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Combinatorial one-pot chemoenzymatic synthesis of heparin

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ABSTRACT

Contamination in heparin batches during early 2008 has resulted in a significant effort to develop a safer bioengineered heparin using bacterial capsular polysaccharide heparosan and recombinant enzymes derived from the heparin/heparan sulfate biosynthetic pathway. This requires controlled chemical *N*-deacetylation/*N*-sulfonation of heparosan followed by epimerization of most of its glucuronic acid residues to iduronic acid and *O*-sulfation of the C2 position of iduronic acid and the C3 and C6 positions of the glucosamine residues. A combinatorial study of multi-enzyme, one-pot, *in vitro* biocatalytic synthesis, carried out in tandem with sensitive analytical techniques, reveals controlled structural changes leading to heparin products similar to animal-derived heparin active pharmaceutical ingredients. Liquid chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy analysis confirms an abundance of heparin's characteristic trisulfated disaccharide, as well as 3-*O*-sulfo containing residues critical for heparin binding to antithrombin III and its anticoagulant activity. The bioengineered heparins prepared using this simplified one-pot chemoenzymatic synthesis also show *in vitro* anticoagulant activity.

1. Introduction

Heparin, the first biopolymeric drug, possesses a wide range of structural heterogeneity owing to its biosynthesis (Bhaskar et al., 2012). Heparin's diverse fine structure is further complicated by an animal-sourced, tissue-based recovery, leading to considerable structural differences within commercial heparin active pharmaceutical ingredients (APIs) (Linhardt & Gunay, 1999). Serious concerns about control of livestock, the primary source of heparin, have been raised since the 1990s following a series of incidents involving bovine spongiform encephalopathy, viral infections and prion contamination (Wilesmith, Wells, Cranwell, & Ryan, 1988). Lack of quality control during initial recovery stages led to adulteration of the pharmaceutical heparin supply with oversulfated chondroitin sulfate (OSCS), resulting in an international crisis in 2008 associated with over 100 deaths reported in US alone (Guerrini et al., 2008; Liu, Zhang, & Linhardt, 2009).

The inherent problems with animal tissue-based heparin production have motivated us to develop a commercially feasible chemoenzymatic heparin preparation process (Zhang et al., 2008). This is based on bacterial fermentation of *Escherichia coli* K5 to generate a capsular polysaccharide heparin precursor, which is then chemically *N*-deacetylated and *N*-sulfonated (Hickey, Bhaskar, Linhardt, & Dordick, 2013; Wang et al., 2011). The iterative application of selected recombinant enzymes, derived from the heparin biosynthetic pathway and expressed in *E. coli*, epimerize uronic acid residues and sulfates the C2, C3 and C6 positions (Hickey et al., 2013; Wang et al., 2011). In summary, C5-epimerase epimerizes uronic acid residues, followed by sulfation at the C2 position by 2-*O*-sulfotransferase (2-*O*ST) in the presence of an aryl sulfotransferase IV (AST IV) based cofactor regeneration system (Bethea, Xu, Liu, & Pedersen, 2008; Burkart, Izumi, Chapman, Lin, & Wong, 2000; Sheng, Xu, Dulaney, Huang, & Liu, 2012; Zhang et al., 2008). This is followed by sulfation at the C6 position by two isoforms of 6-*O*-sulfotransferase (6-*O*ST-1 & -3) in the presence of the cofactor regeneration system leading to generation of non-anticoagulant

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heparin structure (Chen et al., 2005; Restaino et al., 2013; Zhang et al., 2001; Zhang et al., 2008). 3-*O*-sulfotransferase-1 (3-*O*ST-1) then sulfates the C3 position, also in the presence of the cofactor regeneration system to generate anticoagulant heparin (Myette et al., 2002; Zhang et al., 2008). A similar sequential approach led to another version of bioengineered heparin derived from partially *N*-deacteylated/*N*-sulfonated heparosan as substrate (Wang et al., 2011). This chemoenzymatic approach has also been employed to generate an analogue of ultra low molecular weight heparin (ULMWH), Arixtra (Xu et al., 2011).

The total synthesis of full length heparin polysaccharides is considered infeasible owing to large number of modest yield steps and side product formation (Bhaskar et al., 2012; DeAngelis, Liu, & Linhardt, 2013; Driguez, Potier, & Trouilleux, 2014; Petitou et al., 1987). One-pot chemical synthesis in organic chemistry is frequently employed for simplified synthesis of glycoconjugates and sugar building blocks (Koeller & Wong, 2000). One-pot chemical synthesis of heparin oligosaccharides from sugar building blocks with low to moderate overall yield has been previously described (Polat & Wong, 2007; Wang et al., 2010). This one step synthesis enables high speed processing of analogues with increased overall process yield. Combinatorial one-pot synthesis can potentially be used toward preparation of heparin mimetic microarrays for deciphering the effect of structural heterogeneity on structure activity relationship (SAR) and heparin–protein interactions (Capila & Linhardt, 2002; Feizi, 2003; Noti, de Paz, Polito, & Seeberger, 2006; Wang et al., 2010). As an alternative to the sequential process design (Fig. 1), we aimed at development of a one-pot chemoenzymatic synthesis of heparin from *N*-sulfo heparosan leading to generation of biologically active bioengineered heparin products.

