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Expression of heparan sulfate sulfotransferases in *Kluyveromyces lactis* and preparation of 3′-phosphoadenosine-5′-phosphosulfate

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Heparan sulfate (HS) belongs to a major class of glycans that perform central physiological functions. Heparin is a specialized form of HS and is a clinically used anticoagulant drug. Heparin is a natural product isolated from pig intestine. There is a strong demand to replace natural heparin with a synthetic counterpart. Although a chemoenzymatic approach has been employed to prepare synthetic heparin, the scale of the synthesis is limited by the availability of sulfotransferases and the cofactor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Here, we present a novel method to produce secreted forms of sulfotransferases in the yeast cells, *Kluyveromyces lactis*. Five sulfotransferases including *N*-sulfotransferase, 2-*O*-sulfotransferase, 3-*O*-sulfotransferase 1 and 6-*O*-sulfotransferases 1 and 3 were expressed using this method. Unlike bacterial-expressed sulfotransferases, the yeast proteins can be directly used to modify polysaccharides without laborious purification. The yeast-expressed sulfotransferases also tend to have higher specific activity and thermostability. Furthermore, we demonstrated the possibility for the gram-scale synthesis of PAPS from adenosine 5'-triphosphate at only 1/5000th of the price purchased from a commercial source. Our results pave the way to conduct the enzymatic synthesis of heparin in large quantities.

Keywords: heparan sulfate / heparin / *K. lactis* / PAPS / sulfotransferase

Introduction

Heparan sulfate (HS) is a highly sulfated polysaccharide representing a major component on the mammalian cell surface and in the extracellular matrix. HS interacts with a variety of proteins to regulate many different biological systems, including blood coagulation, viral/bacterial infection, tumor metastasis and inflammatory response (Bernfield et al. 1999; Esko and Selleck 2002). Heparin, the specialized form of HS, is a commonly used anticoagulant drug with the worldwide production exceeding 100 t year^{-1} (Aquino et al. 2010). Pharmaceutical heparin is primarily isolated from porcine intestinal mucosa through a long supply chain that posts a potential risk of contaminants and adulteration (Liu et al. 2009). Recent worldwide outbreak of contaminated heparin underscores the vulnerability of heparin supply chain. Therefore, a method that can prepare synthetic heparin in a confined facility is highly desirable (Peterson et al. 2009). Chemical synthesis of short heparin fragments is accomplished via a complex and inefficient scheme. Attempts to synthesize any fragments larger than an octasaccharide have been proved to be extremely challenging (Petitou and van Boeckel 2004). Given the fact that the average size for a full-length heparin is about 50 saccharide units, preparation of heparin from a total synthetic approach is nearly impossible. Preparation of heparin-like polysaccharide from a bacteria capsular polysaccharide known as heparosan using a chemical method has been reported. However, the method yields 3-*O*-sulfated glucuronic acid unit, a residue that is not believed to be present in natural heparin (Lindahl et al. 2005). An enzymatic approach to prepare synthetic heparin has recently been developed to overcome the difficulties encountered by chemical synthesis.

The HS biosynthetic pathway involves multiple enzymes, including HS polymerase, epimerase and sulfotransferases (Figure 1). HS polymerase is responsible for building the polysaccharide backbone, containing the repeating unit of -GlcUA-GlcNAc-. The backbone is then modified by *N*-deacetylase/*N*-sulfotransferase (NDST, having two separate domains displaying the activity of *N*-deacetylase and *N*-sulfotransferase (NST), respectively), C_5-epimerase (converting GlcUA to IdoUA), 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST) and 3-*O*-sulfotransferase (3-OST) to produce the fully elaborated HS. These enzymes can be used to prepare synthetic heparin. The enzymatic synthesis of HS from heparosan was first carried out at the microgram scale (Kuberan et al. 2003). The milligram-scale synthesis of HS was reported by our lab (Chen et al. 2005, 2007; Copeland et al. 2008). The success of a larger-scale

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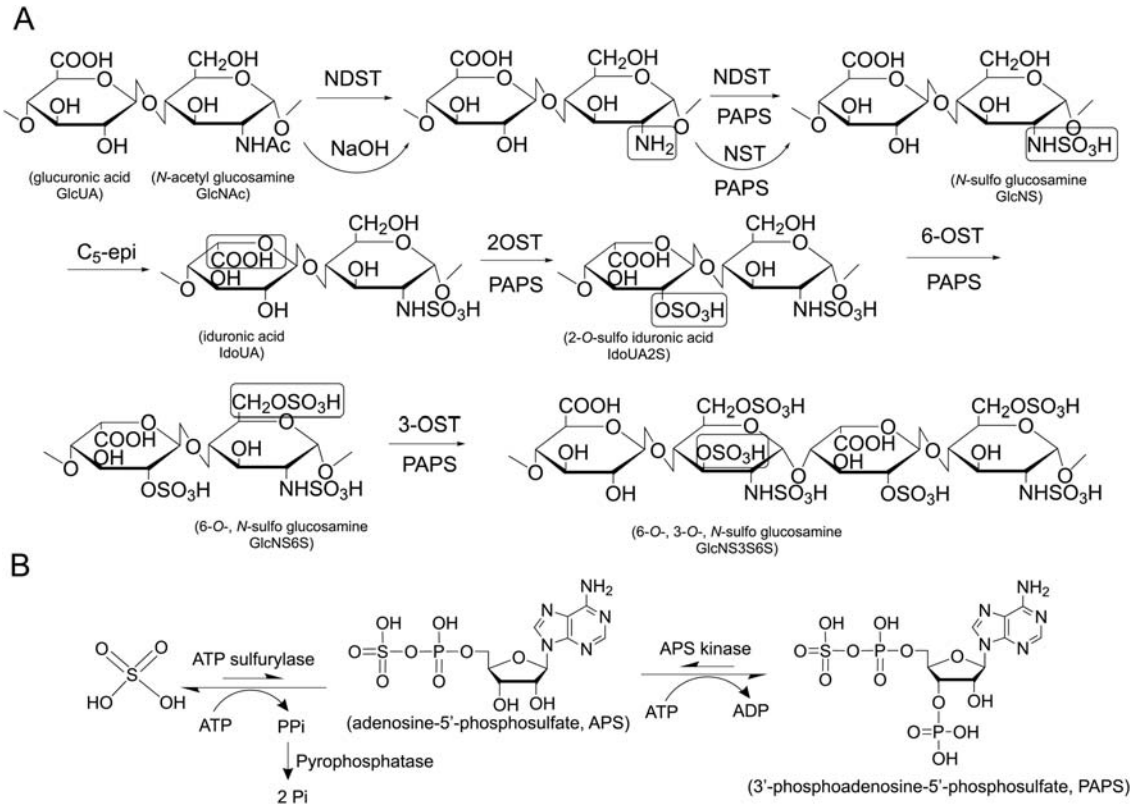

