and the acceptor molecule, identified as UDP-GlcNAc (Chapter 3). Prior to be incubated with PmHS2-$GlcNAc^+$ (column B), the reaction mixture was heat treated (65°C/15 min) to prevent residual enzyme activity. Successive incubation cycles of the reaction mixture (UDP-sugars and oligosaccharide acceptors [heparosan]-GlcNAc-UDP) enable to control the synthesis of heparosan oligosaccharides.

Analysis of the polymerization activity

For the analysis of the polymerization activity, the same procedures as described before have been used (Chapter 2).

Coupled enzyme assay

Samples were quenched by immersion in liquid nitrogen and the UDP-sugars conversion was quantified by measuring the NADH reduction into NAD^+ at 340 nm in a coupled enzyme assay (Gosselin, Alhussaini et al. 1994; Krupa, Shaya et al. 2007).

Gel electrophoresis

Heparosan oligomers/polymers synthesized during the enzymatic reactions were analyzed by Novex 20% Tris Borate-EDTA (TBE) polyacrylamide gel (Invitrogen) and 2% (w/v) agarose gel electrophoresis. The sample preparation, the gel electrophoresis parameters, and the staining procedures were done according to Chavaroche *et al*., (Chapter 3). A selected hyaluronan molecular mass marker ranging from 27 kDa to 495 kDa (Select-HALoLadder, Hyalose) was used to estimate the heparosan molecular weight.

High performance size exclusion chromatography (HPSEC) analysis

The size distribution and the molecular weight of the heparosan polymers were analyzed by HPSEC on an Ultimate 3000 (Dionex, Sunnyvale, USA) using three TosoH Bioscience TSK-gel columns in series (4000, 3000, and 2500 Super AW, 150 x 6.0 mm) preceded by a TSK AW-L guard column (35 x 4.6 mm;TosoH Bioscience, Japan) as previously described (Chapter 3). For each HPSEC analysis, the molecular weight and the size distribution of the samples were determined by fitting to a logarithmic model the elution time of the hyaluronan molecular mass markers (30, 160 and 262 kDa. Hyalose) (Chapter 2).

High performance anion exchange chromatography (HPAEC) analysis

The composition of the reaction mixture (UDP-sugars and heparosan oligosaccharides) was analyzed by HPAEC using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2 x 250 mm), in combination with a CarboPac PA guard column (2 x 25 mm), a VWD-3100 single wave detector (Dionex, Sunnyvale, USA), and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA) in series. The same elution conditions as previously described were used (Chapter 3). The presence of a peak with UV detection, as with electrochemical detection shows the presence of an UDP group (Orellana and Mohnen 1999).

Matrix assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOF MS) analysis

An Ultraflex workstation (Bruker Daltonics, Germany) 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 PmHS2 single action transferases (PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$) were immobilized on Ni(II)-nitrilotriacetic acid (Ni-NTA) agarose during the purification procedure. The effect of the immobilization on the polymerization activity and stability was determined first. The parameters influencing the PmHS2 polymerization process and the heparosan chain elongation were studied. Therefore, non-equimolar amounts of immobilized PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ were incubated together in the presence of UDP-sugars. A fractional factorial design was included to determine the influence of PmHS2-GlcUA$^+$, PmHS2-GlcNAc$^+$, UDP-GlcUA, and UDP-GlcNAc concentrations.
Moreover as a proof of concept, we showed that PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$, immobilized onto separate columns, enabled to control the synthesis of heparosan oligosaccharides.

Enzyme immobilization

By comparing the amount of PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ protein immobilized onto Ni-NTA agarose and recovered after the PD10 purification step it was found that per volume of induced *E. coli* culture the amount of immobilized enzyme and PD10-purified enzymes were comparable based on the protein assay. It was determined that about 0.36 mg of PmHS2 transferases were immobilized per 1 ml of Ni-NTA slurry (50% agarose/50% buffer). The SDS-PAGE gel analysis of the fractions washed from the column after storage at 4°C, revealed the presence of a small quantity of protein, suggesting protein leaching. Thus, in order to avoid possible contamination of each column by undesired PmHS2 enzyme activities, the reaction mixtures were heat inactivated for 15 min at 65°C before being applied from column A to column B, and vise versa (Fig. 1).

The immobilized PmHS2 single action transferases were more stable during storage at 4°C than the PD10-purified single action transferases (Fig. 2). The activity of the immobilized PmHS2 single action transferases was stable during 2 days of storage. After 2 weeks of storage at 4°C, the immobilized PmHS2 single action transferases exhibited about 75% of the polymerization activity observed with the freshly immobilized enzymes (t = 0 h). After 50

days of storage, the PmHS2 single action transferases exhibited about 60% of their initial activity (t = 0 h). The PD10-purified single action transferases were not stable at 4°C; after 1 day of storage the activity decreased to about 50%, and after 1 week only 10% of the activity remained. The addition of ethylene glycol and metal ions ($MgCl_2$ and $MnCl_2$) in the buffer (Tris-HCl 50 mM. pH 7.15) did not improve the enzyme stability of the PD10-purified single action transferases. Furthermore, immobilization did not hamper the polymerization process catalyzed by the PmHS2 single action transferases. Indeed, the specific activity of the PmHS2 single action transferase was comparable whether the enzymes were immobilized or PD10-purified. The polymerization activity of the PD10-purified PmHS2-$GlcUA^+$ and PmHS2-$GlcNAc^+$, incubated together at 32°C for 2 to 8 h in the presence of 5 mM UDP-sugars, was about 0.065 nmol/min/μg protein, similar as previously reported (Chapter 3). Under the same incubation condition, immobilized PmHS2 single action transferases added together exhibited comparable catalytic efficiency, about 0.070 nmol/min/μg protein.

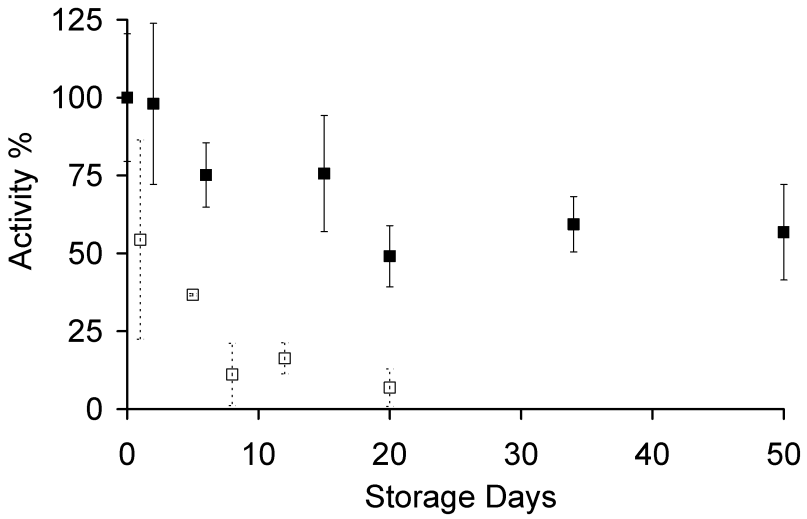

Figure 2. Storage stability of immobilized and PD10-purified PmHS2 single action transferases. The PmHS2 single action transferases (25-30 μg/ml of each transferase) were incubated together for 24 h in the presence of 5 mM of each UDP-sugar (■ immobilized and PD10-purified PmHS2 single action transferases). The amount of UDP-sugar converted after 24 h of incubation at 32°C was quantified by the coupled enzyme assay. The polymerization activity of the fresh immobilized and PD10-purified enzymes (t = 0) was set as 100%.

Influence of the transferase domains on the polymerization activity

We previously reported that the heparosan chain elongation by PmHS2 is influenced by the UDP-sugar concentrations (Chapter 2 and 3). Here, in order to determine the influence of the PmHS2 transferases on the heparosan initiation and elongation, the polymerization process of

non-equimolar amount of immobilized PmHS2 single action transferases (3 to 48 μg/ml of each transferase) incubated with 1 mM or 5 mM of each UDP-sugar was investigated. The polymerization activity observed in the presence of the same amount of immobilized PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ (12 μg/ml of each PmHS2 transferase) was used as reference. The polymerization process was influenced by the respective concentrations of PmHS2-GlcUA$^+$ or PmHS2-GlcNAc$^+$ present in the reaction mixture (Fig. 3 and 4). The results showed that both single action transferases differ in catalytic efficiency.

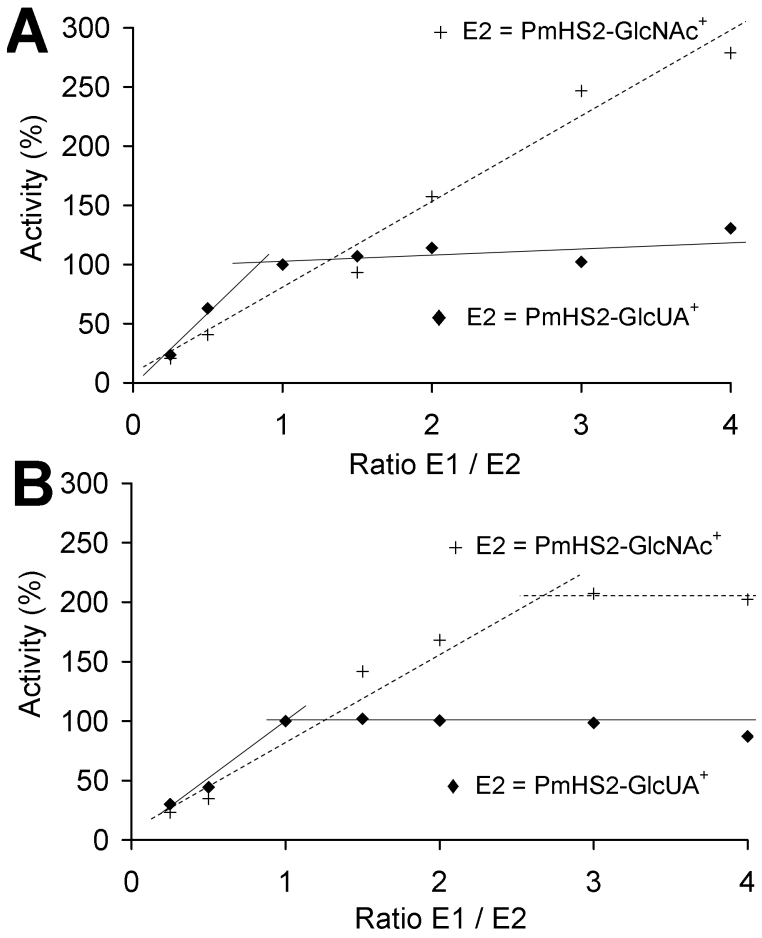

Figure 3. Polymerization activity of non-equimolar amount of PmHS2 single action transferases. Non-equimolar concentration of immobilized PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ (E1/E2 in which E1 is ranging from 3 to 48 μg/ml and E2=12 μg/ml) were incubated for 4 h in the presence of 1 mM (A) and 5 mM (B) of each UDP-sugar (UDP-GlcUA and UDP-GlcNAc). The amount of UDP-sugars converted after 4 h of incubation was determined by the coupled enzyme assay. The polymerization activity observed in the presence of immobilized PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ (12 μg/ml of each transferase, E1/E2 =1) was set at 100% relative activity. These results have been obtained from triplicate experiments; the standard deviation is ± 3 -10%.

When incubated in the presence of PmHS2 single action transferase ratio below 1 (3 to 6 μg/ml E1 and 12 μg/ml E2), the polymerization process was hampered resulting in a low polymerization activity (Fig. 3). In the presence of low concentrations PmHS2-GlcUA$^+$ or PmHS2-GlcNAc$^+$, it is likely that both the initiation and the chain elongation of heparosan polymers are hindered, resulting in the synthesis of heparosan chain concentrations below the detection limit of the agarose gels (Fig. 4).

When the reaction mixtures were incubated in the presence of PmHS2 single action transferase ratio above 1 (E1/E2 >1), the polymerization efficiency was influenced by the concentration of the respective transferases. In the presence of an excess of PmHS2-GlcUA$^+$ (from 12 up to 48 μg/ml) and 1 mM of each UDP-sugar, the polymerization efficiency increased proportionally to the increase of PmHS2-GlcUA$^+$ concentration (Fig. 3A). Indeed, in the presence of 36 μg/ml PmHS2-GlcUA$^+$ and 12 μg/ml PmHS2-GlcNAc$^+$, the polymerization efficiency increased 2.5 times in comparison to the equimolar concentration of PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$. The polymerization efficiency also increased proportionally to the increase of PmHS2-GlcUA$^+$ concentration in the presence of 5 mM of each UDP-sugar (Fig. 3B). However, it was observed that for high PmHS2-GlcUA$^+$ concentrations (36 μg/ml to 48 μg/ml) and a PmHS2-GlcNAc$^+$ concentration of 12 μg/ml, the polymerization efficiency leveled off.

Agarose gel electrophoresis analysis of the reaction mixtures (PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$) showed that increasing amounts of PmHS2-GlcUA$^+$ (below 36 μg/ml) resulted in the synthesis of a higher number of heparosan chains but with the same molecular weight and size distribution (Fig. 4A). This suggests that the incorporation of GlcNAc and GlcUA residues occurred at the same velocity for each heparosan chain. However, heparosan polymers with a lower molecular weight were synthesized in the presence of 5 mM UDP-sugars and 4-fold more the amount of PmHS2-GlcUA$^+$ than PmHS2-GlcNAc$^+$. Probably, the too high number of heparosan chains initiated by PmHS2-GlcUA$^+$ either inhibited or saturated PmHS2-GlcNAc$^+$, resulting in the incapability of the PmHS2-GlcNAc$^+$ to elongate all the chains at the same velocity.

When PmHS2-GlcNAc$^+$ was added in excess to the incubation mixtures (from 12 to 48 μg/ml), the polymerization efficiency did not increase (Fig. 3). The same observation was made in reaction mixtures incubated with 1 mM or 5 mM of each UDP-sugar. Agarose gel electrophoresis analysis showed that independently of the PmHS2-GlcNAc$^+$ concentrations (from 12 to 48 μg/ml), the same number of heparosan chains were initiated and elongated to the same length (Fig. 4B). These results suggest that the PmHS2-GlcUA$^+$ activity is limiting the overall polymerization efficiency.

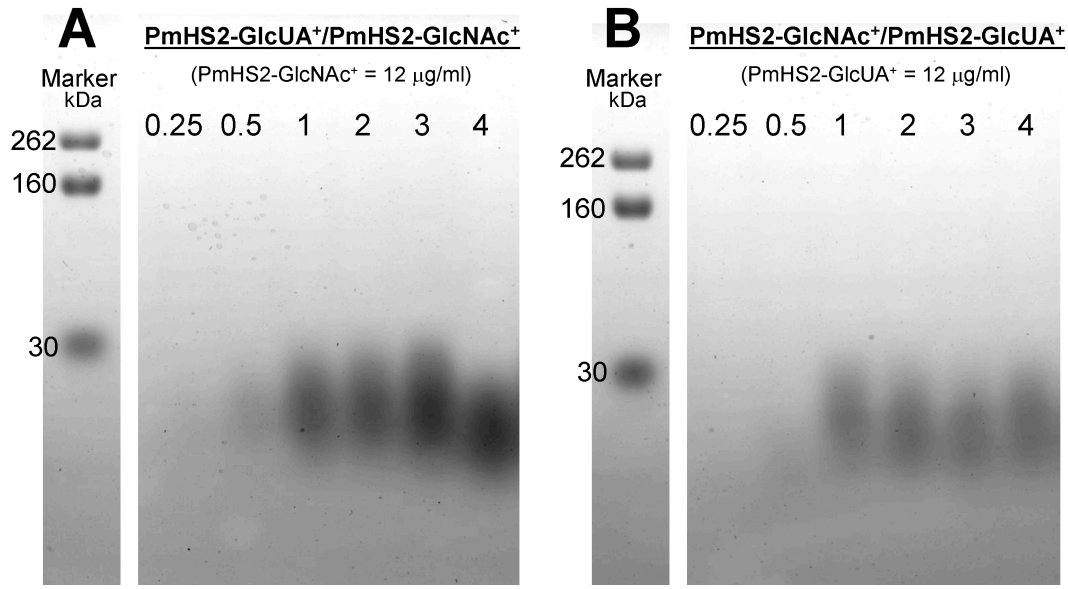

Figure 4. Agarose gel of heparosan polymers synthesized in the presence of non-equimolar amount of immobilized PmHS2 single action transferases. The reaction mixtures were incubated for 4 h in the presence of 5 mM of each UDP-sugar (UDP-GlcUA and UDP-GlcNAc) and non-equimolar concentration of PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ (E1/E2 ratio ranging from 0.25 to 4, in which E1=3 to 48 µg/ml and E2=12 µg/ml, respectively).