2. Methods

2.1. Recombinant enzymes and N-sulfo heparosan preparation

Recombinant *E. coli* strains expressing fusion proteins of C5-epi, 2-*O*ST, 6-*O*ST-1, 6-*O*ST-3, 3-*O*ST-1 and AST IV were grown in LB medium (MP Biomedicals) at 37 °C using rotary air shaker (New Brunswick Scientific Innova 44R) (Burkart et al., 2000; Chen et al., 2005; Chen, Jones, & Liu, 2007; Zhang et al., 2008). Recovered cell pellets were stored at −80 °C until purified. Recombinant enzymes were purified from clarified cell lysates using either MBP- or His-affinity chromatography. Briefly, cell pellets were re-suspended in respective extraction buffers, lysed and centrifuged to obtain a clear cell lysate. The clarified cell lysate was then loaded onto respective affinity column connected to a GE Äkta purifier system. Elution was carried out using either high maltose (for MBP tagged proteins) or high imidazole (for His tagged proteins) containing buffers. The eluted protein was stored at −80 °C with 10–15% glycerol, until further use.

E. coli K5 capsular polysaccharide, heparosan, was purified from the supernatant of fed batch fermentation using ammonium sulfate precipitation (Wang et al., 2011). *N*-sulfo heparosan (*N*SH) was prepared by partial chemical *N*-deacetylation and *N*-sulfonation of heparosan as described earlier (Wang et al., 2011). Titanium dioxide based depolymerization was employed to reduce the molecular weight, if required (Higashi et al., 2011). Analysis of the *N*SH product obtained indicated following characteristics: Number average molecular weight (M_n) = 11,100 ± 200 Da; weight average molecular weight (M_w) = 18,800 ± 200 Da; polydispersity index (PDI) = 1.69 ± 0.01; % N-sulfo groups = 81.4 ± 0.9%.

2.2. Combinatorial one-pot chemoenzymatic synthesis of heparin

Initial combinatorial chemoenzymatic heparin synthesis experiments were carried out using 1 mg of *N*SH as substrate in 50 mM MES, pH 7 buffer. The reaction mixture consisted of 0.1 mg/mL of *N*SH substrate and 300 μM of sulfo group donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS), an essential cofactor for sulfotransferases. A 5 mM concentration of *p*-nitrophenyl sulfate (PNPS, Sigma) was used in the reaction mixture as the sulfo group donor to establish an AST IV based co-factor recycling system for PAPS regeneration (Burkart et al., 2000). The control reaction contained 0.1 mg/mL each of C5-epi, 2*O*ST, 6-*O*ST-1, 6-*O*ST-3 and AST IV. The 3-*O*ST-1 was not included in the initial combinatorial synthesis of heparin to reduce product complexity in order to simplify analysis (3-*O*-sulfo group containing sequences are resistant to heparin lyases and thus do not afford disaccharide products). The concentration of three groups of enzymes was varied combinatorially by either 2-fold (0.2 mg/mL) or 10-fold (1 mg/mL): 1. C5-Epi & 2-*O*ST individually as well as together; 2. 6-*O*ST-1 & 3 individually as well as together; and 3. AST IV alone. All enzymes not varied were maintained constant at 0.1 mg/mL, as was the reaction control. The reaction mixtures were incubated overnight at 37 °C. The resulting products were analyzed using disaccharide analysis and optimal conditions were identified.

In the second set of experiments, 2 mg of the product formed using the two best conditions, identified through the combinatorial experiments, were treated with 3-*O*ST-1 at a final concentration of 0.1 mg/mL, after the end of overnight incubation and the reaction mixture was incubated for an additional day. The product was purified using anion exchange chromatography and further evaluated using chemical and biological assays.

2.3. Strong anion exchange (SAX) purification of bioengineered heparin

Reaction product, obtained after the second enzymatic one-pot preparation, was boiled and centrifuged, and the supernatant was filtered using a 0.22 μm filter. The clarified permeate was then dialyzed using centrifugal ultrafiltration units (Amicon centrifugal filter units, Millipore) and DI water. Dialyzed polysaccharide solution was then loaded onto a 20 mL Q-Sepharose fast flow (GE life sciences) strong anion exchange (SAX) glass column connected to a GE *Äkta* purifier FPLC system. Prior to loading the sample, Q-Sepharose column was washed with 4 column volumes of DI water, 4 column volumes of 20% v/v ethanol and 4 column volumes of DI water. After loading the sample, column was washed using 4 column volumes of buffer A (DI water) and 4 column volumes of 0.4 M NaCl by mixing buffer A and buffer B (2 M NaCl in DI water). This was followed by step elution at 2 M NaCl by buffer B. Fractions eluted with 2 M salt were collected, dialyzed and lyophilized. These samples were used for further analysis.

2.4. Enzymatic digestion for disaccharide analysis and tetrasaccharide mapping

For disaccharides analysis, heparin lyases 1, 2, and 3 (10 mU each) in 5 μL of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) were added to 10 μg heparin sample in 100 μL of distilled water and incubated at 35 °C for 10 h to degrade heparin sample completely (Yang, Chang, Weyers, Sterner, & Linhardt, 2012). The products were recovered by centrifugal filtration using a YM-10 micro-concentrator (Millipore), and the heparin disaccharides were recovered in the flow-through and freeze-dried. The digested heparin disaccharides were dissolved in water to concentration of 50–100 ng/2 μL for liquid chromatography (LC)–mass spectrometric (MS) analysis.

For tetrasaccharide analysis, 40 mU of heparin lyase 2 in 20 μL of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) was added to 50–100 μg heparin sample in 100 μL of distilled water and

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