Fig. 1. The biosynthetic/chemoenzymatic synthesis of heparin and HS-like polysaccharides and PAPS. (**A**) HS biosynthetic and chemoenzymatic synthesis pathway. Polysaccharide backbone is a copolymer of GlcUA-GlcNAc repeating disaccharide unit. The straight arrows indicate the reactions catalyzed by enzymes. The curved arrows indicate the conversion using a chemoenzymatic approach. The reaction sites at each modification step are cycled. The names and abbreviations for the saccharide units are displayed underneath the sugar unit. (**B**) The steps involved in PAPS synthesis. Sodium sulfate and adenosine 5'-triphosphate (ATP) are first converted to adenosine 5'-phosphosulfate (APS), and then the second phospho group is added to produce PAPS. PPi represents pyrophosphate, and Pi represents phosphate.

synthesis was based on using the recombinant biosynthetic enzymes expressed in *Escherichia coli* and a cofactor recycling system. The expression of enzymes in *E. coli* allowed us to have the easy access to the proteins. Coupling the cofactor recycling system with the sulfotransferases reduced the cost for the use of sulfo donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Despite the limited success, using the recombinant proteins from bacterial to prepare heparin raises the concerns of the potential bacteria endotoxin contamination. To this end, a protocol that is devoid of bacterial endotoxin and cost-effective for the large-scale production of HS biosynthetic enzymes is ideal for preparing synthetic heparin.

The yeast *Kluyveromyces lactis* expression system has been successfully used for the industrial-scale production of proteins over decades. Most of the proteins can be produced in the secreted forms, significantly simplifying the down-stream purification process (Van Ooyen et al. 2006). *K. lactis* has been affirmed as a "Generally Recognized As Safe" organism by the US FDA (Bonekamp and Oosterom 1994). Numerous pharmaceutical grade proteins have been produced in *K. lactis*, such as interleukin $1-\beta$, macrophage colony stimulating factor and insulin precursor (Van Ooyen et al. 2006). Additionally, the expression in *K. lactis* does not require the induction by methanol, and thus the explosion-proof equipment for methylotrophic yeasts such as *Pichia pastoris* is not required (Van Ooyen et al. 2006). These advantages make *K. lactis* well suited for the large-scale preparation of HS biosynthetic enzymes.

In this work, we constructed several *K. lactis* strains expressing the secreted form of recombinant sulfotransferases, including NST, 2-OST, 3-OST-1, 6-OST-1 or 6-OST-3. The crude sulfotransferases can be directly used to prepare sulfated polysaccharides. To compare the *K. lactis* enzymes with the bacterial counterparts, the proteins were purified with a heparin-Sepharose column and a 3′-phosphoadenosine-5′-phosphate (PAP)-Agarose column. All these five yeast-expressed enzymes show higher specific sulfotransferase activity, ranging from 2- to 10-fold. Furthermore, we demonstrate that 3-OST-1 and 6-OST-3 have significantly higher thermostability than that of the *E. coli*-expressed enzymes. In addition, we also show the results of PAPS synthesis and purification at gram scale through a simple process, and the product can be used to prepare sulfated polysaccharides. Our results demonstrated clear advancement for synthesizing heparin in large scale.

Results

Optimization of the expression of HS sulfotransferases in K. lactis

Our goal was to prepare a secreted form of recombinant proteins, which will eliminate the purification steps. To this end,

the N-terminal transmembrane domain of sulfotransferases was replaced by *K. lactis* α-mating factor (α-MF) domain, a signal peptide to allow the target proteins to be secreted into the medium (Colussi et al. 2005; Klabunde et al. 2007). The initial expression levels were low, especially for 6-OST-3, which was barely above the detection limit.

We enhanced the expression of 6-OST-3 by three approaches, altering the media composition, decreasing the culture temperature and increasing the pH of media (Figure 2). It was observed that the addition of ethylenediaminetetraacetic acid (EDTA) or casamino acids increased the antiserpin single-chain antibody production by 3–5-fold (Borgenstrom et al. 2003). We observed that the addition of 2% casamino acids into the medium increased the expression level by about 2-fold (Figure 2A). Temperature also played a significant role in the expression of 6-OST-3. The activity of 6-OST-3 in the spent medium was increased about 10-fold by dropping the growth temperature to 16°C from 28°C (Figure 2B). We also found that the expression of 6-OST-3 is sensitive to the pH. Increasing the pH value from 5.0 to 7.0 elevated the expression level by 3-fold (Figure 2C). It is important to note that the growth rate after induction was similar under different assayed conditions. By combining the effects of medium composition, temperature and pH, we improved the expression of 6-OST-3 by more than 30-fold. Using the conditions for 6-OST-3 as a starting point, we briefly optimized the expression of 3-OST-1, 6-OST-1, NST and 2-OST.

Increasing the copy number of expression cassette may increase the expression of protein (Macauley-Patrick et al. 2005). Since all of the expressing cells harbor multiple copies of the expression cassette, the copy number was not an issue in our study. We also compared the expression level of 6-OST-3 in the wild-type strain *K. lactis* 699 vs. that in the commercially available protease-deficient strains *K. lactis* YCT389, YCT390, YCT569 and YCT598 (New England Biolabs) as it is believed that the proteases could degrade the target proteins. Unfortunately, neither of the tested cell lines displayed an improved expression level (data not shown).

The specific activity of sulfotransferases expressed in K. lactis *is higher than that expressed in* E. coli

Totally, five sulfotransferases, NST, 2-OST, 3-OST-1, 6-OST-1 and 6-OST-3, were successfully expressed under the optimal conditions (Figure 3). Our measurement suggests that NST, 2-OST, 3-OST-1 and 6-OST-3 have similar levels of sulfotransferase activities and the level of 6-OST-1 is almost 4-fold higher than that of others. We estimated that the amount of crude enzymes in 1 L of media adequately sulfate 15–100 mg of polysaccharide substrates. The proteins were then purified by heparin-Sepharose and PAP-Agarose chromatography, which method was used to purify the proteins from natural sources (Orellana et al. 1994; Habuchi et al. 1995; Kobayashi et al. 1996; Liu et al. 1996). The recombinant proteins expressed in *E. coli* were obtained by following the procedures described previously (Chen et al. 2005, 2007). Next, we compared the specific activity of the enzymes expressed in *K. lactis* with their counterparts expressed in *E. coli* (Figure 4). Overall, the specific activity of the enzymes expressed in *K. lactis* was higher than those expressed in bacteria although with different extents. Among them, the specific activity of NST and 2-OST expressed in *K. lactis* was increased by 2-fold. The specific activity of 6-OST-1 and 6-OST-3 expressed in *K. lactis* showed the highest value, increased by 8–10-fold. The specific activity of 3-OST-1 expressed in *K. lactis* was increased by 4-fold.