Experimental design analysis

To be able to control heparosan polymer elongation using PmHS2 glycosyltransferases, the effect of combined parameters on PmHS2 polymerization process were investigated using a fractional factorial design with 4 variables (3 levels (3^{4-1}) for each variable). Thus, 27 reaction mixtures were prepared in which different combinations of UDP-sugar and immobilized PmHS2 single action transferase concentrations were added (Table 2). The reaction mixtures were analyzed by the coupled enzyme assay to monitor the polymerization efficiency (data not shown), and they were also analyzed by HPSEC to assess the polymer elongation process. Using the Mallow's Cp criterion, the analysis of the 27 reaction mixtures showed that 8 parameters (R^2 0.848) were needed to obtain the best fitting model. The following equation describes the influence of the single action transferase concentrations and UDP-sugar concentrations on the heparosan elongation process:

heparosan molecular mass = -24.82 + 2.24 [PmHS2-GlcNAc$^+$] + 1.07 [PmHS2-GlcUA$^+$] + 2.34 [UDP-GlcNAc] -0.03 [PmHS2-GlcNAc$^+$]^2 -1.77.E-02 [PmHS2-GlcUA$^+$]^2 - 8.57 [PmHS2-GlcNAc$^+$][UDP-GlcUA] - 0.16 [PmHS2-GlcNAc$^+$][UDP-GlcNAc] + 0.57 [UDP-GlcUA][UDP-GlcNAc]

This model showed that the four variables (PmHS2-GlcUA$^+$, PmHS2-GlcNAc$^+$, UDP-GlcUA, and UDP-GlcNAc concentrations) influenced the polymerization activity with respect to the heparosan molecular weight. It was found that only the PmHS2-GlcNAc$^+$ (x_1), PmHS2-GlcUA$^+$ (x_2), and UDP-GlcNAc (x_4) concentrations are represented as main effect. In addition, the coefficient for the PmHS2-GlcNAc$^+$ concentration is higher than for the PmHS2-GlcUA$^+$ concentration. Despite the fact that the UDP-GlcUA concentration (x_3) was not represented as a main effect, the interaction effects showed that UDP-GlcUA concentration influences the heparosan elongation. The analysis of the contour plot visualization obtained from this analysis is presented in figure 5.

In reaction mixtures incubated with increasing concentrations of PmHS2-GlcUA$^+$ (7, 21, or 34 μg/ml) and a constant concentration of PmHS2-GlcNAc$^+$ (Fig. 5, from left to right), it was observed that the polymer molecular weight was neither influenced by the UDP-sugar concentration nor by the PmHS2-GlcUA$^+$ concentrations (Fig. 5A, 5B, and 5C). Nevertheless, the increase of PmHS2-GlcUA$^+$ concentration resulted in the synthesis of a higher number of heparosan chains and higher polymerization efficiency, as observed using the coupled enzyme assay (data not shown). In the presence of a low PmHS2-GlcUA$^+$ concentration (7 μg/ml), the polymerization was hampered as it can be assessed by the blue color trend (Fig. 5A); this is probably due to the low number of heparosan chain initiated.

In the reaction mixtures incubated in the presence of increasing PmHS2-GlcNAc$^+$ concentrations (7, 21, or 34 μg/ml) in combination with a constant concentrations of PmHS2-GlcUA$^+$ (Fig. 5, from top to bottom), resulted in different polymer elongation profiles as function of the UDP-sugar concentration present in the reaction mixture (Fig. 5D, 5E, and 5F). A constant concentration of PmHS2-GlcUA$^+$ in the reaction mixture implies the initiation of the same number of heparosan chains at a given UDP-sugar concentration. Thus, the polymerization trend observed is mainly the result of the PmHS2-GlcNAc$^+$ concentration. In the presence of a low concentration of PmHS2-GlcNAc$^+$ (7 μg/ml), the longest heparosan polymers were synthesized when incubated with high UDP-sugar concentrations (Fig. 5D). The fact that the polymerization occurred in the presence of a high UDP-sugar concentration suggests that PmHS2-GlcNAc$^+$ required a high concentration of heparosan oligosaccharides to perform its catalytic activity. Therefore, it was assumed that the affinity of the GlcNAc acceptor site is low. For the intermediate concentration of PmHS2-GlcNAc$^+$ (21 μg/ml), it seemed that the synthesis of long heparosan polymers was favored in the presence of either high or low equimolar UDP-sugar concentrations (Fig. 5E). While the production of long heparosan chains and the polymerization activity was hampered in the presence of low UDP-GlcNAc concentrations combined with high UDP-GlcUA concentrations; incubation conditions which were determined to be not suitable to initiate heparosan chains (Chapter 3). In the presence of high PmHS2-GlcNAc$^+$ (34 μg/ml) concentrations, the longest polymers

were obtained when incubated in the presence of low UDP-sugar concentration (Fig. 5F). Low UDP-sugar concentrations result in the synthesis of a lower number of heparosan chains (Chapter 2), which may avoid the saturation of the PmHS2-GlcNAc$^+$ acceptor site, and thus favor the elongation process.

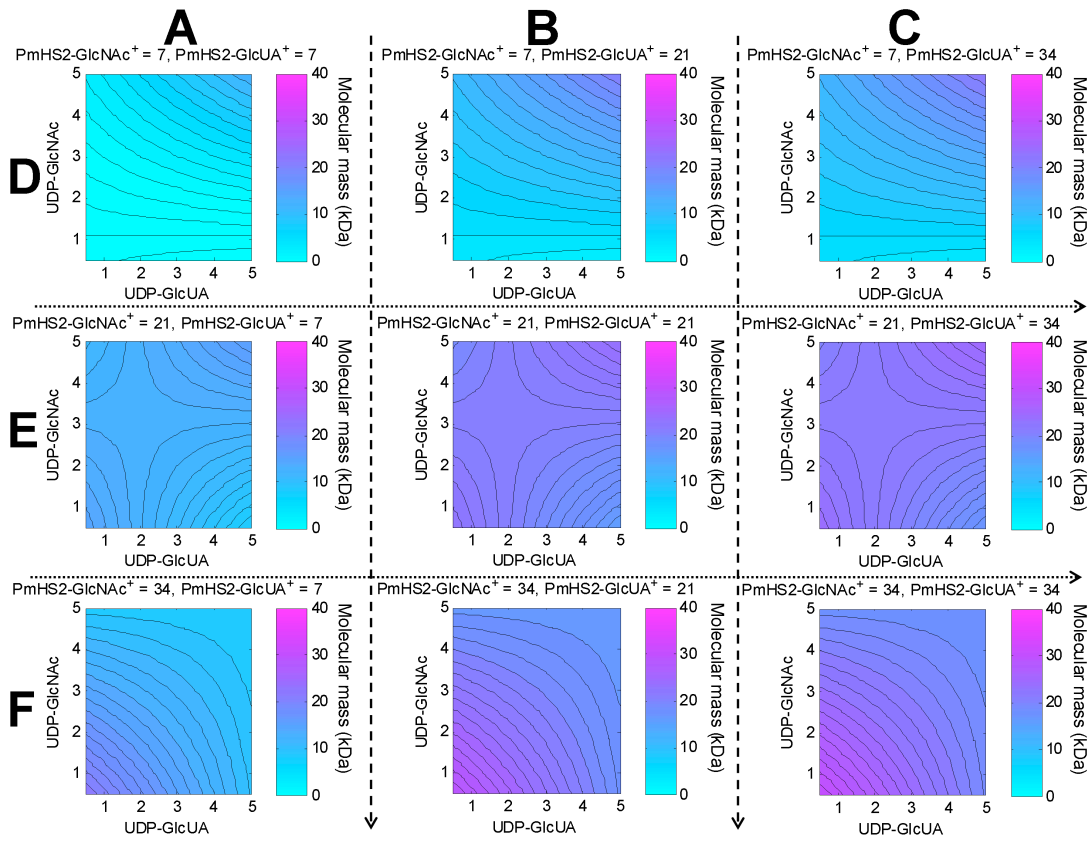

Figure 5. Contour plot visualization of the statistical analysis of the PmHS2 single action transferase polymerization activity with respect to the polymer molecular weight. The reaction mixtures were prepared according to a fractional factorial design with 4 variables and 3 levels (3^{4-1}) for each variable (immobilized PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ (μg/ml), UDP-GlcUA, and UDP-GlcNAc (mM)). After 4 h of incubation at 32°C, the heparosan average molecular mass (kDa, presented in the scale at the right of each graph) was determined by high performance size exclusion chromatography (HPSEC). The data were visualized using the mathematical software package Matlab.

Controlled elongation of heparosan oligosaccharides

It was observed that PmHS2 polymerization process is influenced by both transferase activities and both UDP-sugar concentrations, rendering the control of heparosan chain elongation difficult.

PmHS2-GlcUA$^+$ was reported to initiate heparosan chains when incubated in the presence of both UDP-sugars (Chapter 3). This implies that PmHS2-GlcUA$^+$ is active without forming a protein complex with PmHS2-GlcNAc$^+$. To determine if PmHS2-GlcNAc$^+$ is also active as a

single function transferase, and thus to evaluate if the PmHS2-single action transferases could be used to elongate step by step heparosan oligosaccharides in the absence of template molecules, PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ were immobilized on separate columns. The reaction mixture was incubated in each column and recycled from one column (A) to the other (B) in order to elongate heparosan step by step (Fig. 1). The analysis of the reaction mixture using HPAEC, Maldi-Tof MS, and 20% TBE gel electrophoresis (data not shown) showed that heparosan oligosaccharides with a defined length could be synthesized by the PmHS2 single action transferases. It was observed with HPAEC analysis that the odd numbered oligosaccharides (non-reducing end GlcNAc) eluted earlier than the even numbered oligosaccharides (non-reducing end GlcUA). Indeed, the trisaccharide GlcNAc-GlcUA-GlcNAc-UDP eluted at 25.8 min, while the disaccharide GlcUA-GlcNAc-UDP eluted at 28.8 min. The monitoring of the reaction mixture after each incubation step showed that the oligosaccharides were completely elongated during each incubation step (Fig. 6). Using HPAEC and Maldi-Tof MS analysis, it was observed that at the end of the incubation time with PmHS2-GlcUA$^+$ only odd numbered oligosaccharides were present in the reaction mixture, while only uneven numbered oligosaccharides were found after incubation with PmHS2-GlcNAc$^+$.

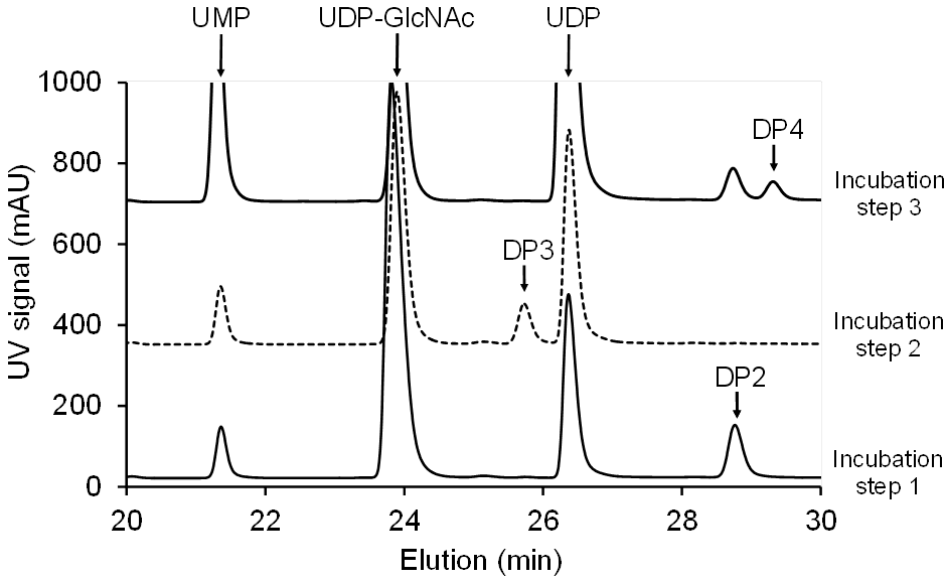

Figure 6. High performance anion exchange chromatography analysis of the first and second incubation cycle on the PmHS2-GlcUA$^+$ and the PmHS2-GlcNAc$^+$ column, respectively. Heparosan disaccharides (DP2) were released from the PmHS2-GlcUA$^+$ column (column A; 0.5 mg PmHS2-GlcUA$^+$/ml reaction, incubation step 1) and heparosan trisaccharide (DP3) from the PmHS2-GlcNAc$^+$ column (column B; 0.5 mg PmHS2-GlcNAc$^+$/ml reaction, incubation step 2), and a mixture of heparosan disaccharides (DP2) and tetrasaccharides (DP4) from the PmHS2-GlcUA$^+$ column (incubation step 3). The UMP, UDP-GlcNAc, UDP, and UDP-GlcUA eluted after 21.1, 23.5, 26.0, and 33.6 min, respectively. The annotation DP$_X$ stands for "degree of polymerization X", in which X represents the number of monosaccharide units.

After nine successive incubation cycles in PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ columns, the reaction mixture released from PmHS2-GlcUA$^+$ column showed that only even numbered heparosan oligosaccharides (disaccharides to octasaccharides, DP2 to DP8 respectively) were produced (Fig. 7). The disaccharides GlcUA-GlcNAc-UDP (DP2) eluted after 28.8 min (*m/z* 782.6) (Chapter 3), the others heparosan oligosaccharides constituted with 4 (*m/z* 1160.3), 6 (*m/z* 1539.0), and 8 (*m/z* 1918.0) monosaccharide units eluted after 29.4, 30.0, and 30.6 min, respectively.

As observed with HPAEC and Maldi-Tof MS, the heparosan disaccharides produced after the first incubation cycle on PmHS2-GlcUA$^+$ (cycle 1) were completely converted into trisaccharide when incubated with PmHS2-GlcNAc$^+$ (cycle 2). The reaction mixture released from the PmHS2-GlcUA$^+$ (cycle 3) contained disaccharides and tetrasaccharides. This result showed that the initiation of new chains occurred during each incubation step on PmHS2-GlcUA$^+$.

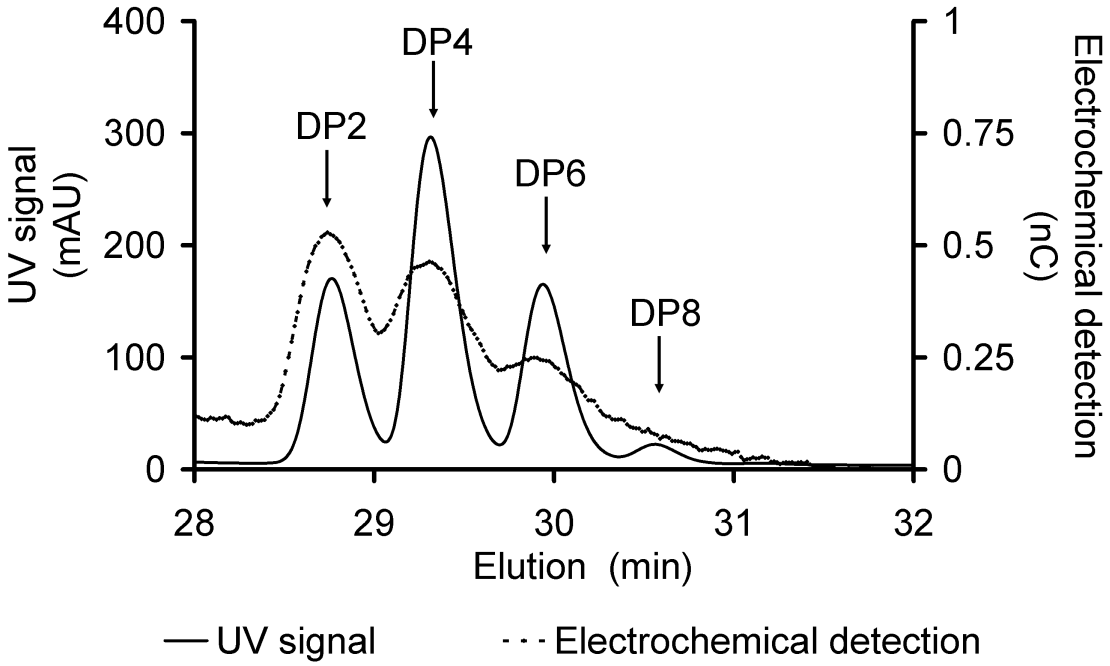

Figure 7. High performance anion exchange chromatography analysis of the reaction mixture released from the PmHS2-GlcUA$^+$ column (column A). Even numbered heparosan oligosaccharide mixture from disaccharides up to octasaccharides was synthesized by the immobilized PmHS2 single action transferases (0.5 mg/ml PmHS2-GlcUA$^+$ and 0.5 mg/ml PmHS2-GlcNAc$^+$), as shown in Fig.1. The reaction mixture was obtained after 9 successive incubation cycles (last incubation cycle on column A). The annotation DP_X stands for "degree of polymerization X", in which X represents the number of monosaccharide units.