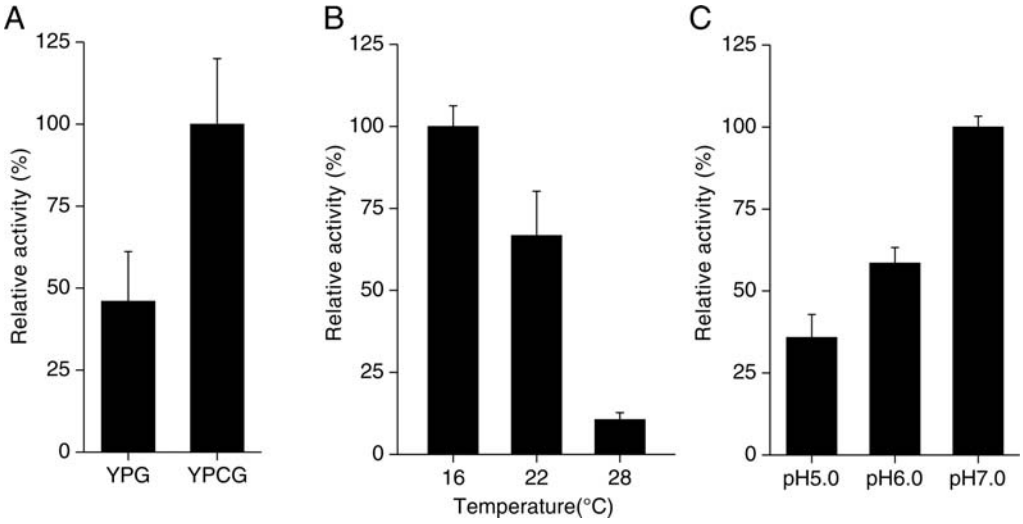

Fig. 2. Optimization for the expression of 6-OST-3 in *K. lactis*. (**A**) Effects of casamino acids on 6-OST-3 expression. YPCG medium is similar to YPG medium with additional 2% casamino acids. The host strain is *K. lactis* 699. The cultures (OD_{600} = 1.0) were induced for 48 h at 28°C, then sulfotransferase activity was determined as described in *Materials and methods*. (**B**) Effects of temperature on 6-OST-3 expression. YPCG medium was used to induce the protein expression with *K. lactis* 699 as the host strain, and the induction was carried out at different temperatures for 48 h. (**C**) Effects of pH on 6-OST-3 expression. YPCGN medium was used to induce the protein expression with *K. lactis* YCT598 as the host strain, and the induction was carried out at 16°C for 48 h. The data presented are the average of three determinations, and the error bars indicate the standard deviations.

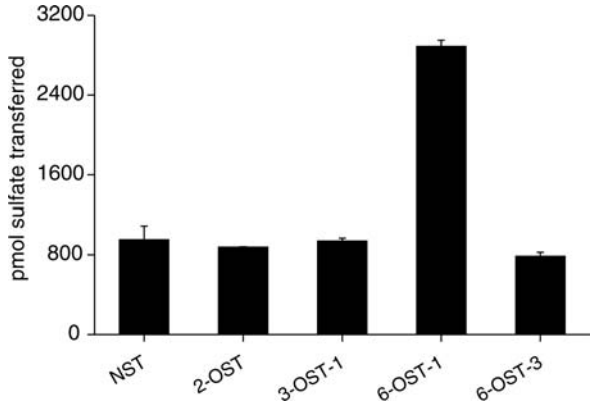

Fig. 3. Sulfotransferase activity in the supernatant. Sulfotransferase expression was carried out in YPCGN medium at 16°C for 48 h. Sulfotransferase activity assay was carried out by incubating 20 μL of filtered supernatant with appropriate substrate in 100 μL of reaction buffer at 37°C overnight. The data presented are the average of two determinations, and the error bars indicate the ranges.

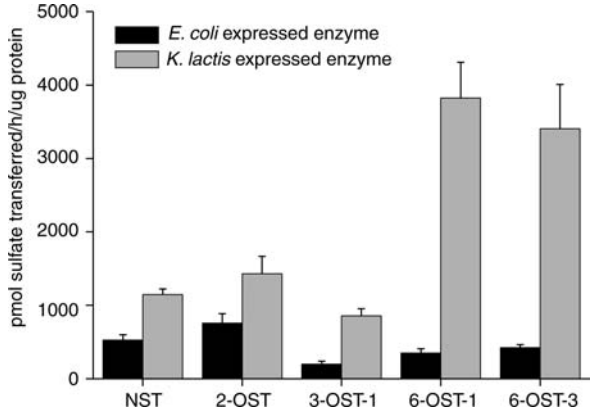

Fig. 4. Comparison of the specific activity between *E. coli*- and *K. lactis*-expressed sulfotransferases. Same amount of purified protein was mixed with appropriate substrate in reaction buffer, and the activity was determined after 1 h of incubation at 37°C. The data presented are the average of two determinations, and the error bars indicate the ranges.

Comparison of the thermostability between E. coli- *and* K. lactis-*expressed sulfotransferases*

In an effort to improve the enzymatic synthesis efficiency, a thermally stable enzyme is desirable. We hypothesized that the sulfotransferases expressed in *K. lactis* are glycosylated and thus have higher thermal stability than bacterial enzymes. To this end, equal amount of purified enzymes expressed in *K. lactis* and *E. coli* was maintained in the reaction buffer at 37°C. At different time points, the enzymes were removed and assayed for the sulfotransferase activities by the addition of ^{35}S-labeled PAPS into the reaction mixture. The results show that the thermostability of sulfotransferases was increased or unchanged with the exception of NST (Figure 5). We found that the most profound effect on the stability was for 6-OST-3. The activity of 6-OST-3 (*K. lactis*) remained unchanged up to 96 h, whereas the activity of 6-OST-3 (*E. coli*) was nearly completely lost after 48 h (Figure 5C). The 3-OST-1 also showed a

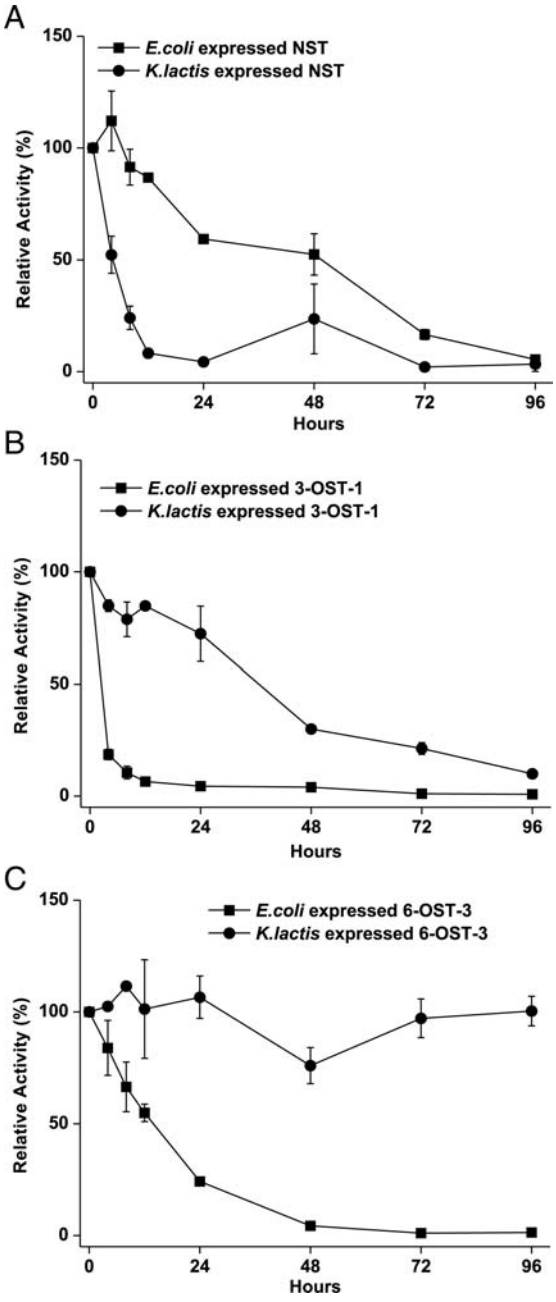

Fig. 5. Comparison of the thermostability between *E. coli*- and *K. lactis*-expressed sulfotransferases. Same amount of protein was mixed with appropriate substrate in reaction buffer without PAPS and incubated at 37°C. At different time points during incubation, 75 μL of aliquots was removed and the sulfotransfer reactions were performed for 1 h at 37°C by adding [^{35}S] PAPS into the aliquots. The data presented are the average of two determinations, and the error bars indicate the ranges.

significant improvement on the thermostability as about 50% of 3-OST-1 activity remained at around 30 h, whereas the activity of 3-OST-1 (*E. coli*) was dropped below the detection limit in 6 h (Figure 5B). Both the *K. lactis* version 2-OST and the *E. coli* version 2-OST were very stable, their activity remained 100% after 96 h of incubation (data not shown). A similar observation was made for 6-OST-1, namely their activity

remained 100% after 48 h of incubation and only decreased to 30–40% after 96 h of incubation regardless of the protein expressed in *K. lactis* or in *E. coli* (data not shown). We observed that NST expressed in *K. lactis* was less stable than the one expressed in *E. coli*. The activity of *K. lactis* version NST dropped to 24% in 8 h, whereas that of *E. coli* version NST only dropped to 52% in 48 h. The observation for the decrease in its stability for NST is somewhat unexpected. It should be noted that NST is only the C-terminal domain of NDST, which is not a natural enzyme present in the mammalian cells. Whether the expression of entire sequence of NDST improves its thermal stability is currently unknown.

The unique properties of the sulfotransferases expressed in *K. lactis* are likely attributed to the glycosylation introduced during the expression. Indeed, N-glycosylation sites in the sulfotransferases are predicted with the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) using artificial neural networks that examine the context of Asn-Xaa-Ser/Thr sequences (Blom et al. 2004). The putative N-linked glycosylation sites are present within the catalytic domain of the sulfotransferases, including NST (667NTT), 2-OST (108NTT and 127NVT), 6-OST-1 (261NMS and 317NST), 6-OST-3 (127NFT, 323NLT and 379NIT) and 3-OST-1(52NGS, 141NPT, 196NRS and 253NKT). The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the sulfotransferases supports this notion. Unlike their counterparts expressed in *E. coli*, the sulfotransferases expressed in *K. lactis* did not migrate as a discrete band rather as a smear. Furthermore, the apparent molecular weights of the yeast-expressed proteins are significantly larger than that of the anticipated molecular weight without glycosylation. These observations suggest that the proteins are glycosylated (Figure 6).

Synthesis, purification and the application of purified PAPS to sulfotransfer reaction

PAPS is a key cofactor for sulfotransferases to synthesize heparin. PAPS is expensive even for the milligram-scale synthesis in an academic lab. Previously, we employed a PAPS regeneration system to prepare heparin (Chen et al. 2005). In this system, arylsulfotransferase IV was used to convert PAP to PAPS using *p*-nitrophenol sulfate as a sulfo donor. However, whether coupling PAPS regeneration system with HS sulfotransferases yields unexpected byproducts is difficult to determine, especially the product is a mixture of polysaccharides. Using PAPS as a sulfo source, we should eliminate this concern. We chose to synthesize PAPS enzymatically using adenosine 5'-triphosphate (ATP) sulfurylase (from *K. lactis*), adenosine 5'-phosphosulfate (APS) kinase (from *Penicillium chrysogenum*) and pyrophosphatase (from *E. coli*) as described in Figure 1B. All three enzymes involved in the synthesis of PAPS were expressed as $(His)_6$-tagged proteins in *E. coli* in high yields. As expected, the preparation of PAPS can be achieved with purified ATP sulfurylase, APS kinase and pyrophosphatase (Figure 7A). The products contained both adenosine 5'-diphosphate (ADP) and PAPS, which were identified by PAMN-HPLC (high-pressure liquid chromatography; Figure 7A). Alternatively, the preparation of PAPS was also achieved using crude extracts from the bacteria expressing the three enzymes. Using crude enzymes, ATP and sulfate were converted to PAPS effectively, although ADP, adenosine 5'-monophosphate (AMP) and trace amount of unreacted ATP were present in the product (Figure 7B). The product was further purified by a diethylaminoethyl (DEAE) column. The purified PAPS exhibited a single peak with polyamine anion exchange (PAMN)-HPLC, suggesting that the preparation was pure (Figure 7C). The molecular mass of product was determined to be 506.6 ± 0.2 Da by electrospray ionization mass spectrometry (MS), very close to the calculated molecular mass of PAPS (507.0; Figure 7D). The purified PAPS was employed to prepare *N*-sulfated and *N*,6-*O*-sulfated heparosan using NST and 6-OST-1, respectively. The disaccharide analysis of *N*-sulfated heparosan confirmed the presence of a disaccharide of ΔUA-GlcNS (Figure 7F); the disaccharide analysis of *N*,6-*O*-sulfated