Discussion

Immobilization of PmHS2 single action transferases

PmHS2-GlcUA$^+$/PmHS2-GlcNAc$^+$ were successfully immobilized on Ni(II)-nitrilotriacetic acid (Ni-NTA) agarose during the purification step. The use of immobilized-metal affinity chromatography (IMAC) such as Ni-NTA agarose is frequently reported for the purification of protein but its usage for immobilization purpose is not common (Liu, Zhang et al. 2002). However, absorption or affinity immobilizations such as IMAC do not strongly bind the catalyst and leaching can occur after storage, and during the washing and the incubation process. An alternative to prevent leaching is the immobilization by covalent coupling, but there is a risk of enzyme activity loss. Immobilization strategies could be further investigated to optimize the process.

Influence of the transferase domains on the polymerization activity

The PmHS2 polymerization process was investigated in the presence of different ratios PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$. It was found that both transferase domains do not exhibit the same catalytic efficiency.

Here we report that the addition of an excess of PmHS2-GlcUA$^+$ in comparison to PmHS2-GlcNAc$^+$ increased the polymerization activity, while an excess of PmHS2-GlcNAc$^+$ did not influence the catalytic efficiency. The results suggested that glucuronyl transferase (PmHS2-GlcUA$^+$) catalytic activity is the limiting step in the overall polymerization process, either by controlling the amount of chain initiated and by exhibiting per heparosan chain a slow and a constant rate in transferring GlcUA residue in comparison to the GlcNAc transfer by PmHS2-GlcNAc$^+$. We previously reported that for a same amount of protein, the specific activity of PmHS2-single action transferases was 2-fold lower than for PmHS2 (Chapter 3). Based on the results presented here, this was due to a 2 times lower concentration of active PmHS2-GlcUA$^+$ in the reaction mixture compared to PmHS2. Thus, the PmHS2 single action transferases exhibit similar polymerization efficiency as PmHS2.

We also observed that *N*-acetylglucosaminyl transferase (PmHS2-GlcNAc$^+$) is either saturated or inhibited in the presence of too high number of chains. This may explain why PmHS2 synthesizes low molecular weight heparosan polymers in the presence of high UDP-sugars concentrations (Chapter 2).

Experimental design analysis

The analysis of the experimental design showed that the 4 variables: PmHS2-GlcUA$^+$, PmHS2-GlcNAc$^+$, UDP-GlcUA, and UDP-GlcNAc concentrations influence the heparosan elongation. This is in agreement with what have been reported previously for the PmHS2 polymerization process (Chapter 2 and 3). It was observed that the polymer chain length and size distribution is mainly determined by the number of chain initiated which depends on the concentration of UDP-sugar concentrations (Chapter 2), on the number of acceptor molecules (UDP-GlcNAc) (Chapter 3), and on the number of acceptor sites (PmHS2-GlcUA$^+$). The heparosan chain length is also influenced by the catalytic efficiency of the transferases to elongate heparosan chains. The PmHS2-GlcNAc$^+$ was found to play a critical role in the heparosan chain length, by being saturated or inhibited in the presence of a too high number of chains.

Controlled elongation of heparosan oligosaccharides

We showed that PmHS2 single action transferases are independent and do not need complex formation with each other to exhibit activity. This represents a great advantage over the mammalians heparosan synthases (EXT1 and EXT2) (McCormick, Duncan et al. 2000; Kim, Kitagawa et al. 2003) and the *E. coli* heparosan synthases (KfiC and KfiA) (Griffiths, Cook et al. 1998; Hodson, Griffiths et al. 2000; Sugiura, Baba et al. 2010) to control the elongation of heparosan.

For the first time, immobilized PmHS2 single action transferases have been used to elongate step by step heparosan oligosaccharides. It was observed for each incubation step that the heparosan oligosaccharides were completely elongated; since only PmHS2-GlcUA$^+$ is capable to initiate heparosan chains (Chapter 3), this resulted in the presence of only even numbered heparosan oligosaccharides with PmHS2-GlcUA$^+$ and only odd numbered oligosaccharides with PmHS2-GlcNAc$^+$. In addition, the controlled synthesis of GAGs have not been reported yet in the absence of template (DeAngelis, Oatman et al. 2003; Sugiura, Shimokata et al. 2008; Liu, Xu et al. 2010). This approach is of interest since the production of template molecules is expensive and laborious. Production of templates either by chemical hydrolysis (Sismey-Ragatz, Green et al. 2007) or enzymatic degradation (Ernst, Langer et al. 1995) of heparosan K5 polysaccharides is not a well controlled process, and thus to obtain monodisperse heparosan molecules a fractionation step is required. In addition, heparin lyases cleave the glycosidic bond via an elimination reaction resulting in the formation of oligosaccharides containing unsaturated uronic acid residues at the non-reducing end

terminus. In order to use the templates for polymerization, the saturated uronic acid residues need to be removed using mercuric salts (Ludwigs, Elgavish et al. 1987).

Synthesis of heparosan disaccharides from nucleotide sugars requires the incubation of PmHS2-GlcUA$^+$ in the presence of both UDP-sugars (Chapter 3). The production of a mixture of heparosan oligosaccharides is due to the initiation of new heparosan chains for each elongation step with PmHS2-GlcUA$^+$. This shows that both UDP-GlcNAc and UDP-GlcUA are present in the reaction mixture. Thus, to synthesize monodisperse heparosan oligosaccharides, the incubation conditions enabling to reach the complete conversion of the UDP-GlcNAc by PmHS2-GlcNAc$^+$ should be determined to avoid the presence of both UDP-sugars when incubated with PmHS2-GlcUA$^+$. Furthermore, at the end of the second incubation step, the purification of the heparosan trisaccharides to be used as templates, could facilitate the production of monodisperse heparosan polymers during the step by step elongation.

Conclusion

To summarize, we found that both UDP-sugars and both transferases influence the polymerization with respect to the catalytic efficiency and the polymer molecular weigh. The glucuronyl transferase (PmHS2-GlcUA$^+$) catalytic activity is limiting the overall polymerization process activity and the *N*-acetylglucosaminyl transferase (PmHS2-GlcNAc$^+$) influences the polymer elongation depending on the number of chains to elongate and the UDP-sugar concentrations present in the reaction mixture. A more detailed analysis of the kinetic parameters should help to further optimize the polymerization conditions to control heparosan chain length and size distribution.

We also showed, for the first time, that immobilized PmHS2 single action transferases incubated on two separate columns in the absence of template molecules are capable to elongate step by step heparosan oligomers. PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$ are not only independent transferases, but they can also use modified UDP-sugars to elongate heparosan (Sismey-Ragatz, Green et al. 2007; Liu, Xu et al. 2010). These properties are of interest to facilitate the *in vitro* synthesis of Hep/HS-like products (Liu, Xu et al. 2010) and to enlarge the potential of heparin and heparan sulfate biological activity (Sismey-Ragatz, Green et al. 2007).

Abbreviations

The abbreviations used are: UDP, uridine diphosphate; UDP-GlcUA, UDP-glucuronic acid; UDP-GlcNAc, UDP-N-acetylglucosamine; PmHS2, *Pasteurella multocida* heparosan synthase 2, HS1, heparosan synthase 1; HAS, hyaluronan synthase; CS, chondroitin synthase; HPAEC, High performance anion exchange chromatography; HPSEC, High performance size exclusion chromatography; MALDI-TOF MS, Matrix-assisted laser desorption-ionisation time of flight mass spectrometry; PmHS2-GlcUA^{+}, PmHS2 glucuronyl transferase; PmHS2-GlcNAc^{+}, PmHS2 *N*-acetylglucosaminyl transferase.

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Chapter 5

Agarose gel electrophoresis assay to screen *Pasteurella multocida* heparosan synthases library for thermostable PmHS2 variants

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Abstract

Agarose gel electrophoresis is commonly used to analyze negatively charged polysaccharides such as glycosaminoglycans. Here, we describe for the first time the use of an agarose gel electrophoresis assay to screen a *Pasteurella multocida* heparosan synthase -PmHS2- mutant library for its ability to polymerize heparosan. The PmHS2 mutant library was created by error prone PCR and about 1000 mutants were screened for increased thermostability. Some of the identified PmHS2 variants showing improved thermostability compared to the parental PmHS2 enzyme were purified, and further characterized. It was found that the *C*-terminus amino acid tag is influencing the stability of PmHS2 when incubated above 40°C. In the absence of a *C*-terminus tag, PmHS2 optimum temperature was increased by 10°C and the catalytic efficiency was 2 times higher at the optimum temperature.

Introduction

Glycosaminoglycans (GAGs) are un-branched and negatively charged polysaccharides that are involved in many physiological processes such as cell adhesion, chemokine signaling, biochemical cascades, signal transduction, and even pathogen recognition (Linhardt and Toida 2004). These polysaccharides are present in most of the vertebrate cells, where they are polymerized by glycosyltransferases (GTs) from activated nucleoside diphosphate monosaccharides (UDP-sugars) (DeAngelis 2002). GAGs such as hyaluronan, chondroitin sulfate, and heparin are commonly used in health care and have a large therapeutic potential. Since GAGs are present in vertebrate cells, the pharmaceutically grade GAG products have been, and are still, extracted from animal tissues. However, due to the harsh process conditions needed to recover the active compounds, the amount of waste produced, the shortage in raw materials of animal origin (Petitou and van Boeckel 2004), and the potential safety risk that represents the use of animal derivatives (Guerrini, Beccati et al. 2008; Liu, Zhang et al. 2009), the trend is to replace this traditional production system by sustainable bio-processes (Liu, Zhang et al. 2009). Nowadays, the pharmaceutically grade hyaluronic acid is already produced in a bioprocess using recombinant bacteria (Widner, Behr et al. 2005). To produce GAG oligo and polymers the (chemo)enzymatic synthesis of GAGs is also being investigated (Kuberan, Beeler et al. 2003; Kuberan, Lech et al. 2003).

In order to enzymatically polymerize GAG-like polymers at industrial scale, recombinant GAG-synthases exhibiting improved catalytic properties should be developed. The aim for industrial purposes is to obtain GAG-synthases exhibiting a higher catalytic efficiency, using a broader substrate range (Weïwer, Sherwood et al. 2008), having an increased stability to elevated temperatures, solvent, and pH, and also being less sensitive to inhibitors such as UDP (Tlapak-Simmons 2004; Baggenstoss and Weigel 2006) (chapter 2). Applying directed evolution to improve biocatalyst characteristics is commonly used (Bornscheuer and Pohl 2001) and was shown to be successful with some GTs (Aharoni, Thieme et al. 2006; Persson and Palcic 2008; Yang, Rich et al. 2010). The development of suitable assays for the screening of mutant libraries is important to identify mutated enzymes of interest.
Several procedures are available to study the enzymatic activity of GTs. Biochemical assays such as coupled enzyme assay (Gosselin, Alhussaini et al. 1994; Krupa, Shaya et al. 2007), pH sensitive/colorimetric assay (Deng and Chen 2004; Persson and Palcic 2008), and assays using radiolabelled UDP-sugars (Palcic and Keiko 2001) enabled to assess GTs catalytic activity. The GTs that catalyze the formation of oligo- and polysaccharides, such as the GAG-

synthases, can also be characterized by analyzing the polymer formation using high performance size exclusion chromatography (HPSEC) (Chapter 2) in combination with MALLS (Sismey-Ragatz, Green et al. 2007), high performance anion exchange chromatography (HPAEC) (Chapter 3), agarose and polyacrylamide gel electrophoresis (Lee and Cowman 1994; Ikegami-Kawai and Takahashi 2002; Volpi and Maccari 2006), fluorophore-assisted carbohydrate electrophoresis (FACE) (Calabro, Hascall et al. 2000; Kooy, Ma et al. 2009), thin layer chromatography (TLC) (Abeling, Rusch et al. 1996; Zhang, Xie et al. 2007), or mass spectrometry (Sismey-Ragatz, Green et al. 2007). Among all these analytical procedures, only a few are suited for the screening of mutant libraries, which represents the analysis of a large number of samples. The use of an ultra high throughput method using fluorescence activated cell sensing (FACS) (Aharoni, Thieme et al. 2006; Yang, Rich et al. 2010), and a pH-indicator high-throughput assay (Persson and Palcic 2008), were reported for the screening of GTs that catalyze the transfer of one monosaccharide to an acceptor molecule. However, screening of GTs mutant libraries involved in the synthesis of polysaccharides such as the GAG-synthases has not been described yet.

Here, we report for the first time the screening of a GAG-synthase mutant library. Biocatalysts with increased thermostability are generally also more stable to other denaturing factors (Eijsink, Gáseidnes et al. 2005), therefore it is of interest to increase the thermostability of biocatalysts for industrial purposes. The negatively charged GAG polymers are known to migrate as a function of their charge during gel electrophoresis, and can be stained to determine their molecular weight and amount (Volpi and Maccari 2006). Thus, we used agarose gel electrophoresis to screen a *Pasteurella multocida* heparosan synthase PmHS2 mutant library for variants with increased thermostability. The PmHS2 variants showing improved stability to elevated temperatures were characterized in more detail to obtain information about the thermostability of PmHS2.

Experimental Procedures

Mutant library construction

Random mutagenesis was performed using the error-prone PCR GeneMorph II Random Mutagenesis Kit (Stratagene), known to exhibit a minimal mutational error bias. The error-prone PCR (epPCR) reaction mixture was prepared according to the manufacturer's instructions. The *pm*hs2 (*pm*hssB. GenBank acc. No. AY292200) gene ligated into pET101-TOPO (Invitrogen) was used as template (about 5 ng/µl PCR reaction) and amplified by 30 cycles (95°C/30 sec, 59°C/1 min, 72°C/2 min).

To amplify *pm*hs2, the forward primer 5´-CGTAGGATCCATGAAGGGAAAAAAA GAGATG-3´ and the reverse primer 5´-GCATGAGCTCTAAAAAATAAAAAGGT AAACAGG-3´ (gold quality primers. 80% purity. Eurogentec) adding *Bam*HI and *Sac*I restriction sites, respectively, were used. The PCR product was digested, gel purified, and ligated into pET101 vector (1:1 molar ratio) using the *Bam*HI and *Sac*I restriction sites. Note that the commercially available pET101-TOPO (Invitrogen) has been adapted for this experiment in order to contain the *Bam*HI restriction site.

The ligation mixture was electroporated in *E. coli* electrocompetent TOP10 cells (Invitrogen) and plated on LB/ampicilin (100 μg/ml) agar plates. After overnight incubation at 37°C, about 4500 colonies were obtained. The mutants were pooled by washing the plates with LB broth, and the cell suspension was diluted to reach a 1.6 OD_{600}. After centrifugation, the supernatant was discarded and the pellets (2 ml aliquot) were stored at -20°C.

Plasmid DNA was isolated from the pellet, gel purified and used to transform *E. coli* BL21* (DE3) expression cells (Invitrogen). Ampicillin tolerant colonies were checked by PCR and positive colonies were selected to constitute the PmHS2 mutant library (1100 ±50 mutants).

The mutation rate of this PmHS2 library was determined by sequencing 95 randomly selected *pm*hs2 mutants (800 bp/gene) (Base Clear, The Netherlands).

Screening of the PmHS2 mutants: polymerization activity and thermal tolerance

To each 96-wells microtiterplates (U shape bottom, Greiner) containing 200 μl LB broth/ampicillin (50 μl/ml), 94 individual PmHS2 mutants and two parental PmHS2 were transferred and were grown overnight at 37°C/200 rpm (preculture). Fresh cultures (183 μl) were inoculated with 7 μl of the preculture and incubated for 3.5 h at 37°C/240 rpm. The protein expression was induced with IPTG (0.5 mM) and conducted for 3 h at 30°C under the same shaking conditions. In order to facilitate the handling after induction, the cultures were transferred from the microtiterplates to PCR-plates (96-wells, Greiner), centrifuged for 10 min at 6000x*g*, washed with 100 μl Tris-HCl (pH 7.15), and centrifuged a last time before being stored at -80°C.

To each thawed cell pellet obtained from 200 μl culture, 20 μl of polymerization reaction mixture was added (40 mM Tris-HCl (pH 7.15), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 5 mM UDP-GlcUA, 5 mM UDP-GlcNAc) and incubated during 18 h at 32°C/80 rpm. At the end of the polymerization reaction, 8 μl of 70% glycerol/loading dye was added to each reaction mixture; the PCR-plates were shortly centrifuged and stored at -20°C. For the analysis, the reaction mixtures (8 μl) were loaded on 2% (w/v) agarose gel using a multi-channel pipette, and were run for 40 min at 50 V (migration of approximately 2.5 cm). Each agarose gel (104

wells. 12 cm x 12 cm. i-MyRun.N/Cosmo Bio.Japan) enabled to analyze 96 samples (94 PmHS2 mutants + 2 parental PmHS2) for the presence of synthesized heparosan polymers. The 2% (w/v) agarose gels were stained by Stains-all and destained as previously described to assess the heparosan product synthesis (Chapter 2).

The PmHS2 variants exhibiting a polymerization activity after 18 h at 32°C, were selected in order to proceed to a second selection round to isolate thermostable PmHS2 variants. For this purpose, the crude enzymes were exposed for 15 min at 56°C, and then incubated 18 h at 32°C to perform polymerization of heparosan (referred as polymerization conditions). The heat exposure was done in a PCR machine with a hot sealing to avoid condensation.