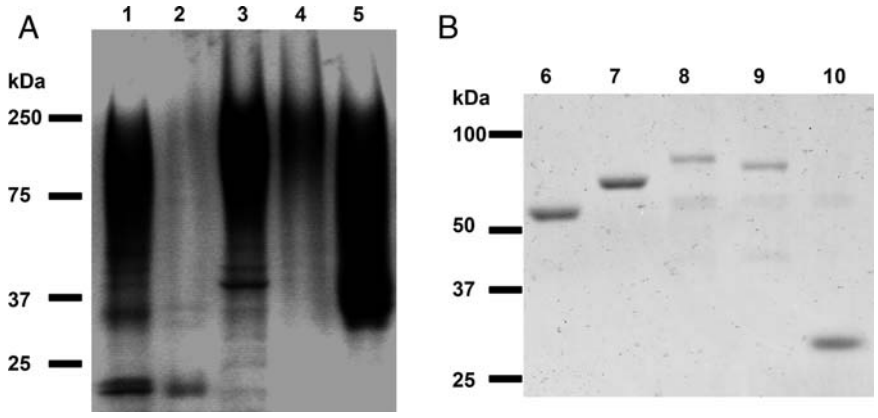

Fig. 6. SDS–PAGE analysis of sulfotransferases. (**A**) Sulfotransferases expressed in *K. lactis*. Lane 1, NST; lane 2, 2-OST; lane 3, 6-OST-1; lane 4, 6-OST-3; lane 5, 3-OST-1. The anticipated molecular weight of sulfotransferases expressed by *K. lactis* without glycosylation is: 35 kDa for NST, 36 kDa for 2-OST, 41 kDa for 6-OST-1, 41 kDa for 6-OST-3 and 34 kDa for 3-OST-1. (**B**) Sulfotransferases expressed by *E. coli*. Lane 6, NST (in glutathione *S*-transferase fusion protein form); lane 7, 2-OST (in maltose-binding protein fusion protein form); lane 8, 6-OST-1 (in maltose-binding protein fusion protein form); lane 9, 6-OST-3 (in maltose-binding protein fusion protein form); lane 10, 3-OST-1 (in $(His)_6$-fusion protein form). The anticipated MW for bacterial-expressed protein is: 61 kDa for NST, 70 kDa for 2-OST, 83 kDa for 6-OST-1, 81 kDa for 6-OST-3 and 35 kDa for 3-OST-1.

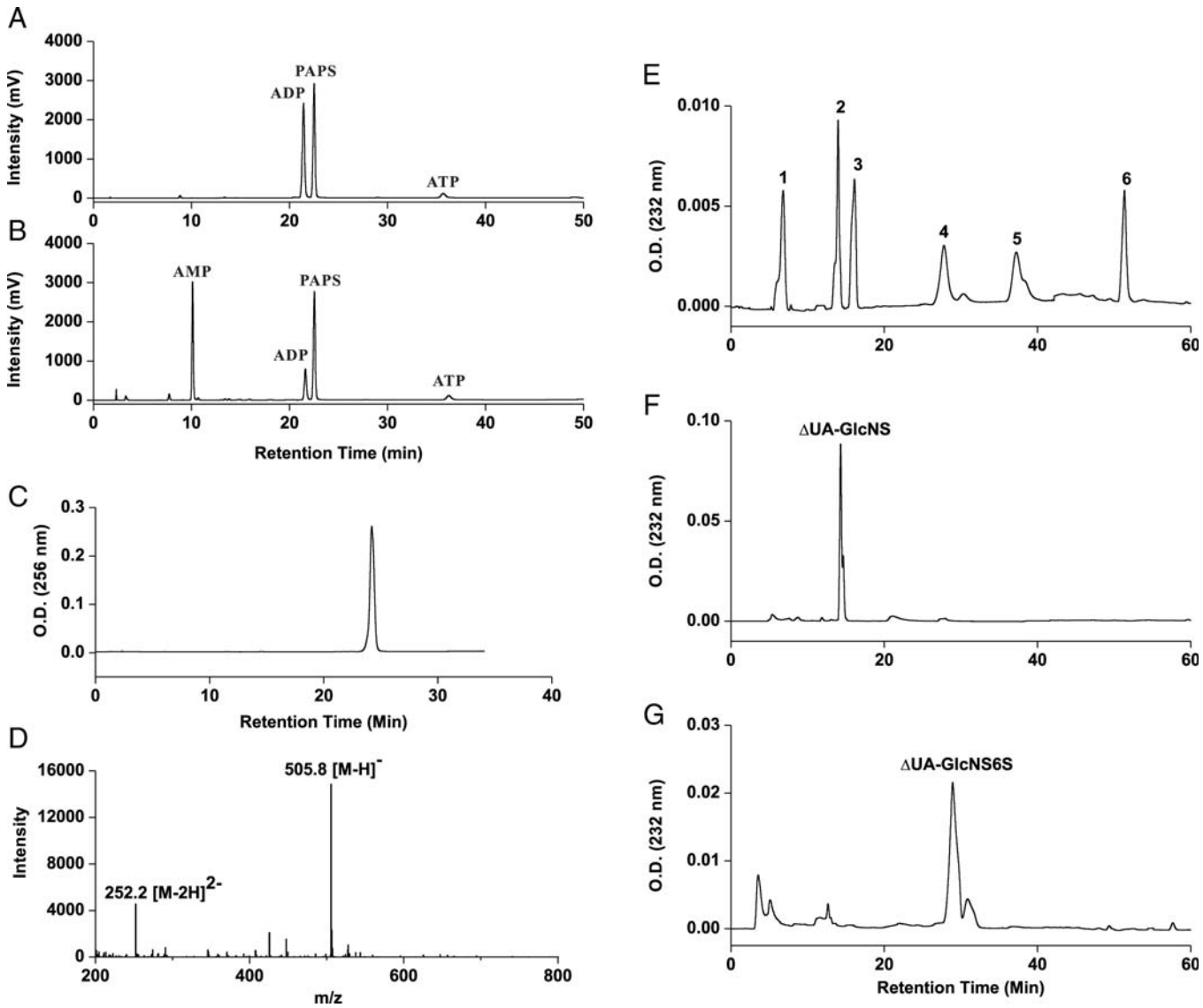

Fig. 7. PAPS synthesis, purification and the application of purified PAPS to sulfotransfer reaction. (**A**) HPLC analysis of the products from PAPS synthesis reaction catalyzed by purified enzymes. The area of PAPS peak (Area = 701 units) and that of the adenosine 5'-diphosphate (ADP) peak (Area = 685 units) are similar to each other. (**B**) HPLC analysis of the products from PAPS synthesis reaction catalyzed by crude enzymes. ATP and sulfate were converted to PAPS (Area = 625 units), ADP (Area = 214 units) and adenosine 5'-monophosphate (AMP) (Area = 503 units). Our data suggest that close to 95% of ATP was converted to the product in 6 h reaction. The elution positions of PAPS, ATP, ADP and AMP were determined by injecting authentic standard compounds. (**C**) PAPS purity determination by HPLC analysis. (**D**) MS analysis of the purified PAPS. (**E**–**G**) Disaccharide analysis. The purified PAPS was applied to NST (F) or 6-OST-1 (G) catalyzed sulfotransfer reaction, respectively. (E) The eluted positions of authentic disaccharide standards, where 1 represents ΔUA-GlcNAc, 2 represents ΔUA-GlcNS, 3 represents ΔUA-GlcNAc6S, 4 represents ΔUA-GlcNS6S, 5 represents ΔUA2S-GlcNS and 6 represents ΔUA2S-GlcNS6S.

heparosan confirmed the presence of a disaccharide of ΔUA-GlcNS6S (Figure 7G). The results of disaccharide analysis demonstrated that the synthetic PAPS can be utilized by HS sulfotransferases to prepare sulfated polysaccharides. Figure 7E shows the eluted positions of authentic disaccharide standards. It is worthwhile to mention that we obtained 5 g of PAPS from 1 L reaction with a cost of about $50, estimated to be at $0.01 mg^{-1}. The price of PAPS on Sigma catalog of 2010 is $36–$85 mg^{-1}, almost 3600–8500-fold higher than the synthetic PAPS using our method.

Discussion

Heparin is an important anticoagulant drug, which is currently isolated from the porcine intestine. Developing a method to prepare synthetic heparin will not only eliminate the potential sources of contamination and adulteration. The method will also offer the possibility to alter the sulfation patterns to synthesize the heparin without side effects. For example, a common side effect that is associated with the use of heparin is heparin-induced thrombocytopenia because heparin binds

to platelet factor 4 (Arepally and Ortel 2006). Using a synthetic approach, we could potentially separate the anticoagulant activity and the binding affinity to platelet factor 4 to reduce the risk of heparin-induced thrombocytopenia. The high regioselectivity of HS biosynthetic enzymes provides a unique advantage to prepare synthetic heparin by eliminating protecting/unprotecting steps used in chemical synthesis. Despite our initial success in preparing milligram-scale synthesis, the availability of sulfotransferases and PAPS remains two obstacles for the cost-effective synthesis in a large scale. Here, we described the expression of HS sulfotransferases in *K. lactis*, permitting us to harvest the enzymes directly from conditioned medium. We notice that the genes encode the sulfotransferases we expressed from three different species. This is because the genes were available in our lab when the study was initiated. The purified enzymes expressed in *K. lactis* have favorable properties for conducting the enzymatic synthesis of heparin: higher specific activity and improved thermostability. In addition, an enzymatic approach was successfully employed to synthesize a gram scale of PAPS at low cost.

Our results demonstrated that *K. lactis* system is an excellent expression approach to prepare HS sulfotransferases. HS sulfotransferases are membrane-bound sulfotransferases and glycosylated (Esko and Selleck 2002; Saribas et al. 2004). Expression of recombinant HS biosynthetic enzymes can be achieved in insect cells using a baculovirus expression approach and in bacteria (Kuberan et al. 2003; Chen et al. 2005). Given the complexity of using the baculovirus expression approach, bacterial expression offers an efficient method to obtain the recombinant enzymes. However, *E. coli* has no capability to introduce glycosylation that could reduce the specific enzymatic activity and decrease the thermostability. Furthermore, the expression was carried out in cytosol and the recombinant proteins are likely to contain endotoxin. Procedures for purifying the proteins and to remove endotoxin are laborious and costly. Therefore, the expression of recombinant HS sulfotransferases in yeast should be an ideal alternative because yeast cells do not produce endotoxin and are cheap to grow. Up to date, only NDST1 has been reportedly expressed in *Saccharomyces cerevisiae* (Saribas et al. 2004). However, the expression was achieved intracellularly, and the level of expression is not sufficiently high. By constructing a secreted form of recombinant protein, we can now harvest HS sulfotransferases from the conditioned medium of *K. lactis*. We should be able to use the enzymes to carry out the synthesis without purification.

Our results suggest that optimization has significant impact on improving the protein expression in *K. lactis*. Proteolytic degradation is a serious problem for yeast expression. Addition of amino acid rich supplements to the culture medium, such as casamino acids, can enhance the stability of recombinant proteins, possibly by acting as alternative and competing substrates for the undesired proteases, or repressing protease induction caused by nitrogen limitation (Macauley-Patrick et al. 2005). In our study, the activity of 6-OST-3 in the spent culture increased about 2-fold by addition of 2% casamino acids in the induction medium. Lower cultivation temperature can also influence yield of recombinant proteins (Macauley-Patrick et al. 2005). A previous work shows that the activity of laccase was increased about 4-fold by induction at 20°C instead at 30°C (Hong et al. 2002). The mechanisms behind the temperature effect may be due to the decrease in the stability of the recombinant protein at higher temperatures, release of more proteases from dead cells and folding problems at higher temperature (Hong et al. 2002; Macauley-Patrick et al. 2005). In our work, dropping the induction temperature from 28°C to 16°C increased the expression of 6-OST-3 by about 10-fold. The pH value is another key factor affecting the yield of recombinant protein production, mainly due to the influence on proteases activity by variable pH values (Flores et al. 1999). In our work, when the pH value increased from 5.0 to 7.0, the activity of 6-OST-3 increased about 3-fold (Figure 2C).

It has been recognized for decades that post-translational modification, especially glycosylation, can improve proper folding, biological activity and stability of recombinant proteins (Stanley 1992). Comparing the sulfotransferases expressed in two different sources, we observed unique properties of sulfotransferases due to the glycosylation of the sulfotransferases carried out in *K. lactis*. The specific activities of the five sulfotransferases expressed in *K. lactis* were increased by 2–10-fold higher than those expressed in *E. coli*. We observed a range of effects on the thermostability of the enzymes. For 3-OST-1 and 6-OST-3, the enzymes were clearly more stable, while 2-OST and 6-OST-1 remained the same. For NST-1, the thermostability is decreased. These interesting findings also offer us a potential opportunity to modulate the thermostability of the enzyme by controlling the glycosylation.

In summary, we report new methods to prepare sulfotransferases and PAPS for the enzymatic synthesis of heparin. The enzymes can be expressed in a secreted form in *K. lactis* in high yields. The recombinant sulfotransferases can be directly harvested from media, and some proteins display improved thermostability and specific activity. Furthermore, an enzymatic approach was also developed to synthesize PAPS in one pot reaction at very low cost. Our results could contribute significantly toward the large-scale synthesis of heparin drugs.