Characterization of thermostable PmHS2 mutants

PmHS2 variants found to synthesize more heparosan polymers than the crude parental PmHS2 after 15 min exposure at 56°C and 18 h polymerization, were expressed in a larger culture (100 ml) and purified in order to be characterized as previously described (Chapter 2). The enzymatic activity and thermostability of the PmHS2 mutants were determined using the coupled enzyme assay (Gosselin, Alhussaini et al. 1994) and the heparosan products were analyzed with 2% (w/v) agarose gel electrophoresis (Chapter 2). The amount of purified protein was evaluated by SDS-PAGE analysis and by a bicinchoninic acid (BCA) assay (Pierce) using bovine serum albumin (BSA) as the standard (Chapter 2).

Directed mutagenesis of parental PmHS2

In order to investigate which mutation(s) contributed in the increase of thermal stability, primer sets were designed to modify step by step the parental PmHS2 according to the mutations observed with the PmHS2 mutants.

The nucleotide modifications were introduced into the *pmhs*2 gene using the primer sets presented in Table 1. Each PCR fragment was ligated into the pET101-TOPO vector, transformed in *E. coli* Top10 and subsequently in *E. coli* BL21*(DE3) expression strain (Invitrogen). Amino acid changes and deletions of the mutants were confirmed by DNA sequencing (Base Clear, The Netherlands).

Table 1. Primer sets for mutagenesis of the parental PmHS2.

The PmHS2-(S182C) was constructed using PCR and overlapping PCR techniques such as previously described (Chapter 3). The primers FW_1/RV_1 were used to amplify Fragment 1, the FW_2/RV_2 for Fragment 2, and *pm*hs2-(S182C) was obtained by overlapping Fragments 1 and 2 using $FW_{HS2\text{-}tag}/RV_{HS2\text{-}tag}$.

PmHS2 variants	Forward and reverse primer sets (FW an RV, respectively)
PmHS2-tag	FW. 5´-CACCATGAAGGGAAAAAAAGAGATG-3´ RV. 5´-TAAAAAATAAAAAGGTAAACAGGGG-3´
PmHS2-(Δ6aa *N*-terminus)	FW. 5´-CACCAGAAGGGAAAAAAAGAGATG-3´ RV. 5´-TAAAAAATAAAAAGGTAAACAGGGG-3´
PmHS2-(S182C)	FW_1. 5´-CACCATGAAGGGAAAAAAAGAGATG-3´ RV_1. 5´-GACTTTGCATGTCGTATTCGC-3´ FW_2. 5´-GCGAATACGACATGCAAAGTC-3´ RV_2. 5´-TAAAAAATAAAAAGGTAAACAGGGG-3´
PmHS2-(Y649I, ΔL651,Δ32aa-tag *C*-terminus)	FW. 5´-CACCATGAAGGGAAAAAAAGAGATG-3´ RV. 5´-TAAAAAATAAAAGGTAAACAGGGG-3´
PmHS2-(Δ32aa-tag *C*-terminus)	FW. 5´-CACCATGAAGGGAAAAAAAGAGATG-3´ RV. 5´-TTATAAAAAATAAAAAGGTAAACAGGG-3´
PmHS2-(Y649I, DL651)	FW. 5´-CACCATGAAGGGAAAAAAAGAGATG-3´ RV. 5´- AAAAATAAAAGGTAAACAGGGG-3´

Results

Set up of the screening assay

Purification of enzymes is laborious and costly; therefore it cannot be applied for the screening of a mutant library harboring a large number of variants. Thus, assays using crude enzymes instead of purified enzymes are of interest to screen mutant libraries. Here, both the crude and purified PmHS2 were found to be capable to synthesize heparosan polymers when incubated in polymerization conditions, based on agarose gel analysis.

The polymerization activity (18 h at 32°C) of the crude and purified PmHS2 after 15 min exposure between 40°C and 54°C were analyzed. The inactivation of the enzyme was determined using coupled enzyme assay (data not shown). It was observed that the polymerization activity of purified PmHS2 decreased by about 50% when the reaction mixture was exposed at 49°C, and it was completely inactivated at temperatures above 51.6°C. The inactivation of the crude PmHS2 was also investigated by the coupled enzyme assay but it was not possible to measure the amount of UDP-sugars converted due to the

presence of a high background signal. The fact that the coupled enzyme assay is not suitable to analyze the polymerization activity of crude GTs was also mentioned by Persson and Palcic (2008).

Agarose gel electrophoresis is a good method to determine the molecular weight and the size distribution of heparosan products synthesized by purified PmHS2 (Fig. 1A) (Chapter 2). Here, it was observed that crude (PmHS2 present in *E. coli* cell crude extract) is capable to synthesize heparosan polymers when incubated in the presence of both UDP-sugars. While the negative control, *E. coli* cells that do not express PmHS2, did not synthesize any polymers when incubated in the presence of UDP-GlcUA and UDP-GlcNAc. In contrast with the purified PmHS2, the polymer molecular weight and size distribution of heparosan polymers synthesized by crude PmHS2 cannot be determined; but it was found that agarose gels can be used to asses the presence and the quantity of heparosan polymers in the reaction mixture, and also to investigate the thermal stability of PmHS2 (Fig. 1B).

Based on the heparosan product synthesized, it was found that the purified PmHS2 was more sensitive to heat than the crude PmHS2. The activity of the purified PmHS2 was completely inactivated after an exposure at 51.6°C (Fig. 1A), while heparosan polymers were still synthesized by crude PmHS2 after an exposure at 54.4°C (heparosan polymer = dark spot) (Fig. 1B). This result shows that agarose gel electrophoresis can be used to analyze the polymerization activity and to investigate the thermal stability of crude PmHS2.

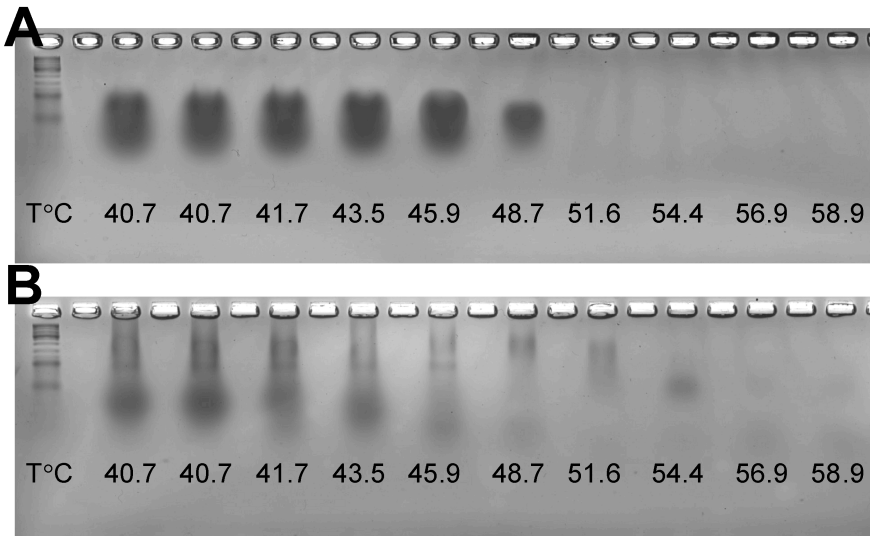

Figure 1. Thermostability of crude and purified parental PmHS2. The polymerization activity of purified (A) and crude (B) parental PmHS2 in the standard polymerization condition (32°C/18 h) was evaluated after exposure of the reaction mixture for 15 min between 40.7°C and 58.9°C (first lane, DNA marker). The synthesized heparosan product was analyzed by 2% agarose gel (40 min/50 V)/Stains-all staining. In both agarose gels (A and B), the dark spots observed are heparosan polymers.

Library mutation rate

A PmHS2 mutant library of approximately 1000 mutants was created. The sequencing of 95 randomly selected active and inactive mutants (800 bp from the FW-T7 priming site) showed that about 1.45 mutations per Kb occurred. Based on sequencing results, it was assumed that about 2 amino acid substitutions were present per PmHS2 protein. In addition, sequencing revealed the presence of mutants (about 7%) exhibiting a deletion of a base pair in the forward primer site due to the quality of the purchased primers, and that 3% of the parental *pm*hs2 (DNA template *pm*hs2-TOPO cloning sites) were present in this PmHS2 library. Furthermore, it was observed that due to the preference for certain mutations or the enrichment of the library because of the two steps cloning, about 6% duplicates of PmHS2 variants were present in this PmHS2 library.

Screening of the mutant library

Since agarose gel electrophoresis was demonstrated to be a suitable method to analyze the polymerization activity and thermal inactivation using crude PmHS2, this method was used to screen the PmHS2 mutant library for thermostable variants.

In order to improve the thermal stability of enzymes without loosing their polymerization activity at 32°C, two screening rounds were carried out (Eijsink, Gáseidnes et al. 2005). The first round aimed to distinguish the active from the non-active PmHS2 mutants; thus the PmHS2 library was only incubated in polymerization conditions (18 h at 32°C), without heat exposure. It was found that about 33% of the 1000 PmH2 mutants were active; for the other 67% the absence of polymerization product suggested their inactivation in the tested conditions. Among the active PmHS2 mutants, approximately 20% were found to be at least as active as the crude parental PmHS2 based on the amount of heparosan product stained with Stains-all.

For the second round only the 330 active PmHS2 mutants were selected and their polymerization activity was analyzed after an exposure at 56°C prior to 18 h incubation for polymerization. When exposed at 56°C, the crude parental PmHS2 did not synthesize heparosan, while about 70 PmHS2 mutants were still able to synthesize heparosan (Fig. 2). From the 70 thermostable PmHS2 mutants, nine mutants showing the highest thermostability based on the amount of heparosan products synthesized, were further analyzed. These enzymes, such as PmHS2-M2A8, PmHS2-M2D10, PmHS2-M2E4, and PmHS2-M2H5, were purified and characterized for thermal stability.

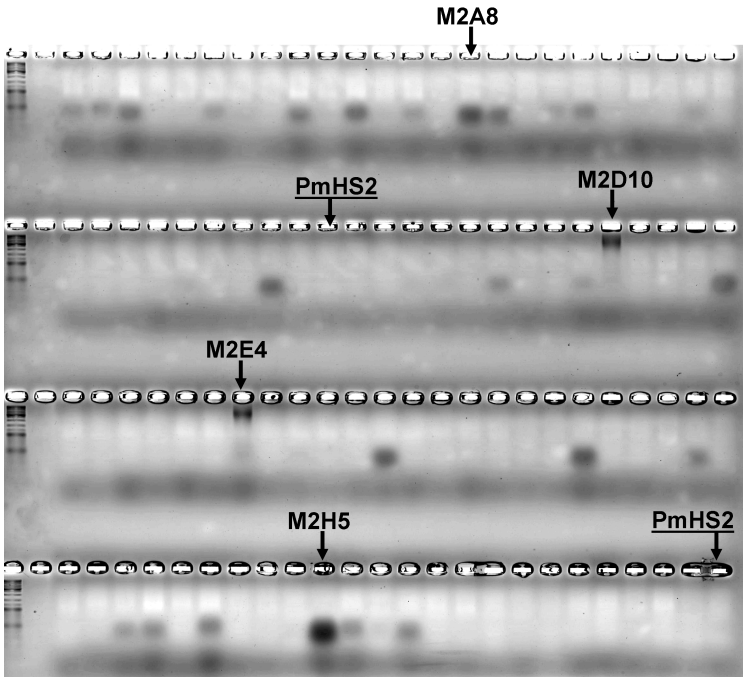

Figure 2. Screening of the PmHS2 mutant library for thermostable variants. Example of a 2% agarose gel electrophoresis (12 cm x 12 cm; first lane. DNA marker) obtained from the screening of 94 crude PmHS2 mutants and 2 crude parental PmHS2. The polymerization activity (18 h/32°C) of PmHS2 variants exposed for 15 min at 56°C was analyzed. The thermostable PmHS2 variants showing the highest product yield were selected for further analysis; they are indicated with arrows.

Characterization of PmHS2 thermo stable mutants

The PmHS2 mutants showed comparable expression and recovery levels as the parental PmHS2. The polymerization activity of the purified PmHS2 mutants was investigated by the coupled enzyme assay at incubation temperatures ranging from 32°C to 64°C. Most of the selected PmHS2 mutants had an increased optimal temperature in comparison to PmHS2. The temperature optimum after 4 h of incubation was found to be 38°C for parental PmHS2 and PmHS2-M2A8, 40°C for PmHS2-M2H5, and 45°C for PmHS2-M2D10 and PmHS2-M2E4. Thus, the optimal temperature was about 7°C higher for PmHS2-M2D10 and PmHS2-M2E4, than for the parental PmHS2 (Fig. 3). PmHS2-M2A8, PmHS2-M2E4, and PmHS2-M2H5 converted at least 2 times less UDP-sugars than PmHS2, indicating a decrease in the polymerization activity in comparison to the parental PmHS2. The polymerization efficiency of PmHS2-M2D10 and PmHS2 was comparable when incubated at their respective optimum temperatures, revealing that PmHS2-M2D10 overall catalytic efficiency was not decreased but was only shifted as a function of the temperature. PmHS2-M2D10 was used for further analysis.

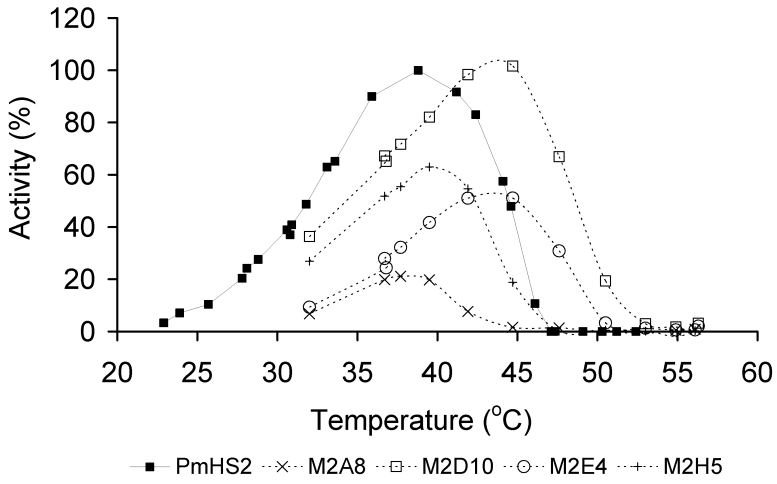

Figure 3. Temperature optimum of PmHS2 and the PmHS2 mutants. The purified PmHS2 and the PmHS2 mutants (M2A8, M2D10, M2E4 and M2H5) were incubated for 4 h at temperatures in between 32°C and 56°C. The amount of UDP-sugar converted after 4 h of incubation was assessed by the coupled enzyme assay. The PmHS2 activity at 38°C was set to 100% activity for comparison.

The thermal stability of the purified parental PmHS2 and PmHS2-M2D10 was also compared after 15 min exposure between 32°C and 56°C. Using the coupled enzyme assay it was determined that the parental PmHS2 polymerization activity decreased about 60% when pretreated for 15 min at 50.5°C, while PmHS2-M2D10 still exhibited 90% of its activity. PmHS2 was completely inactivated after 15 min incubation at 53°C; PmHS2-M2D10 pretreated at 53°C still exhibited 50% of polymerization activity. The analysis of the polymerization product on 2% agarose gel confirmed these results (Fig. 4).

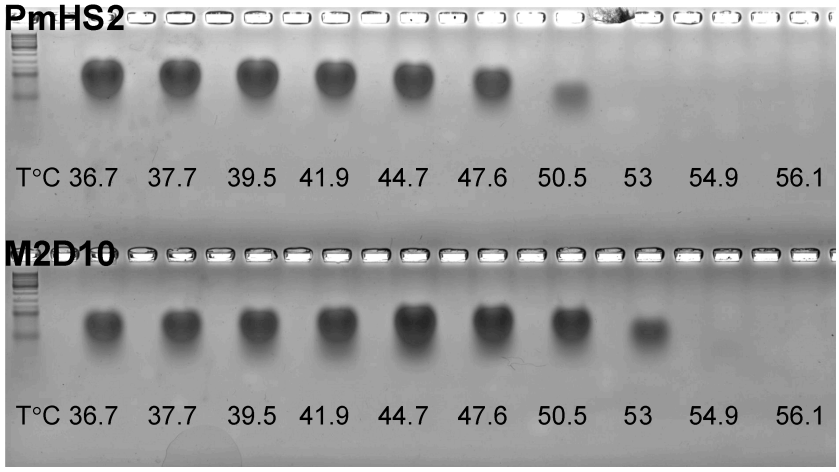

Figure 4. Thermal stability of parental PmHS2 and PmHS2-M2D10. Agarose gel electrophoresis analysis of the polymerization reaction mixture (18 h/32°C) of purified PmHS2 and PmHS2-M2D10 after 15 min exposure between 32°C and 56°C (first lane. DNA marker).