Materials and methods

Yeast and bacterial strains and plasmid construction

Strains and plasmids used in this study are listed in Table I. *E. coli* strains were grown in Luria-Bertani (LB) medium or on LB plates containing 1.5% agar. Unless otherwise stated, *K. lactis* GG799 or *K. lactis* YCT598 were grown in YPG medium (1% yeast extract, 2% peptone and 2% galactose) for propagation, YCB medium (30 mM Tris–HCl, pH 7.0, 11.7 g L^{-1} of yeast carbon base and 5 mM acetamide) for selection or YPCGN medium (1% yeast extract, 2% peptone, 2% casamino acids, 2% galactose and 5 g L^{-1} of ammonium sulfate) for expression. Carbenicillin and streptomycin were added at 50 μg mL^{-1} when necessary.

To construct the secreted forms of recombinant proteins, NST domain of *Rattus norvegicus* NDST1 (H591-R882), *Gallus gallus* 2-OST (D69-N356), *Mus musculus* 3-OST-1 (G48-H311), *G. gallus* 6-OST-1 (P46-H378) or *M. musculus*

Table I. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
E. coli strains		
DH5α	Cloning host	Invitrogen
BL21 Star (DE3)	Expression host	Invitrogen
Yeast Strains		
K. lactis GG799		New England Biolab
K. lactis YCT598		New England Biolab
Plasmids		
pKLAC2	Expression vector, Ap^R	New England Biolab
pET-15b	Expression vector, Ap^R	Novagen
pCDFDuet-1	Expression vector, Sm^R	Novagen
pETDuet-1	Expression vector, Ap^R	Novagen
APSK-pET23a(+)	pET-23a(+) carrying APS kinase gene of *P. chrysogenum*, Ap^R	MacRae et al. (1998)
APSK-pETDuet-1	pETDuet-1 carrying APS kinase gene of *P. chrysogenum*, Ap^R	This work
KAST-APSK-pETDuet-1	pETDuet-1 carrying His-tagged ATP sulfurylase gene of *K. lactis* and APS kinase gene of *P. chrysogenum*, Ap^R	This work
APSK-pET-15b	pET-15b carrying His-tagged APS kinase gene	This work
PPA-pCDFDuet-1	pCDFDuet-1 carrying His-tagged pyrophosphatase gene of *E. coli*, Sm^R	This work

APS, adenosine 5'-phosphosulfate; ATP, adenosine 5'-triphosphate.

6-OST-3 (P120-P449) was cloned into pKLAC2 downstream of the *K. lactis* α-MF domain. Both the DNA sequences of NST and 3-OST-1 were subjected to yeast codon optimization, and the optimized DNAs were synthesized by GenScript. The constructs were linearized with *Sac*II, recovered using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) and transformed into *K. lactis* competent cells according to the manual of *K. lactis* Protein Expression Kit (New England Biolabs Inc.). Colonies that were grown on YCB agar medium were inoculated into 1 mL of YCB medium and incubating at 28°C overnight with shaking at 250 rpm. The overnight culture (50 μL) was expanded into 1 mL of YPCGN medium and induced by galactose for 48 h at 28°C. The supernatant was subjected to sulfotransferase activity assay. Clones showing the highest activity were chosen for further analysis.

To construct APSK-pETDuet-1, APSK-pET23a(+) (MacRae et al. 1998) was digested with restriction enzyme *Nde*I and *Xho*I. Fragments carrying APS kinase gene were purified and ligated into the *Nde*I–*Xho*I site of pETDuet-1 (Novagen). To construct KAST-APSK-pETDuet-1 plasmid, the ATP sulfurylase was amplified from *K. lactis* GG799 genome and cloned into the *Pst*I–*Not*I site of APSK-pETDuet-1 using primers with the sequences of 5′-primer AAAA**CTGCAG**ATGC CTTCTCCTCATGGTGGT and 3′-primer ATAAGAAT **GCGGCCGC**TCAGAATTGGAAAAAGCCTTGGT, where characters in bold face represent the restriction site. APSK-pET-15b was constructed by inserting the fragment with APS kinase gene into the *Nde*I–*Xho*I site of pET-15b. Pyrophosphatase gene was amplified from *E. coli* K12 genome using primers (5′-primer AGCCGC**GAATTC**GATGAGCTT ACTCAACGTCCCTG and 3′-primer GTACCC**AAGCTT**TT ATTTATTCTTTGCGCGCTCG, where characters in bold face represent the restriction site) and cloned into the *Eco*RI–*Hin*dIII site of pCDFDuet-1 to form the PPA-pCDFDuet-1 plasmid. All constructs were sequenced at the UNC genomic analysis facility to verify the reading frame and free from mutations.

Protein expression and purification

The expression was carried out in YPCGN medium at 16°C for 48 h with *K. lactis* YCT598 as the host strain, unless otherwise stated. *K. lactis* cells carrying the NST, 2-OST, 3-OST-1, 6-OST-1 or 6-OST-3 expression cassette were grown in YCB medium and then inoculated into YPG medium. The cultures were incubated at 28°C overnight with shaking at 250 rpm until the OD_{600} reached to 10 U. To induce protein expression and secretion, overnight cultures were expanded into 500 mL YPCGN medium to a density of 1.0 at OD_{600} in a 2.8 L flask and induced for 48 h at 16°C, where galactose served as an inducer. The culture was harvested by centrifugation, adjusted to pH 5.5, and the supernatant was filtered through a Whatman glass microfiber filter (1.2 μm, Fisher).

All subsequent protein purification procedures were performed at 4°C. The supernatant was applied to a heparin-Sepharose (GE Healthcare) column and washed with buffer W1 (50 mM sodium acetate, 150 mM NaCl, 10% glycerol, pH 5.5). The protein was eluted with buffer E1 (50 mM sodium acetate, 1 M NaCl, 10% glycerol, pH 5.5). Fractions containing 3-OST-1, 6-OST-1 or 6-OST-3 were pooled and dialyzed against buffer W2 (50 mM MOPS, 50 mM NaCl, 5% glycerol, pH 7.0). Fractions containing NST or 2-OST were pooled and dialyzed against buffer W3 (50 mM MOPS, 5% glycerol, pH 7.0). The dialyzed samples were applied to a PAP-Agarose (Sigma) column and washed with buffer W2 (for 3-OST-1, 6-OST-1 and 6-OST-3 purification) or W3 (for NST and 2-OST purification), respectively, at a flow rate of 0.5 mL min^{-1}. The protein was eluted with a linear gradient of 50 mM–1 M NaCl in buffer W3 for 60 min. Fractions were pooled and stored at −80°C until use. *E. coli*-expressed NST, 2-OST, 3-OST-1, 6-OST-1 and 6-OST-3 were purified as described previously (Kakuta et al. 1999; Edavettal et al. 2004; Chen et al. 2005, 2007; Abdel-Banat et al. 2010). The protein concentration was determined by Bradford Reagent (Sigma) according to the manufacturer's introduction.

The enzymes involved in synthesizing PAPS, including ATP sulfurylase, APS kinase and pyrophosphatase, were expressed in *E. coli* BL21 Star(DE3) (Invitrogen). Briefly, the bacteria were grown in LB to the OD_{600nm} value of 0.6. After decreasing the temperature to 22°C, the culture was induced with 0.2 mM IPTG overnight. His-tagged ATP sulfurylase (expressed by KAST-APSK-pETDuet-1), APS kinase (expressed by APSK-pET-15b) and pyrophosphatase (expressed by PPA-pCDFDuet-1) were purified with Ni-Agarose (GE Health) column. All purification procedures were performed at 4°C. The cultures were harvested and resuspended in 50 mL of buffer A (20 mM Tris–HCl, 500 mM NaCl, pH 7.6). The cells were lysed by sonication, and then 1 mM phenylmethanesulfonyl fluoride was added immediately to prevent protein degradation. After centrifugation at 12,000 rpm for 20 min, the supernatant was filtrated through a 0.45 μm membrane and applied to a Ni-Agarose column at a flow rate of 3.0 mL min^{-1}. The column was eluted with a linear gradient of 50–300 mM imidazole in buffer A for 30 min. Fractions containing the protein were pooled, dialyzed against dialysis buffer (20 mM Tris–HCl, pH 7.6, at 4°C; 500 mM NaCl; 10% glycerol) and stored at −80°C. The purity of protein was analyzed by 10% precasted SDS–PAGE (BioRad).

PAPS synthesis and purification

The PAPS preparation was achieved by crude enzymes. The bacterial cells expressing ATP sulfurylase (expressed using KAST-APSK-pETDuet-1 plasmid), APS kinase (expressed using KAST-APSK-pETDuet-1plasmid) and pyrophosphatase (expressed using PPA-pCDFDuet-1plasmid) were lysed as described in protein expression and purification. After centrifugation, filtration and addition of 10% glycerol, the crude enzyme solutions were stored at −80°C until use. With a modified method described previously (Burkart et al. 2000), the reaction included 22.5 mM ATP disodium, 100 mM Na_2SO_4, 10 mM $MgCl_2$, 10 mM LiCl, 0.6 mg mL^{-1} of crude enzymes including ATP sulfurylase and APS kinase, 0.3 mg mL^{-1} of crude enzyme including pyrophosphatase and 50 mM Tris–HCl at pH 8.0 and incubated at 30°C for 6 h. The completion of PAPS synthesis was checked by PAMN-HPLC (polyamine II column, YMC America, Inc.) as follows: 100% water for 10 min, followed with a linear gradient of 0–100% of 1 M KH_2PO_4 for 30 min, followed by 100% 1 M KH_2PO_4 for 15 min at a flow rate of 1 mL min^{-1} with UV 254 nm detection. Large-scale purification of PAPS was achieved on a DEAE-Sepharose fast flow column (GE Health; 1.5 × 60 cm). The DEAE column was washed with water, and PAPS was eluted with a gradient of 0–500 mM NaCl at 5.0 mL min^{-1} for 200 min. Fractions containing PAPS as determined by PAMN-HPLC were pooled and stored at −80°C.

Analysis of PAPS by MS

Purified PAPS was dialyzed using the Spectra/Por dialysis membrane (MWCO 1000 Da) against 20 mM ammonium acetate for 4 h. A syringe pump (Harvard Apparatus) was used to introduce the sample via direct infusion (10 μL min^{-1}) into an Agilent 1100 MSD-Trap at the ADME Mass Spectrometry Center. Experiments were performed in a negative ionization mode with the electrospray source set to 2500 V and 200°C, and the compound stability set to 30%. Nitrogen was used for both nebulizer (5 L min^{-1}) and drying gas (15 psi). Helium was used for collision-induced dissociation. The MS and MS/MS data were acquired and processed using Bruker Trap Software 4.1.

Sulfotransferase activity assay

Sulfotransferase activity was determined by incubating 3-OST-1 with 10 μg of HS (from bovine kidney; ICN) in 100 μL of reaction buffer (50 mM 2-(N-Morpholino)ethanesulfonic acid, pH 7.0, 5 mM $MgCl_2$, 50 μM PAPS and 1–10×10^5 cpm of [^{35}S] PAPS (∼2 μM)). Assays for other sulfotransferases were almost the same, while different substrates were used. Deacetylated heparosan (1 μg) was used for NST (Lindahl et al. 2005), and completely desulfated *N*-sulfated heparin (1 μg; designated as DE-OS-Heparin from Neoparin Inc.) was used as a substrate for 6-OST-1, 6-OST-3 and 2-OST. The reaction was incubated at 37°C for 1 h and quenched by the addition of UPAS buffer (50 mM sodium acetate, pH 5.5, 150 mM NaCl, 6 M urea, 1 mM EDTA and 0.01% Triton X-100). The samples were then subjected to 200 μL DEAE-Sepharose chromatography to purify the [^{35}S] products.

Disaccharide analysis

The polysaccharides (100 μg) were purified by a 200 μL DEAE-Sepharose column, then dialyzed against deionized water, dried by speed vacuum and degraded by a mixture of heparin lyases including *Flavobacterium heparinum* heparin lyases I, II and III. The resultant disaccharides were desalted on BioGel P-2 column (0.5 × 200 cm) eluted with 0.1 M ammonium bicarbonate with a flow rate of 3 mL h^{-1}. The disaccharides were analyzed on reverse phase-ion pairing HPLC by using a C_{18} reversed phase column (4.6 × 250 mm; Vydac) with UV 232 nm detection as described previously (Kobayashi et al. 1996). The column was eluted with acetonitrile as follows: 7% for 30 min followed by 15% for 15 min, followed by 19.5% for 15 min, followed by 50% for 15 min and followed by 7% for 10 min, in a solution containing 38 mM ammonium phosphate monobasic, 2 mM phosphoric acid and 1 mM tetrabutylammonium phosphate monobasic at a flow rate of 0.5 mL min^{-1}.

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Conflict of interest

None declared.

Abbreviations

ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; APS, adenosine 5'-phosphosulfate; ATP, adenosine 5'-triphosphate; α-MF, α-mating factor; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; HS, heparan sulfate; LB, Luria-Bertani; NDST, *N*-deacetylase/*N*-sulfotransferase; NST, *N*-sulfotransferase; 2-OST, 2-*O*-sulfotransferase; 3-OST, 3-*O*-sulfotransferase; 6-OST, 6-*O*-sulfotransferase; PAP, 3′-phosphoadenosine-5′-phosphate; PAMN, polyamine anion exchange; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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