Analysis of the PmHS2-M2D10 mutations contributing to the increase of thermal stability

The PmHS2-M2D10, found to be more thermostable than the other mutated PmHS2 enzymes, was sequenced. Sequencing revealed that PmHS2-M2D10 exhibited several mutations (Table 2). PmHS2-M2D10 contained a cysteine (C) instead of a serine (S) at position 182 (S182C). In addition, the forward primer (5´-CGTAGGATCCATGAAGGGAAAAAAAGAGATG-3´) had a deletion of the base thymine (T) in the "ATG" start codon. This was not due to a mistake during the design of the primers but was due to the quality of the primers. About 6% of the primers seemed to contain this mistake. Fortunately, the open reading frame was conserved, and this deletion resulted only in the deletion of the first six amino acids (aa) at the *N*-terminus (Δ6aa *N*-terminus). The reverse primer (5´-GCATGAGCTCTAAAAAATAAAAGGTAAACAGG-3´) contained also a deletion due to the primer quality which leaded in the removal of an adenine (A) in the underlined region. It resulted in the change of a tyrosine (Y) into isoleucine (I) at position 649 (Y649I), the deletion of a leucine (L) at position 651 (ΔL651), followed by the insertion of a stop codon. Thus, the PmHS2-M2D10 mutant did not have a *C*-terminus composed of 32 amino acids including a V5-epitope and a His-tag (Δ32aa-tag *C*-terminus).

Site directed mutagenesis was applied on the parental PmHS2 (PmHS2-tag) in order to investigate which mutation was responsible for the increase in thermal stability of PmHS2-M2D10. The following PmHS2 enzymes were constructed: PmHS2-(Δ6aa *N*-terminus), PmHS2-(S182C), PmHS2-(Y649I, ΔL651, Δ32aa-tag *C*-terminus), and PmHS2-(Y649I, ΔL651). All mutated enzymes were expressed, purified and characterized for their polymerization activity and thermal stability (data not shown). When incubated in polymerization conditions (32°C/24 h), the PmHS2-(Δ6aa *N*-terminus), the PmHS2-(Y649I, ΔL651), and the PmHS2-(Y649I, ΔL651, Δ32aa-tag *C*-terminus) showed comparable activity as the parental PmHS2, while the PmHS2-(S182C) showed a lower activity. At the exception of the PmHS2-(Y649I, ΔL651, Δ32aa-tag *C*-terminus), all the PmHS2 variants showed a lower thermostability than PmHS2-M2D10. Thus, the analysis of all the PmHS2 variants mentioned above showed that the decrease of activity at 32°C of PmHS2-2D10 is due to the amino acid change in position 182 (S182C), and that the increase of thermal stability is related to the deletion of the *C*-terminus tag (Fig. 5). Thus, PmHS2-(Δ32aa-tag *C*-terminus) was cloned and characterized for its thermostability. PmHS2-(Δ32aa-tag *C*-terminus) is in fact not a mutated version of PmHS2, but the native PmHS2 and is referred to as PmHS2-wild type (PmHS2-WT) (Table 2).

Table 2. Details of the PmHS2-M2D10 mutations compared to the parental PmHS2.
The parental PmHS2 (PmHS2-tag) was used as template for the error prone PCR. The changes and deletions of amino acid are underlined.

	***N*-terminus**	**S182C**	***C*-terminus**
PmHS2 (PmHS2-tag)	MKGKKEMTQI	ANTTSKVRV	FTDLIPCLPFYFL-KGEL(aa)$_n$-V5epitope-6His-Stop
PmHS2-M2D10	Δ6aa - MTQI	ANTTCKVRV	FTDLIPCLPFIFΔ-Stop
PmHS2-WT (PmHS2-Δ32aa-tag *C*-terminus)	MKGKKEMTQI	ANTTSKVRV	FTDLIPCLPFYFL-Stop

Characterization of the PmHS2-WT (PmHS2-(Δ32aa-tag *C*-terminus))

Despite the absence of the His-tag, the PmHS2-WT could be purified on a Ni-NTA resin (Fig. 5A). This result suggests that the 19 histidines present in PmHS2 enable the protein to bind non-specifically to the resin. In addition, the purified PmHS2-WT was found to have a higher purity than the parental PmHS2 (PmHS2-tag) after the purification step on Ni-NTA column.

PmHS2-WT exhibited the same polymerization behavior as PmHS2-tag with respect to the polymer elongation when incubated at 32°C in the presence of equimolar UDP-sugar concentrations ranging from 0.25 to 20 mM of each UDP-sugars (Fig. 5B) (Chapter 2). The PmHS2-tag and PmHS2-WT specific activity were similar, about 0.14 nmol of UDP-sugar converted/µg PmHS2/min, during 2 to 8 h of incubation at 32°C. However, the thermostability was improved; PmHS2-WT was completely inactivated after 15 min exposure at temperature 61°C, while PmHS2-tag was inactivated after heat exposure at 53°C (Fig. 5C).

The characterization of the PmHS2-WT enzyme activity at temperatures above 32°C showed that the absence of a *C*-terminus tag increased the temperature optimum of PmHS2-WT by at least 10°C (Fig. 6D). After 4 h incubation at temperatures below 40°C, the polymerization activity was similar for PmHS2-WT and PmHS2-tag. When incubated above 40°C, PmHS2-WT polymerization increased with the temperature, while PmHS2-tag activity decreased untill being completely inactivated at 47°C. The PmHS2-WT enzyme activity was inactivated at 60°C, and the optimum incubation temperature of PmHS2-WT was found at 50°C. PmHS2-WT converted twice the amount of UDP-sugar when incubated at its optimum temperature, resulting in the accumulation of about 9 mM of UDP in the reaction mixture. This suggests that PmHS2-WT is less sensitive to UDP inhibition than PmHS2-tag (Chapter 2).

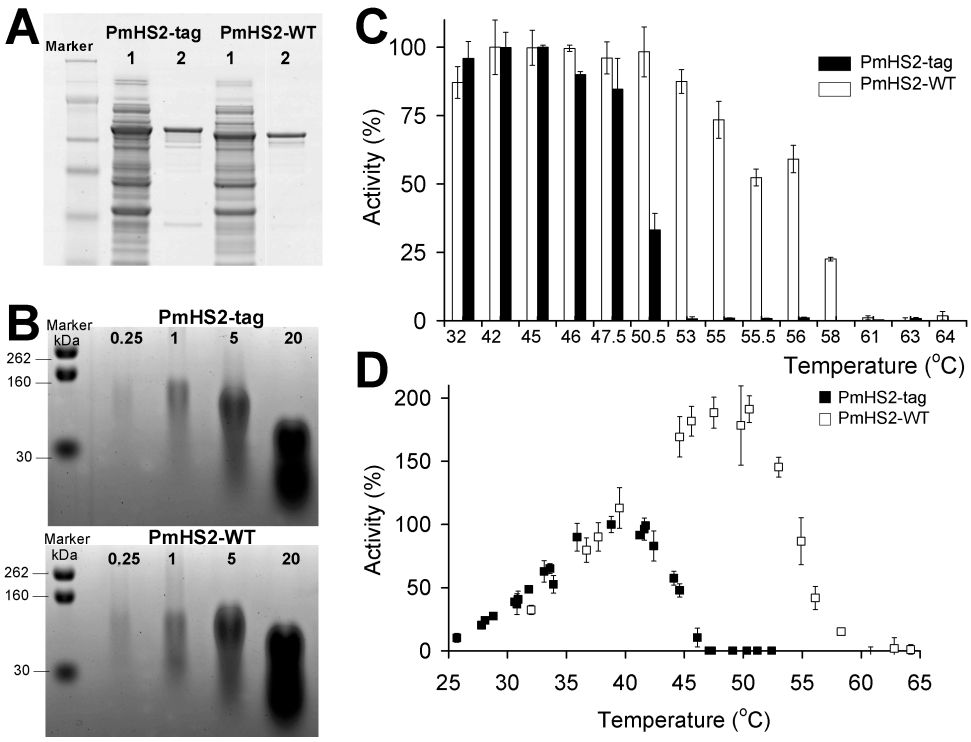

Figure 5. Characterization of the purified PmHS2-(Δ32aa-tag *C*-terminus) referred as PmHS2-WT in comparison with PmHS2-tag. A). 20% SDS-PAGE gel of PmHS2-tag and PmHS2-WT. Lane 1 crude cell extract and lane 2 soluble fraction, respectively. B). 2% agarose gel electrophoresis analysis of the polymerization product synthesized after 24 h of incubation at 32°C in the presence of equimolar UDP-sugar concentrations (0.25 to 20 mM of each UDP-sugar). C). The amount of UDP-sugar, converted by the PmHS2-tag and PmHS2-WT after 15 min exposure between 42°C up to 64°C prior to 24 h of incubation at 32°C for polymerization, was quantified by the coupled enzyme assay. The polymerization activity observed at 32°C was set to 100% activity for both the PmHS2-tag and PmHS2-WT. D). Temperature optimum of PmHS2-tag and PmHS2-WT. Enzymes were incubated for polymer synthesis at temperatures in between 32°C and 64°C for 4 h. The amount of UDP-sugar converted was assessed by the coupled enzyme assay. The polymerization activity of the PmHS2-tag at 39°C was set to 100% activity.

Discussion

GAG-synthase mutant libraries have not been reported yet due to the difficulty to have a good assay to screen the mutants of interest. Here, we report that heparosan polymers synthesized by crude PmHS2 can be analyzed by agarose gel electrophoresis. Thus, it was possible to screen 1000 PmHS2 mutants obtained by error prone PCR for improved thermostability using agarose gel electrophoresis. Despite the low rate in mutations, the level of inactivation of PmHS2 variants after the first screening round was high since 70% of the PmHS2 mutants were inactivated. This observation may suggest that PmHS2 integrity is highly sensitive to amino acid changes. For the second screening round, the analysis of the polymerization

activity of crude enzymes exposed at 56°C for 15 min, enabled to identify 70 PmHS2 mutants with an increased resistance to heat in comparison to the parental PmHS2. Among the nine selected and further analyzed PmHS2 mutants, it was observed that not all the crude PmHS2 variants selected showed an improved thermostability after purification. The cell extract was found to protect against the denaturation of PmHS2 when exposed to elevated temperatures, thus it is critical to characterize the mutants isolated with the screening assay after their purification. Nevertheless, it seems that the amount of heparosan product observed by agarose gel electrophoresis analysis is a good indication of the increased stability. Crude PmHS2 variants capable to synthesize large amount of heparosan were found to be more thermostable. Only the PmHS2-M2D10 mutant showed comparable polymerization efficiency as the parental PmHS2, and had a polymerization temperature optimum 7°C higher. Furthermore, PmHS2-M2D10 stability to heat exposure increased about 4°C, in comparison to PmHS2. DNA sequencing of PmHS2-M2D10 revealed the presence of several mutations: Δ6aa *N*-terminus, S182C, Y649I, ΔL651, and Δ32aa *C*-terminus resulting in the deletion of the *C*-terminus protein tag. Directed mutagenesis was applied on the parental PmHS2 to determine which amino acid changes increased the thermostability. For example, PmHS2-(S182C) had a reduced polymerization activity and a lower thermostability, suggesting the role of the cystein in the incorrect folding of PmHS2 (Kumari, Tlapak-Simmons et al. 2002). The deletion of the *C*-terminus tag enabled to increase the thermostability and the catalytic activity of PmHS2 (PmHS2-WT) at higher temperatures. It was found that in the absence of a *C*-terminus tag, PmHS2 optimum temperature was increased by 10°C, and that at this temperature the polymerization activity was 2 times higher. The overall polymerization mechanism between 32°C and 40°C was not influenced by the presence or the absence of the *C*-terminus tag. The use of immobilized-metal affinity chromatography (IMAC), such as Ni-NTA agarose, is frequently reported for the purification of proteins (Liu, Zhang et al. 2002). The His-tag is commonly used at the *N*-/*C*-terminus of recombinant enzymes since it is referred as being nondestructive, usable under mild elution conditions and having little interference on protein folding (Gaberc-Porekar and Menart 2001). Here, we observed that the *C*-terminus tag (KGEL(aa)$_n$-V5epitope-6His-tag) did not influence the polymerization activity of PmHS2 at temperature below 40°C, but that it decreased PmHS2 thermostability when incubated above 40°C. Thus, this suggests that at elevated temperature the *C*-terminus tag affects the folding of PmHS2, leading to its denaturation.

Here we have shown that agarose gel electrophoresis assay is a suitable method for the screening of GAG-synthase mutant libraries. At this moment only few methods have been developed to screen GTs mutant libraries either using recombinant *E. coli* cell-based assays

(Aharoni, Thieme et al. 2006; Yu, Tyo et al. 2008; Park, Park et al. 2009; Yang, Rich et al. 2010), crude cell extract (Persson and Palcic 2008), or purified enzymes (Gosselin, Alhussaini et al. 1994). For the screening of mutant libraries there is a real interest to have an assay with a limited amount of steps to reduce the labor and the cost. Ultra high throughput assay using cell-based assays are very efficient (10^8 mutants/day), they require little labor and are relatively cheap (Aharoni, Thieme et al. 2006). However, such *in vivo* systems cannot be used to select for enzymes with improved stability, and they seem to be limited for screening of GAG-synthases since only the chain initiation step could be assessed (Aharoni, Thieme et al. 2006). A general pH-indicator assay using crude enzymes has been reported for the screening of an α-1,3-galactosyltransferase mutant library (Persson and Palcic 2008). This assay, described by the authors as a general method for the screening of any GT mutant library, could be suitable for the screening of GAG-synthases after optimization of the polymerization reaction buffer. In our opinion, agarose gel electrophoresis assay is highly valuable for the screening of GAG-synthases mutant libraries. Indeed, the analysis of the crude enzyme polymerization reaction by agarose gel electrophoresis has many advantages. Firstly, the use of crude enzyme to assess the activity of a library of mutants saves a lot of labor and reduces the experimental cost. The fact that there is not a prerequisite for the reaction buffer enables to perform the polymerization reaction in the biocatalysts' optimal conditions. In addition, this screening strategy seems to be suitable for the screening of mutant libraries for increased stability to temperature, pH, and solvent stability, and as well for their capability to use modified UDP-sugars as substrate. For example, agarose gel electrophoresis assay could be used to select for heparosan synthases capable of transferring both IdoUA and GlcUA residues (Weïwer, Sherwood et al. 2008), or modified sugar residues (Liu, Xu et al. 2010). Furthermore, agarose gel assay is easy, does not require the use of expensive equipments, and it can be performed in any molecular biology and biochemistry laboratories. Therefore, this assay has a large potential for the screening of GAG-synthases, and probably also for the screening of any glycosyltransferases (GTs) involved in the synthesis of negatively charged polysaccharides.

To summarize, we have developped a general assay using agarose gel electrophoresis analysis to screen a library of mutated PmHS2 for increased thermostability. The screening of a GAG-synthase mutant library is reported here for the first time. Our method has a large potential and could be used for the screening of any GAG-synthase mutant libraries for improved catalytic properties. In addition, we showed that PmHS2 enzymes are not stable to amino acid changes and that the *C*-terminus tag (V5 epitope and His-tag), generally considered as having no influence on the protein stability, has a strong effect on PmHS2 thermal stability and catalytic efficiency at elevated temperatures.

Abbreviations

UDP, uridine diphosphate; UDP-GlcUA, UDP-glucuronic acid; UDP-GlcNAc, UDP-N-acetylglucosamine; PmHS2, *Pasteurella multocida* heparosan synthase 2; epPCR, error-prone PCR.

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Chapter 6

General discussion

A part of this Chapter is in preparation for publication as a Review article.

Introduction

Heparin (Hep) and heparan sulfate (HS) are important bioactive polysaccharides due to their involvement in many physiological processes (Linhardt and Toida 2004; Bishop, Schuksz et al. 2007). In addition to the well described antithrombotic activity of heparin, *in-vitro* studies showed that heparin, as well as heparan sulfate molecules have a potential for the treatment of cancer (Yip, Smollich et al. 2006) and for the prevention of virus infections (Rusnati, Vicenzi et al. 2009). The biological activity of Hep/HS is determined by their polymer chain length, saccharide unit composition, and sulfation pattern.

Currently, anticoagulant heparin is obtained from animal derivatives or chemical synthesis which results in the production of a limited range of molecules. Therefore, for the development of well defined Hep/HS-based drugs, (chemo)enzymatic production systems controlling tightly each of the Hep/HS synthesis steps are of interest.

In this research, the polymerization of heparosan has been studied in order to obtain new insight in the controlled enzymatic synthesis of heparin and heparan sulfate. Among the described heparosan synthases (Chapter 1), the glycosyltransferase heparosan synthase 2 – PmHS2– from *Pasteurella multocida* was the biocatalyst of choice due to its ability to exhibit both acetylglucosaminyl and glucuronyl transferase activities (DeAngelis and White 2004) and its capability to use modified UDP-sugars which is of interest for the synthesis of defined Hep/HS-like polymers (Sismey-Ragatz, Green et al. 2007; Liu, Xu et al. 2010)

In this chapter is discussed how PmHS2 can be used to control the heparosan synthesis. The PmHS2 polymerization mechanism is compared with the polymerization mechanism of other GAG-polymerases and glycosyltransferases. Then, the modification steps following the polymerization of heparosan polysaccharide are described and it is explained briefly how they can be used in a (chemo)enzymatic system to synthesize well defined Hep/HS polysaccharides. As an example, the outline of a (chemo)enzymatic system to produce anticoagulant Hep/HS-like compounds is described. Finally, the perspectives for the synthesis of novel and defined Hep/HS molecules using alternative production systems are discussed based on the recent advancements and discoveries in this field.

Control of heparosan chain length and size distribution

Parameters influencing the polymerization process

PmHS2 is a member of the glycosyltransferase (GT) family, more specifically of the glycosyltransferase-A (GT-A) superfamily due to its requirement for divalent metal ions and its conserved D*X*D amino acid domains (Coutinho, Deleury et al. 2003) (Chapter 2). Polymerases belonging to the GT family are separated in two classes depending on their polymerization mechanism: processive and non-processive GTs (Weigel and DeAngelis 2007). Processive GTs release the polymer chain only when the elongation is accomplished. This implies that the polymer cannot bind anymore to the acceptor site once the chain termination has taken place. While with non-processive GTs, the polymer chain is released from the acceptor site after the transfer of each sugar residue. Thus, the polymer chain needs to bind again to the acceptor site in order to be elongated with an additional sugar unit (Weigel 2002). In theory, with non-processive GTs the polymer could be elongated indefinitely (Weigel 2002).

To control the elongation of GAG polymers, it is important to obtain more knowledge about the different elongation processes. Among the GTs, the polymerases involved in the synthesis of the GAG hyaluronan are an exception since both processive and non-processive GTs are reported, depending on the organisms from which they are isolated (Weigel and DeAngelis 2007). The synthesis of the GAG heparosan has only been reported with non-processive heparosan synthases.

Processive glycosyltransferases

As mentioned before, for processive GTs, once the polymer chain is released from the acceptor site, the polymer elongation cannot take place any longer. Thus, the polymer molecular weight is determined by the chain termination, which depends on the polymer binding/retention energy at the acceptor site. Thus, Weigel (2002) suggested that modifications of the binding retention/energy would dramatically influence the molecular weight of the product (Weigel 2002). In addition, the ratio between the acceptor site and the UDP-sugar concentration also influences the polymer molecular weight by determining the number of polymer chains initiated (Jing and DeAngelis 2004), and the number of sugar units distributed among the chains. It was also observed that unbalanced concentration of UDP-

sugars (Cartee, Forsee et al. 2000; Forsee, Cartee et al. 2000; Ventura, Cartee et al. 2006; Chen, Marcellin et al. 2009) induces chain termination, and thus play a crucial role in the polysaccharide chain length.

Non-processive glycosyltransferases

Unlike with the processive GTs, for the non-processive GTs the polymer molecular weight is not determined by the chain termination since the polymer chains can re-bind to the acceptor site (Weigel 2002).

It was observed that the UDP-sugar concentration present in the reaction mixture also influences the polymer molecular weight and size distribution. In the presence of low UDP-sugar concentrations, *P. multocida* PmHS2 initiated only a small number of chains, resulting in polymers with a high molecular weight and a narrower dispersity (Chapter. 2). At higher UDP-sugar concentrations, more chains but with a lower molecular weight were synthesized. This might be explained by the fact that PmHS2-GlcNAc^{+} acceptor/donor sites are saturated with a too high number of heparosan chains (Chapter 4).

In addition, PmHS2 initiates heparosan chains only by transferring a GlcUA to a UDP-GlcNAc acceptor molecule. Thus, the glucuronyl catalytic site concentration (Chapter 4) and the UDP-GlcNAc concentration (Chapter 3) influences the number of heparosan chain synthesized. In the presence of an excess of UDP-GlcUA in comparison to UDP-GlcNAc concentration, less chains were synthesized resulting in a higher molecular weight product. The opposite was observed in the presence of an excess of UDP-GlcNAc (Chapter 3). The influence of non-equimolar UDP-sugar concentrations on the amount of polymer chains initiated and as consequence on the polymer chain length, was also reported for the synthesis of heparosan in *E. coli* K5 (Roman, Roberts et al. 2003).

For the non-processive *P. multocida* GAG synthases PmHS1 (heparosan synthase) (Sismey-Ragatz, Green et al. 2007) and PmHAS (hyaluronan synthase) (Jing and DeAngelis 2004), it was reported that the ratio between UDP-sugar and oligosaccharide template concentrations had a large effect on the polymer chain length and size distribution. PmHS1 and PmHAS exhibited a higher affinity at the acceptor site for short oligosaccharides than for UDP-sugars. Thus, the addition of oligosaccharide templates in the reaction mixture enabled to avoid the polymer chain initiation step, and favored the polymer elongation resulting in a product with a narrower chain length distribution (Jing and DeAngelis 2004) (Sismey-Ragatz, Green et al. 2007). By controlling the amount of PmHAS/UDP-sugar/hyaluronan templates, PmHAS synthesized defined hyaluronan polymers with a molecular weight in the range of 16 kDa to 2 MDa and a polydispersity of about 1.0 - 1.2 (Jing and DeAngelis 2004). In contrast with PmHS1 and PmHAS, it was observed that PmHS2 did not exhibit a higher affinity for the

templates than for the UDP-sugars (Sismey-Ragatz, Green et al. 2007) (Chapter 3). With PmHS2, it appeared that the polymer chain initiation is not controlled by the affinity of the acceptor binding site for short oligosaccharides, but by the affinity for UDP-GlcNAc present in the polymerization reaction (Chapter 3). Based on the results obtained in Chapter 3, it is believed that PmHS2 incubated in the presence of short heparosan oligosaccharide templates and an excess of UDP-GlcUA will only elongate the short templates resulting in heparosan polymers with a narrower size distribution.

The polymer elongation is also influenced by the increase of the reaction mixture viscosity (Chapter 2) (Weigel 2002).

Step by step elongation for controlled synthesis of heparosan oligosaccharides

For the GTs belonging to the GT-A superfamily (Coutinho, Deleury et al. 2003) it has been shown that the substitution of both aspartic acids (D) by asparagines (N) in the D*X*D amino acid motif, results in the inactivation of the catalytic domains. The fact that bi-functional glycosyltransferases can be engineered into two single action transferases has been reported for GAG-synthases (Jing and DeAngelis 2000; Jing and DeAngelis 2003; Kane, White et al. 2006; Sugiura, Shimokata et al. 2008).

In this study it was observed that PmHS2-GlcUA^{+} (glucuronyl transferase) and PmHS2-GlcNAc^{+} (acetyglucosaminyl transferase) incubated together elongated heparosan polymers in the absence of template molecules (Chapter 3). In addition, as a proof of concept, it was shown that PmHS2-GlcUA^{+} and PmHS2-GlcNAc^{+} immobilized on separate columns can synthesize step by step defined heparosan oligosaccharides (Chapter 4). This showed that PmHS2-GlcUA^{+} and PmHS2-GlcNAc^{+} do not need to form a complex together to exhibit catalytic activity. In this respect, the *P. multocida* heparosan synthases are an exception among the known heparosan synthases (Table 1). Indeed, the glucuronyl transferases EXT2 (Senay, Lind et al. 2000; Busse and Kusche-Gullberg 2003) and KfiC (Sugiura, Baba et al. 2010) are not capable to transfer the GlcUA residue to a template molecule when they do not form a protein complex with their acetyglucosaminyl transferases EXT1 and KfiA, respectively.

Table 1. Comparison of the polymerization processes of heparosan polymerases.

Source	Heparosan synthases	Recombinant expression in *E. coli*	Template requirement	Complex formation	Heparosan kDa	Ref
Mammalians	EXT1 & EXT2	—	peptide synthetic aglycon	+	200	(Senay, Lind et al. 2000; Busse and Kusche-Gullberg 2003)
***E. coli* K5**	KfiA & KfiC	+	heparosan K5	+	20	(Sugiura, Baba et al. 2010)
***P. multocida* Type D**	PmHS1	+	—	—	800	(Kane, White et al. 2006)
	PmHS2	+	—	—	130	Chapter 2
	PmHS2 GlcUA+ PmHS2 GlcNAc+	+	—	—	30-100	Chapter 3

The synthesis of heparosan oligosaccharides during the step by step synthesis, described in Chapter 4, revealed that heparosan oligomers can be synthesized in a controlled manner and in the absence of template molecules by PmHS2 single action transferases. The absence of templates complicates the synthesis of monodisperse heparosan oligosaccharides due to the incubation of PmHS2-GlcUA$^+$ in the presence of both UDP-sugars to initiate heparosan chains. This contributes in the synthesis of a mixture of heparosan oligosaccharides. Therefore, the use of oligosaccharide templates (DeAngelis, Oatman et al. 2003; Sugiura, Shimokata et al. 2008) or UDP-disaccharides (Liu, Xu et al. 2010), avoiding the chain initiation during the step by step elongation, should also favor the production of monodisperse heparosan products using PmHS2 single action transferases in contrast with the native PmHS2 (double action transferase). Nevertheless, being able to synthesize step by step heparosan in the absence of templates represents advantage since templates molecules do not need to be synthesized or produced. Thus, to avoid the initiation of new heparosan chains during the elongation cycles, either in the presence or in the absence of templates, it is important to ensure that the reaction mixture is UDP-GlcNAc free when incubated with PmHS2-GlcUA$^+$ (Chapter 3). The incubation step in the presence of PmHS2-GlcNAc$^+$ does not influence the monodispersity of the product since PmHS2-GlcNAc$^+$ cannot initiate heparosan chains.

What to be investigated next in the heparosan synthesis field?

PmHS2 appeared to exhibit a large potential to control heparosan polymerization with respect to the polymer molecular weight and the incorporation of modified sugar residues. High

molecular mass heparosan chains (100 - 130 kDa) with a narrow distribution (PDI = 1.03 - 1.10) can be obtained by incubating PmHS2 in the presence of 0.25 mM to 1 mM of each UDP-sugar (Chapter 2 and Chapter 3). Heparosan oligosaccharides of about 2 to 10 sugar units, can be synthesized using immobilized PmHS2 single action transferases (Chapter 4). In addition, PmHS2 is able to elongate heparosan using modified sugar residues such as UDP-GlcNAc with modified acyl chain (Sismey-Ragatz, Green et al. 2007) and UDP-GlcNTFA (Liu, Xu et al. 2010). These modified heparosan polymers exhibit different behavior towards heparosan lyase (Sismey-Ragatz, Green et al. 2007) and enable to control the sulfation pattern facilitating the *in vitro* synthesis of heparin and heparan sulfate (Liu, Xu et al. 2010).

Thus, PmHS2 and the PmHS2 single action transferases (PmHS2-GlcUA^{+}, PmHS2-GlcNAc^{+}) can be used to synthesize heparosan with specific chain length and modified sugar residues. However, in order to further enlarge the potential of PmHS2 biocatalysts for the synthesis of well defined heparosan polymers and to successfully produce them at large scale and low costs, new hurdles have to taken.

Reduction of heparosan synthesis cost

UDP-GlcUA and UDP-GlcNAc used to synthesize heparosan are commercially available but expensive (€ 1000/g, sigma catalog). Chemical synthesis of UDP-sugars is laborious due to the high number of catalytic steps involved that results in a low yield and turns the kilogram scale production in a non-economically feasible process (Zhao and van der Donk 2003).
In nature, the UDP-sugars pathways are present in almost any living organisms offering a large diversity of biocatalysts (Bülter and Elling 1999). To reduce the synthesis costs inherent to UDP-sugars, several strategies using biocatalysts have been explored to produce UDP-sugars (De Luca, Lansing et al. 1995; Liu, Zhang et al. 2002; Shao, Zhang et al. 2002; Zhao and van der Donk 2003) and to recover the released UDP. Instead of using recombinant enzymes to produce UDP-sugars, the yeast *Saccharomyces cerevisiae* (Ying, Chen et al. 2009) and engineered *E. coli* were used as cell factory (Mao, Shin et al. 2006). The coupling of metabolically engineered bacteria, *E. coli* and *Corynebactrium ammoniagenes*, also has been reported to produce UDP-sugars (Tabata, Koizumi et al. 2000).

Up to now, systems to recover UDP and synthesize UDP-sugars are not economically feasible enough to produce UDP-sugars in high yield and at industrial scale. The production of UDP-GlcUA and UDP-GlcNAc at low cost should be one of the main focuses in the development of (chemo)enzymatic Hep/HS production systems.

Protein engineering of PmHS2

As discussed before, the production of modified heparosan polymers is of interest for the synthesis of a large variety of defined Hep/HS (Sismey-Ragatz, Green et al. 2007; Liu, Xu et al. 2010). Recombinant heparosan synthases exhibiting improved catalytic properties should be developed for the enzymatic synthesis of Hep/HS-like polymers at industrial scale. These enzymes should have a higher catalytic efficiency, an increased stability to temperature, solvent, pH and also to be less sensitive to inhibitor compounds such as UDP (Chapter 2). In addition, there is interest to enlarge the substrate range of heparosan synthases in order to synthesize novel well defined polysaccharides (Weïwer, Sherwood et al. 2008). The improvement and evolvement of biocatalysts can be done either by random (Chapter 5) or site directed mutagenesis. In Chapter 5, an agarose gel electrophoresis assay based on the detection of the synthesized heparosan polymers in the reaction mixture was used to screen a library of crude PmHS2 mutants. Analysis of the polymerization mixture enables to incubate biocatalysts in their optimal buffer condition, and the use of crude enzymes saves time and lowers the cost inherent to protein purification. This assay has a large potential to screen any GAG-synthase mutant libraries for increased stability and for the capability to use modified UDP-sugars as substrates (Weïwer, Sherwood et al. 2008).

PmHS2 versatility

It was observed that PmHS2 is capable of hydrolyzing UDP-sugars into UDP and monosaccharide residue (Chapter 3). When PmHS2 single action transferases are immobilized in the presence of UDP-sugars in order to elongate step by step heparosan; it results in UDP-sugars hydrolysis. UDP-sugars are expensive, thus it is critical to determine the optimal incubation conditions in which the hydrolysis is reduced, and the elongation is favored. On the other hand, the fact that PmHS2 exhibits glycosyltransferase and glycoside hydrolase activity suggests its versatility, which might be of interest to synthesize valuable compounds (Chapter 3) (Hu and Walker 2002; Perugino, Trincone et al. 2004; Zhang, Griffith et al. 2006; Shaikh and Withers 2008). Therefore, the potential of PmHS2 to synthesize UDP-sugars could be investigated.

Chemo/enzymatic synthesis of defined Hep/HS products

The different catalytic steps

The (chemo)enzymatic synthesis of heparin and heparan sulfate polymers is of interest to replace the currently used production systems and to synthesize well defined Hep/HS chains for other medical applications. Hep/HS alternative production systems such as the (chemo)enzymatic synthesis, can be seen as a "construction game" in which building blocks, in this case catalytic steps (Chapter 1), can be added and removed in order to obtain a large variety of defined polymers. In each of the catalytic steps, enzymes and their isoforms modify the polysaccharide chains in a stereo and regio specific manner, which results in the complex and unique structure of heparin and heparan sulfate. All the catalytic steps of the Hep/HS synthesis have been identified and many of biocatalysts involved have been isolated from mammalian cells, expressed in *E. coli* as recombinant proteins (Peterson, Frick et al. 2009), and have been characterized with respect to their potential to be used in an *in vitro* system to produce defined Hep/HS polymer chains. A brief overview of each of the (chemo)enzymatic steps that could be used to produce defined Hep/HS compounds is given in this paragraph.

Synthesis of heparosan

The first step in the synthesis of heparin and heparan sulfate is the polymerization of the polysaccharide backbone heparosan $[\text{-4GlcUA}\beta\text{1-4GlcNAc}\alpha\text{1-}]_n$. Currently, heparosan can be obtained by extraction from the bacterial polysaccharide capsule or using biocatalytic synthesis (Chapter 1). To produce defined heparosan polymers with respect to the control of the polymer molecular weight (Chapter 2, 3, 4) and the incorporation of modified UDP-sugars (Sismey-Ragatz, Green et al. 2007; Liu, Xu et al. 2010), the use of biocatalysts is the most promising method; their application is described in the previous paragraphs.

N-deacetylation and N-sulfation of heparosan

The first step of the post-polymerization modification of heparosan polysaccharide is the *N*-deacetylation/*N*-sulfation of the *N*-acetylglucosamine (GlcNAc) groups. This step is catalyzed by the dual action *N*-deacetylase/*N*-sulfatase (NDST) enzyme in the presence of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as sulfate donor. The PAPS concentration determines the *N*-deacetylation and *N*-sulfation pattern of the Hep/HS polymer defined as

highly *N*-sulfated (NS), non *N*-sulfated (NA) and intermediately *N*-sulfated (NS/NA) (Carlsson, Presto et al. 2008). However, the control of the degree of *N*-deacetylation/*N*-sulfation by adding PAPS to the reaction mixture is not economically feasible (Zhao and van der Donk 2003).

By applying site-directed mutagenesis on NDST, single action *N*-deacetylase (NDase) (Kakuta, Sueyoshi et al. 1999; Duncan, Liu et al. 2006) and single action *N*-sulfotransferase (NST) (Berninsone and Hirschberg 1998) were obtained. These single action NDase and NST have the potential to be use to modulate *in vitro* the *N*-deacetylation/*N*-sulfation step.

Yet, to successfully catalyze at industrial scale a controlled *N*-deacetylation/*N*-sulfation using biocatalysts, incubation conditions in which the NDST step and the regeneration of PAPS can simultaneously be performed have to be optimized (Saribas, Mobasseri et al. 2004). When heparin-like polymers (highly *N*-deacetylated and *N*-sulfated) are required, the *N*-deacetylation and *N*-sulfation can be chemically catalyzed (Kuberan, Beeler et al. 2003; Lindahl, Li et al. 2005).

Glucuronyl C5-epimerization of the N-deacetylated and N-sulfated heparosan

After the NDST step, some of the GlcUA residues are converted into IdoUA by glucuronyl C5-epimerase. The *in vitro* catalysis of this step is difficult to control using biocatalysts due to the fact that soluble C5-epimerase catalyzes a reversible reaction leading to a mixture of GlcUA (65%) and IdoUA (35%) residues (Hagner-Mcwhirter, Lindahl et al. 2000; Li and Lijuan 2010). In addition, the C5-epimerase recognition for the substrate is highly dependent on GlcUA neighboring residues, which limits the variety of epimerization patterns obtained, and it is still unclear what the C5-epimerase substrate prerequisites are in order to convert GlcUA into IdoUA (Li and Lijuan 2010).

Since, the IdoUA residue is important to ensure the biological activity of Hep/HS by increasing the flexibility of the polymer chains, it is critical to control the incorporation of IdoUA into the Hep/HS chains. The discovery or the protein engineering of glucuronyl C5-epimerases capable to catalyze a larger substrate range, such as observed for alginate epimerase (Valla, Li et al. 2001), is of interest to diversify the epimerization pattern for the production of novel and defined Hep/HS molecules. On the other hand, since the chemical catalysis of GlcUA into IdoUA does not represent a useful method, an alternative is to use chemically synthesized UDP-IdoUA as a substrate in the synthesis of heparosan (Weïwer, Sherwood et al. 2008).

O-sulfation of the epimerized heparosan

The last catalytic step in the production of Hep/HS polymers is the *O*-sulfation of the epimerized heparosan by *O*-sulfotransferes: the 2-*O*ST, 6-*O*ST, and 3-*O*ST. The *O*-sulfotranferases require the sulfate donor PAPS to catalyze the sulfation at the oxygen groups. It was shown that immobilized *O*-sulfotransferases catalyze successfully the *O*-sulfation of the Hep/HS polymers when incubated in the presence of the PAPS regeneration system (aryl sulfotransferase-IV and *p*-nitrophenyl sulfate (Burkart, Izumi et al. 2000)) (Chen, Avci et al. 2005).

Although the *O*-sulfation can also be performed chemically (Naggi, De Cristofano et al. 2001), the use of *O*-sulfotransferases to catalyze this step is favored to obtain defined sulfation patterns. A large number of *O*-sulfotransferase isoforms is available; they all catalyze different substrates resulting in the synthesis of a variety of defined sulfation patterns (Habuchi, Tanaka et al. 2000).

Outline of a (chemo)enzymatic system to produce anticoagulant Hep/HS

Anticoagulant "neoheparin" has been produced by applying chemical modifications, at the exception of the enzymatic C5-epimerization, on *E. coli* K5 heparosan (Lindahl, Li et al. 2005). Since chemical modifications are not regio- and strereo-specific, there is an interest to use biocatalysts to tightly control the modifications of heparosan polysaccharides (Kuberan, Beeler et al. 2003; Chen, Avci et al. 2005). Here, an outline of a (chemo)enzymatic system for the production of anticoagulant Hep/HS products, is presented.

The role of IdoUA is dependent on the chain length of the Hep/HS polysaccharides. Although the presence of IdoUA residues is critical for the antiviral activity (Rusnati, Vicenzi et al. 2009) and for the anticoagulant activity of the heparin pentasaccharides (Chen, Jones et al. 2007), it was found that heparin-like molecules composed of 8 to 10 monosaccharides exhibit anticoagulant activity without the presence of IdoUA residues (Kuberan, Beeler et al. 2003; Chen, Jones et al. 2007). The *O*-sulfation of the Hep/HS polymers can occur even in the absence of the IdoUA residues within the chain (Kuberan, Beeler et al. 2003; Chen, Jones et al. 2007). Thus for the production of anticoagulant heparin-like product, the C5-epimerization catalytic step is not critical and can be omitted.

The first step in the (chemo)enzymatic process (Fig. 1) is the polymerization of heparosan oligomers with a length of 8 to 10 monosaccharides (Chen, Jones et al. 2007) by immobilized PmHS2 single action transferases (Chapter 4). Immobilized PmHS2-GlcUA^{+} initiate

heparosan oligosaccharides (Chapter 3). To guarantee the synthesis of monodisperse heparosan oligosaccharides, the complete conversion of the UDP-sugars and removal of the oligosaccharides has to be carefully monitored during the different alternating incubation cycles with PmHS2-GlcUA$^+$ and PmHS2-GlcNAc$^+$. Since anticoagulant heparin is highly *N*-deacetylated and *N*-sulfated, the heparosan oligosaccharides are chemically *N*-deacetylated by hydrazinolysis at 100°C or alkaline treatment with 2 M NaOH at 60-65°C. *N*-sulfation is done using trimethylamine sulfure trioxide (Kuberan, Beeler et al. 2003; Lindahl, Li et al. 2005). The NDST step is followed by the 6-*O* and 3-*O* sulfation of the oligosaccharides by immobilized sulfotransferases (Chen, Avci et al. 2005). During the *O*-sulfation step, the sulfate donor PAPS is regenerated by the use of aryl sulfotransferase-IV (AST-IV EC. 2.8.2.1.) and p-nitrophenyl sulfate (Burkart, Izumi et al. 2000). The PAPS regeneration at the same time as the *O*-sulfation occurs enables to reduce the production cost by about 1000-fold (Chen, Avci et al. 2005).

After each step (polymerization, NDST and *O*-sulfation) the heparosan oligosaccharides are recovered by anion exchange chromatography (Kuberan, Beeler et al. 2003) and lyophilized prior to be desalted using gel filtration (DeAngelis, Oatman et al. 2003).

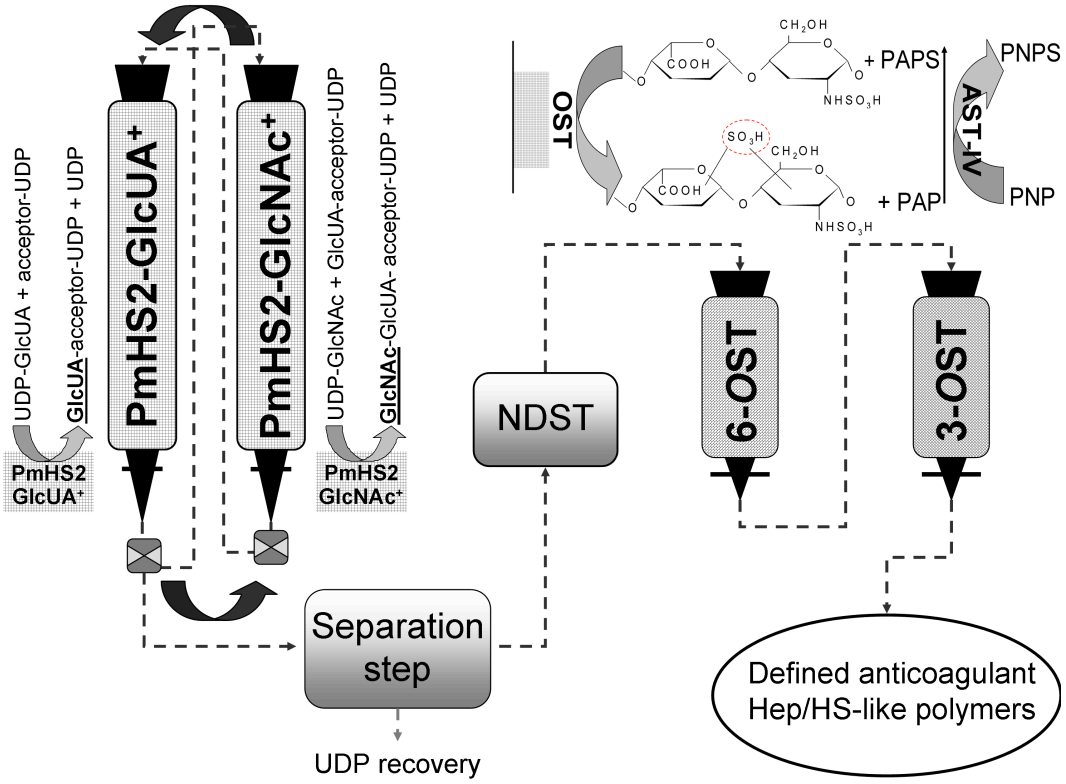

Figure 1. Schematic representation of a (chemo)enzymatic process for the production of anticoagulant heparin-like products.

General conclusions and perspectives

Nowadays, there is still a need for a better understanding of the heparin and heparan sulfate structure/activity relationship. The Hep/HS structures involved in physiological pathways are being identified and elucidated in order to set up new therapeutic strategies for the treatment of cancer and the prevention against virus infection. For this purpose, large libraries of unusual Hep/HS-like molecules are screened for their biological activities using newly developed microarray devices (De Paz and Seeberger 2008; Park, Lee et al. 2008; Liu, Palma et al. 2009; Powell, Zhi et al. 2009). Chinese hamster ovary (CHO) cells are also used to synthesize and study the biological interactions of heparan sulfate in physiological processes (Zhang, Lawrence et al. 2006). Moreover, an artificial Golgi apparatus is currently being developed for nano-scale production of unusual Hep/HS molecules (Martin, Gupta et al. 2009).

In the past decades, biosynthetic pathways of Hep/HS polymers have been extensively investigated. The enzymes involved in the Hep/HS biosynthesis have been isolated, cloned, expressed as recombinant proteins, and characterized for their catalytic activity (Peterson, Frick et al. 2009). The use of these biocatalysts in order to (chemo)enzymatically synthesize defined Hep/HS polymers has been studied.

Here, the potential of heparan synthases to synthesize in a controlled manner heparosan has been evaluated. For this purpose, the *P. multocida* heparosan synthase PmHS2 polymerization mechanism was studied, and it resulted in major contributions in the field of heparosan synthesis. It was found that the heparosan chain length and size distribution is mainly determined by the number of chains initiated which depends on the concentration of UDP-sugars (Chapter 2), on the number of acceptor molecules (UDP-GlcNAc) (Chapter 3), and on the number of acceptor sites (PmHS2-$GlcUA^{+}$) (Chapter 4). It was also found that PmHS2-$GlcNAc^{+}$ plays a critical role in the heparosan chain length by being saturated or inhibited in the presence of a too high number of chains (Chapter 4). A fine tuning of all the parameters could enable to control heparosan polysaccharide elongation. In addition, it was shown that immobilized PmHS2 single action transferases can be used to synthesize step by step heparosan oligosaccharides in the absence of template molecules (Chapter 4). This is an advantage since templates do not need to be produced, thus reducing the cost and the labor of the heparosan oligosaccharide synthesis. Although immobilized PmHS2 single action transferases are suitable to synthesize at industrial scale heparosan oligosaccharides, the optimization of the step by step synthesis is required to avoid the production of a mixture of oligosaccharides (Chapter 4). Finally, an agarose gel electrophoresis assay based on the analysis of the heparosan products synthesized by crude enzymes was evaluated for the screening of a PmHS2 mutant library for improved thermostability. This agarose gel assay has

a large potential to screen heparosan synthase mutant libraries with improved characteristics for the large scale production of novel and defined Hep/HS molecules.

The production of anticoagulant heparin using (chemo)enzymatic systems, instead of using the traditional production method is an interesting approach. However, some hurdles have to be taken to be able to produce large amount of anticoagulant heparin products at industrial scale. One of the biggest challenges in the (chemo)enzymatic synthesis is to reduce the production cost of heparosan, either by recovering UDP for regenerating UDP-sugars or by producing UDP-sugars at low price. Furthermore, it is critical to optimize the step by step elongation of heparosan oligosaccharides in order to synthesize monodisperse products.
For the production of Hep/HS-based molecules to be used in novel therapeutic applications, both the elucidation of interesting Hep/HS structures and the optimization of each (chemo)enzymatic synthesis step is needed. A special effort should be made to understand and control the *N*-deacetylation/*N*-sulfation step, as well as the C5-epimerization step. This will result in the synthesis of well defined Hep/HS polysaccharides to be used in the treatment of cancer and in the prevention against virus infection.

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Summary

Heparin (Hep) and heparan sulfate (HS) are highly sulfated and complex glycosaminoglycan polysaccharides involved in many physiological processes. There is a close relationship between the Hep/HS structures and their biological activity; and it was reported that these polymers have a large potential for medical applications. Heparin is worldwide used as an anticoagulant compound to prevent blood clotting during surgery. Heparan sulfate, an analog of heparin, is not used yet for therapeutic purposes.

The utilization of Hep/HS-based drugs in new therapeutic settings requires the synthesis of well defined heparin and heparan sulfate-like molecules. Since neither the extraction from animal derivates, nor the chemical synthesis, are suitable for the production of a large variety of defined Hep/HS polymers, there is a general interest in developing alternative systems enabling to tightly control Hep/HS synthesis. Heparin and heparan sulfate alternative production systems can be seen as a "construction game" in which the building blocks or catalytic steps can be incorporated in order to obtain polymers with specific and defined final structure.

In mammalian cells, the heparin and heparan sulfate polymers are synthesized by a cascade of enzymatic reactions, in which enzymes and their isoforms catalyze stereo- and regio- specifically the modification of polysaccharide chains resulting in the complex and unique Hep/HS structure (Chapter 1). Up to now, all the catalytic steps of the Hep/HS synthesis have been identified; a good control of each of them will enable the production of defined Hep/HS polymers. During the synthesis of heparin and heparan sulfate, the polymerization of the polysaccharide backbone, known as heparosan, determines the chain length and the size distribution of these polymers. Here, the *Pasteurella multocida* heparosan synthase PmHS2, a bacterial enzyme catalyzing the formation of heparosan polymers, was studied in detail in order to develop methods to control the polymer elongation.

Recombinant PmHS2 enzyme obtained after expression in *Escherichia coli* was characterized for its polymerization mechanism. In Chapter 2, parameters influencing the PmHS2 polymerization activity are described. It was observed that the metallic ions Mn^{2+}/Mg^{2+} are required for the PmHS2 polymerization activity, and that UDP, a by-product of the polymerization reaction, is an inhibitor of PmHS2. In addition, it was shown that the UDP-sugar concentrations influenced the PmHS2 polymerization process with respect to the polymer chain length and size distribution. In the presence of low UDP-sugar concentrations, PmHS2 synthesized heparosan polymers with a high average molecular weight and a narrow size distribution. While, in the presence of high UDP-sugar concentrations, low molecular heparosan polymers with broader distribution were synthesized by PmHS2.

Using site directed mutagenesis techniques, two functional and active PmHS2 single action transferases (PmHS2-$GlcUA^{+}$ and PmHS2-$GlcNAc^{+}$) were obtained. These two single action transferases were used to investigate in detail the heparosan polymerization process catalyzed by PmHS2. In Chapter 3 is described that only the UDP-GlcNAc is used as acceptor molecule to initiate

heparosan chains. As a consequence, it was observed that not only the UDP-sugar concentration influences the polymer molecular weight but also the proportion of each UDP-sugar. In addition, it was found that PmHS2 is able to hydrolyze UDP-sugars; suggesting the versatility of PmHS2.

In Chapter 4, it was observed that each of the two transferase domains have a different influence on the overall polymerization process with respect to catalytic efficiency and polymer elongation. In addition, the PmHS2 single action transferases were immobilized and the elongation of heparosan oligosaccharides was controlled step by step by re-circulating the reaction mixture from the PmHS2-$GlcUA^{+}$ to PmHS2-$GlcNAc^{+}$ columns, respectively. The synthesis of heparosan oligosaccharides was successful, showing that PmHS2 single action transferases do not need to form a complex to be active and to elongate heparosan.

In Chapter 5, a PmHS2 mutant library created by error-prone PCR was screened for increased tolerance to elevated incubation temperatures, using a general agarose gel electrophoresis assay. The identification of thermostable PmHS2 variants validated this screening method. The influence of a *C*-terminus tag on PmHS2 thermal stability and polymerization activity was investigated.

In Chapter 6, the results obtained are discussed with respect to the control of heparosan elongation and size distribution. In addition, an overview is given on each of the catalytic step needed to synthesize heparin and heparan sulfate in a (chemo)enzymatic system. Recent advancements and discoveries in this field, as well as the perspectives concerning the synthesis of heparin and heparan sulfate molecules are presented. It is clear that the future will be an interesting and an exciting time concerning research on the synthesis of well defined Hep/HS oligomers and polymers.

Samenvatting

Heparine (Hep) en heparan sulfaat (HS) zijn veelvuldig gesulfateerde en complexe glycosaminoglycaan polysachariden, die in vele fysiologische processen betrokken zijn. Er is een nauwe verwantschap tussen de Hep/HS structuren en hun biologische activiteit, en het is beschreven dat deze polymeren een grote potentie hebben voor medische toepassingen. Heparine wordt wereldwijd gebruikt als een antistollingsmiddel dat het klonteren van bloed voorkomt gedurende de operatie. Heparan sulfaat, een variant van heparine, is nog niet gebruikt voor therapeutische doeleinden.

Voor het gebruik van Hep/HS-gebaseerde medicijnen in nieuwe therapeutische toepassingen is de synthese van goed gedefinieerde heparine en heparan sulfaat-achtige moleculen noodzakelijk. Aangezien noch de extractie uit dierlijk weefsel noch de chemische synthese geschikt zijn voor de productie van een grote variëteit van gedefinieerde Hep/HS polymeren, is er een algemene interesse in het ontwikkelen van alternative systemen die nauwkeurig gecontroleerde synthese van Hep/HS mogelijk maken. Alternative productie systemen van heparine en heparan sulfaat kunnen gezien worden als een "bouw spel", waarbij de bouwstenen of katalytische stappen kunnen worden gecombineerd om zo polymeren te verkrijgen met specifieke en gedefinieerde eindstructuren.

In dierlijke cellen worden heparine en heparan sulfaat polymeren gesynthetiseerd door een reeks van enzymatische reacties, waarin enzymen en hun isovormen stereo- en regio-specifieke modificaties op de polysacharideketen katalyseren, resulterend in de complexe en unieke Hep/HS structuur (Hoofdstuk 1). Tot nu toe zijn alle katalytische stappen van de Hep/HS synthese geïdentificeerd; een goede controle van elk van hen zal de productie van gedefinieerde Hep/HS polymeren mogelijk maken. Gedurende de synthese van heparine en heparan sulfaat is het de polymerisatie van de polysaccharide keten (bekend als heparosan), die de de keten lengte en de distributie van de grootte van deze polymeren bepaalt. In dit proefschrift was de *Pasteurella multocida* heparosan synthase PmHS2, een bacterieel enzym dat de formatie van heparosan polymeren katalyseert, in detail bestudeerd om zo een methode te onwikkelen om polymeerverlenging te reguleren.

Recombinant PmHS2 enzym, verkregen na expressie in *Escherichia coli,* werd gekarakteriseerd op het polymerisatiemechanisme. In Hoofdstuk 2 zijn de parameters beschreven die de PmHS2 polymerisatieactiviteit beïnvloeden. Er werd geobserveerd dat de metaalionen Mn^{2+}/Mg^{2+} nodig zijn voor de PmHS2 polymerisatieactiviteit en dat UDP, een bijproduct van de polymerisatiereactie, een remmer is van PmHS2. Daarnaast werd getoond dat de UDP-suikerconcentraties de PmHS2 polymerisatieproces, met betrekking tot de polymeerketenlengte en de distributie van de ketenlengte, beïnvloeden. In de aanwezigheid van lage UDP-suikerconcentraties synthetiseerd PmHS2 heparosan polymeren met een groot gemiddelde molecuulgewicht en een smalle ketenlengtedistributie.

Daarentegen worden er in de aanwezigheid van hoge UDP-suikerconcentraties kleine moleculaire heparosan polymeren met bredere distributie gesynthetiseerd door PmHS2.

Via site directed mutagenesis technieken zijn twee functionele en actieve PmHS2 transferase mutanten met enkelzijdige activiteit (PmHS2-GlcUA^{+} en PmHS2-GlcNAc^{+}) verkregen. Deze twee 'single action' transferases werden gebruikt om in detail de heparosan polymerisatieproces, gekatalyseerd door PmHS2, te onderzoeken. In Hoofdstuk 3 is beschreven dat alleen de UDP-GlcNAc wordt gebruikt als acceptormolecuul om heparosan ketens te initiëren. Dit heeft tot gevolg dat niet alleen de UDP-suikerconcentratie het molucuulgewicht van de polymeer beïnvloedt, maar dat ook de proportie van elke UDP-suiker van belang is. Verder werd er gevonden dat PmHS2 in staat is om UDP-suikers te hydrolyseren; dit geeft de veelzijdigheid van PmHS2 aan.

In Hoofdstuk 4 werd er geobserveerd dat elk van de twee transferase domeinen een verschillende invloed heeft op het gehele polymerisatieproces met betrekking tot de katalytische efficiëntie en polymeerverlenging. Vervolgens werden de PmHS2 'single action' transferases geïmmobiliseerd en de verlenging van heparosan oligosachariden werd stap voor stap gecontroleerd door het re-circuleren van het reactiemengsel van de PmHS2-GlcUA^{+} naar de PmHS2-GlcNAc^{+} kolom, respectievelijk. De synthese van heparosan oligosachariden was succesvol, wat aantoonde dat PmHS2 'single action' transferases niet een complex hoeven te vormen om actief te zijn en om heparosan te verlengen.

In Hoofdstuk 5 was een PmHS2 mutant bibliotheek, gecreëerd via error-prone PCR, gescreend voor de toegenomen tolerantie van verhoogde incubatietemperaturen via een algemene agarose gel electrophorese assay. De identificatie van thermostabiele PmHS2 varianten valideerde deze screening methode. De invloed van een C-terminus tag op de thermostabiliteit van PmHS2 en polymerisatieactiviteit werd onderzocht.

In Hoofdstuk 6 worden de verkregen resultaten bediscussiëerd met betrekking tot de controle op heparosanverlenging en de distributie van de ketenlengte. Daarnaast wordt er een overzicht gegeven op elk van de katalytische stappen die nodig zijn om heparine en heparan sulfaat in een (chemo)enzymatisch systeem te synthetiseren. Recente vooruitgang en ontdekkingen in dit veld, naast de perspectieven die de synthese van heparine en heparan sulfaat moleculen biedt, zijn getoond. Het is duidelijk dat de toekomst een interessante en spannende tijd zal zijn omtrent het onderzoek in de synthese van goed gedefinieerde Hep/HS oligomeren en polymeren.

Résumé

L'héparine et le sulfate d'héparan sont des polysaccharides appartenant à la famille des glycosaminoglycanes (GAG). Ils sont présents en grande quantité dans notre organisme où ils sont impliqués dans plusieurs fonctions physiologiques. Il a été observé que leur structure définit leur activité biologique.

L'héparine est connue pour son activité anticoagulante; elle est utilisée en médecine et en chirurgie afin d'éviter les thromboses. Le sulfate d'héparan n'est pas encore utilisé à des fins thérapeutiques, mais il est, au même titre que l'héparine, considéré comme ayant un grand potentiel en médecine.

Lorsque l'on considère les méthodes traditionnelles de production de l'héparine, soit par extraction à partir de tissus animaux ou soit par synthèse chimique, aucune de ces méthodes n'est adaptée pour la production de molécules bien définies et spécifiques. Il est pour cette raison, important de développer des méthodes alternatives de productions qui permettent de contrôler chaque étape de la synthèse de l'héparine et du sulfate d'héparan pour produire des molécules définies et ainsi assurer l'utilisation de l'héparine et du sulfate d'héparan dans de nouvelles thérapies.

Chez les mammifères, l'héparine et le sulfate d'héparan sont synthétisés par une cascade de réactions enzymatiques dans lesquelles les enzymes et leurs isoformes catalysent de façon spécifique les polysaccharides; ce qui résulte en la synthèse de molécules définies et complexes (Chapitre 1). Jusqu'à présent toutes les étapes de la synthèse ont été identifiées; un bon contrôle de chacune d'entre elles rendra possible la synthèse de molécules spécifiques d'héparine et de sulfate d'héparan. Ainsi, les méthodes alternatives de production de l'héparine et du sulfate d'héparan peuvent être vues comme un jeu de construction dans lequel chaque « block » peut être ajouté ou non, afin d'obtenir la parfaite structure à la fin de la synthèse. La synthèse commence par l'élongation du polysaccharide précurseur de l'héparine et du sulfate d'héparan connu sous le nom d'héparosan. L'héparosan détermine la longueur de la chaîne et la distribution des polymères d'héparine et de sulfate d'héparan.

Ici, l'enzyme *Pasteurella multocida* heparosan synthase PmHS2, qui synthétise l'héparosan a été étudiée afin de contrôler l'élongation des chaines d'héparosan. L'enzyme recombinante PmHS2 a été obtenue après expression dans *Escherichia coli* et a été caractérisée pour ses capacités à contrôler la synthèse d'héparosan. Dans le Chapitre 2, les paramètres qui influencent l'activité de polymérisation de PmHS2 sont décrits. Il a été reporté que les ions métallique Mg^{2+} et Mn^{2+}sont indispensables à l'activité de PmHS2, et que l'UDP (uridine diphosphate) produit pendant la consommation du substrat, inhibe la réaction de PmHS2. De plus, la concentration en substrat (UDP-monosaccharide) influence la longueur des chaînes d'héparosan et la distribution des polymères synthétisés. En présence de peu de substrat,

PmHS2 synthétise de longs polymères d'héparosan ayant une faible distribution, alors qu'en présence d'une grande quantité de substrat, des polymères de plus petite taille et avec une plus large distribution sont synthétisés.

En utilisant des méthodes de mutagenèse dirigée sur PmHS2, deux actives et fonctionnelles mono transférases (PmHS2-GlcUA^{+} et PmHS2-GlcNAc^{+}) ont été obtenues. Ces deux enzymes ont permis d'étudier en détail le mécanisme de polymérisation de PmHS2. Le Chapitre 3 décrit le fait que le substrat UDP-GlcNAc (UDP-N-acetylglucosamine) est le premier accepteur utilisé pour initier la synthèse des chaînes d'héparosan, et que le substrat UDP-GlcUA (UDP-acid glucuronique) est le premier monosaccharide donneur. Ainsi la concentration en substrat n'est pas le seul paramètre à influencer la réaction, mais la proportion de chacun des UDP-monosaccharide (UDP-GlcNAc et UDP-GlcUA) est aussi importante. Il a été aussi observé que l'enzyme PmHS2, en plus d'être une glycosyltransférase, est aussi une UDP-hydrolase. Cette caractéristique suggère la versatilité de PmHS2; ce qui pourrait être intéressant pour la synthèse d'importantes molécules.

Dans le Chapitre 4, les mono transférases PmHS2 (PmHS2-GlcUA^{+} et PmHS2-GlcNAc^{-}) ont été immobilisées sur deux différentes colonnes. Après leur immobilisation, les mono transférases ont été utilisées pour allonger étape par étape la chaîne d'héparosan en re-circulant la réaction d'une colonne à l'autre. La synthèse d'oligomères d'héparosan est possible et démontre que les mono transférases PmHS2-GlcUA^{+} et PmHS2-GlcNAc^{+} n'ont pas besoin de former un complexe enzymatique pour être active et allonger héparosan. Il a été aussi montré que les deux mono transférases influencent différemment l'activité enzymatique et l'élongation des chaînes d'héparosan.

Le Chapitre 5 décrit l'utilisation de gel d'agarose pour analyser une librairie de mutants de PmHS2 créée en utilisant la technique de PCR à erreur. L'isolation de variants de PmHS2 plus stables à une hausse de la température d'incubation a permis de valider cette méthode. L'influence du «tag» protéique présent à l'extrémité *C*-terminale de PmHS2, sur la stabilité thermale et l'activité enzymatique de PmHS2 a été évaluée.

Les résultats obtenus sont discutés en fonction de leur contribution pour contrôler l'élongation d'héparosan (Chapitre 6). Toutes les étapes nécessaires pour synthétiser (chemo) enzymatiquement l'héparine et le sulfate d'héparan sont détaillées. Les récentes découvertes faites dans ce domaine sont présentées avec une attention particulière concernant le contrôle de la synthèse de l'héparine et du sulfate d' héparan. Il semble évident que les dix prochaines années vont être fleurissantes dans le domaine de la synthèse de molécules spécifiques d'héparine et de sulfate d'héparan.

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Anaïs

About the author

Curriculum Vitae

Anais Aliette Emile was born on 13th September 1982 in Libourne (France). She grew up in Port Saint Foy, in a nice village in the country side of Dordogne. After finishing High School in 2000, she moved to La Rochelle, to conduct a Bachelor in Biology before going to Montpellier to undertake a Master degree in Food Sciences and Technology and a Master degree in Biotechnology. Meanwhile, she spent 3 months in Burgos (Spain) where she discovered the world of cheese food factory working on the optimization of a production plant, and helped in the development of new dairy products. After this Spanish experience, she worked for about 1 year at DSM-Delft (The Netherlands). During that time she investigated nisin as food preservative against Gram-negative bacteria. After a short period back in Montpellier to finish her master courses, she took off for Wageningen (The Netherlands) to do her master thesis within AFSG, the department of food microbiology (WUR) and TIFN. The aim was to characterize high pressure resistant *Listeria monocytogenes*, known as a food pathogen.

During that time, she found a PhD position that she started in September 2006 in between the bioconversion group at AFSG and the Bioprocess engineering department (WUR) under the supervision of Prof. dr. Gerrit Eggink. This PhD thesis aimed to control the synthesis of heparosan using the PmHS2 glycosyltransferases; the resulting work is presented here.

She is now working as a postdoc at Torrey Pines Institute for Molecular Science (TPIMS) in Florida (USA) for the development of anticancer drugs under the supervision of Dr. Dmitriy Minond.

Publications

Van Boeijen, I.K., Chavaroche A.A. Valderrama, W.B., Moezelaar, R., Zwietering, M.H., Abee, T. (2010) Population diversity of *Listeria monocytogenes* LO28: phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. *Applied Environmental Microbiology*. 76(7): 2225-33.

Chavaroche, A.A.E., Springer, J., Kooy, F., Boeriu, C., and Eggink, G. (2010) In vitro synthesis of heparosan using recombinant *Pasteurella multocida* heparosan synthase PmHS2. *Applied Microbiology and Biotechnology*. 85: 1881-1891.

Chavaroche, A.A.E., van den Broek, L.A.M., Springer, J., Boeriu, C., and Eggink, G. (2011) Analysis of the polymerization initiation and activity of Pasteurella multocida heparosan synthase PmHS2, an enzyme with glycosyltransferase and UDP-sugar hydrolase activity. *Journal of Biological Chemistry*. 286: 1777-1785.

Chavaroche, A.A.E., van den Broek, L.A.M., Springer, J., Boeriu, C., and Eggink, G. (2011) Controlled synthesis of heparosan oligosaccharides by *Pasteurella multocida* PmHS2 single action transferases. *Submitted for publication*.

Chavaroche, A.A.E., Springer, J., van den Broek, L.A.M., and Eggink, G. (2011) Agarose gel electrophoresis assay to screen *Pasteurella multocida* heparosan synthases library for thermostable PmHS2 variants. *Submitted for publication*.

Overview of the competed training activity

Discipline specific activities

Courses

Bioinformatics - a user's approach (WUR. 2007)
Polysaccharides as Food Colloids and Biomaterials (VLAG. 2007)
Food enzymology (VLAG. 2008)
Summer course Glycoscience (VLAG. 2008)
Masterclass "Directed Evolution and Protein Engineering for Biocatalysis" (GBB. 2009)

Symposia and International conferences

B-Basic symposium (The Netherlands. 2007, 2008, 2009, and 2010)
Netherlands Biotechnology Congress (The Netherlands. 2008)
Carbohydrate bioengineering Meeting CMB8 (Italy. 2009)
Biotrans-International Symposium on Biocatalysis and Biotransformations (Switzerland. 2009)

General courses

Literacy and End Note (WUR. 2006)
Vlag PhD week (VLAG. 2007)
The art of writing (CENTA. 2007)
Radiation course (VanHall Larenstein. 2008)
Mobilizing your - scientific – network (SENSE Education desk. 2010)

Optional

PhD Trip BioProcess Engineering (WUR. 2008)
Brain and Game day Process Engineering (WUR. 2008)

Presentations

Poster presentation

B-Basic symposium (The Netherlands. 2007, 2008, 2009, and 2010)
Netherlands Biotechnology Congress (The Netherlands. 2008)
Japan PhD trip (2008)
International conference Carbohydrate bioengineering Meeting CMB8 (Italy. 2009)

Oral presentation

B-Basic symposium (The Netherlands. 2008 and 2010)

The research described in this thesis was performed at the business unit Biobased Product, Food and Biobased research, Wageningen University and Research Center, The Netherlands. It was part of a joint project with the department of Bioprocess Eginnering of Wageningen University and MSD Oss (formely Organon N.V.), which was financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public-private NWO-ACTS program (ACTS, Advanced Chemical Technologies for Sustainability).

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