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High yield, purity and activity of soluble recombinant *Bacteroides thetaiotaomicron* GST-heparinase I from *Escherichia coli*

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Abstract

Heparinase I from *Flavobacterium heparinum*, a source of diverse polysaccharidases, suffers from low yields, insufficient purity for structural studies and insolubility when expressed as a recombinant product in *Escherichia coli* that is devoid of glycosaminoglycan polysaccharidases. In this study, cDNA coding for the orthologue of *F. heparinum* heparinase I was constructed from genomic information from the mammalian gut symbiont *Bacteroides thetaiotaomicron* and expressed in *E. coli* as a fusion protein with GST at the N-terminus. This resulted in high yield (30 mg/g dry bacteria) of soluble product and facilitated one-step affinity purification to homogeneity. Purified heparinase I bearing the GST fusion exhibited a K_m of 2.3 μM and V_{max} of 42.7 μmol/min with a specific activity of 164 U/mg with heparin (average 12,000 Da) as substrate. The results indicate a 2-fold improvement in yield, specific activity and affinity for heparin as substrate over previous reports. The data suggest that the heparinase I from the gut symbiont exhibits a higher intrinsic affinity for heparin than that from *F. heparinum*. The purified GST fusion enzyme exhibited a requirement for Ca^{2+} and a pH optimum between 6.7 and 7.3 that was similar to the enzyme freed of the N-terminal GST portion. Our study revealed that catalytic activity of heparinase I requires a reducing environment. The GST facilitated immobilization of heparinase I in solid phase either for clinical purposes or for structural studies in absence of interference by contaminating polysaccharidases.
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Heparan sulfate (HS)[1] glycosaminoglycan (GAG) is a cell surface and extracellular matrix component in vertebrates and invertebrates. In addition to roles in tissue organization, HS binds and impacts biological activities of numerous proteins that regulate diverse physiological and pathological processes that span cell growth, differentiation, migration and defense against infection [1–3]. HS and heparin GAG polysaccharides are comprised of structurally related uronate-glucosamine (UA-GlcN) disaccharide repeats that vary in length and degree and disposition of epimerized, acetylated or sulfated side groups that impart to HS strong anionic polyelectrolyte character. Variations in sulfation, size and topography of HS chains impacts function of diverse proteins [3–6]. However, it remains unclear whether the regulation is mediated by relatively non-specific variations in overall charge density or a specific arrangement of modified residues for a structurally specific motif within the HS chains [3,4,7]. To resolve this question requires isolation and characterization of

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[1] *Abbreviations used: B. thetaiotaomicron*, *Bacteroides thetaiotaomicron*; DTT, dithiothreitol; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; *F. heparinum*, *Flavobacterium heparinum*; GAG, glycosaminoglycan; GlcN, glycosamine residue that can be *N*-acetylated (Ac) or multiple sulfated (S); GST, glutathione-S-transferase; HS, heparan sulfate; UA, uronic acid residue including glucuronic acid (GlcA) and Iduronic acid (IdoA) that can be sulfated at 2-OH (2S).

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potentially rare and specific oligosaccharide motifs from complex mixtures with highly specific bioprobes followed by microsequence analysis using group specific HS degradative enzymes and gel electrophoresis or mass spectrometry. Recently we have used members of the FGF polypeptide family as bioaffinity reagents to extract extremely rare oligosaccharide motifs that exhibit FGF-specific activity in support of FGFR signal transduction [5,6]. An indirect deduction of structure indicated that such motifs may be undersulfated and exhibit a unique distribution of sulfated and non-sulfated sidegroups.

In experiments to demonstrate FGF-specific oligosaccharides by fragmentation of long chain heparin and heparan sulfates, we found that carefully controlled enzymatic reduction by heparinase I was the most promising compared to other methods [5]. However, currently available heparinase preparations yielded equivocal results and hampered generation of yields sufficient for routine structural analyses. Heparinase 1 (EC 4.2.2.7) cleaves primarily at α-D-GlcNS(±6S) (1 → 4) α-L-IdoA/GlcA2S linkage in the highly *N*-sulfated domains. Commercial heparinase I is a heparin-induced product derived from the soil bacterium *Flavobacterium heparinum* that was selected for use of heparin as sole carbon, nitrogen and sulfur source [8]. *F. heparinum* is a natural source of diverse polysaccharidases including heparinases 1, 2, and 3 and sulfatases that degrade heparin and heparan sulfates at distinct positions [9]. More recently other bacteria strains have been examined as sources of heparinases [10–13]. However, the variability in inductive yields, complexity of purification procedures and potential co-purification with multiple heparin lyases of distinct specific activities have generally hampered both analytical and commercial applications of heparinase I and made them costly. The presence of trace amounts of other enzymes with different specificity compromises accurate microsequencing of rare oligosaccharide motifs [8,9]. To bypass these limits, attempts have been made to express and recover recombinant heparinases in *Escherichia coli* that do not express GAG polysaccharidases [14,15]. However, active soluble product was in low yield and solubilization and refolding was required [14–16]. Sequencing of the complete genomes of new bacterial strains has revealed homologues to *F. heparinum* polysaccharidases. One of the first to emerge was the genome of the human and mouse gut symbiotic *Bacteroides thetaiotaomicron* that exhibits one of the richest adaptive proteomes of polysaccharidases of any bacterium to date [17]. These include a variety of enzymes that target host-derived glycans such as chondroitin sulfates, mucins, hyaluronate and heparin/heparan sulfate. The *B. thetaiotaomicron* polysaccharidase repertoire is thought to be part of a finely tuned evolutionary niche that is symbiotic with humans and mice [18,19]. We posited that *B. thetaiotaomicron* gene products might be a superior source of conventional and potentially novel variants of lyases for heparin and heparan sulfate, as well as other glycosaminoglycans.

In this report in the first of a series to express, recover in high yield and purity and characterize *B. thetaiotaomicron* heparan sulfate lyases, we describe the cloning, recombinant expression and high yield recovery from *E. coli* and enzymatic characterization of *B. thetaiotaomicron* heparinase I, and compare the data to that of heparinase I encoded by *F. heparinum*. Both enzymes were soluble when expressed in *E. coli* with GST fused at the N-terminus. The GST fusion protein facilitated one-step affinity purification and exhibited similar kinetic parameters and co-factor requirements to heparinase I alone after removal of the GST. The approach represented improvements in yield and activity of purified heparinase I preparations. The GST-fused enzyme with high purity and specific activity may be useful in solid phase clinical applications and sequence characterizations of structurally specific heparin and heparan sulfate motifs.

Experimental procedures

Materials

Heparin (porcine intestinal mucosa, 170 USP U/mg), heparan sulfate (bovine kidney), chondroitin sulfate (bovine cartilage), dithiothreitol (DTT) and reduced glutathione were from Sigma (St. Louis, MO). Thrombin, HiTrap Glutathione-Sepharose, Benzamidine-Sepharose and Superose 12 columns were from GE HealthCare Bio-Sciences Corp (Piscataway, NJ). *B. thetaiotaomicron* (#29148) and *F. heparinum* (#13125) were from the American Type Culture Collection (Manassas, VA).

Culture of bacteria and recovery of DNA

Bacteroides thetaiotaomicron was cultured anaerobically in airtight tubes wrapped with aluminum foil containing chopped meat glucose broth medium (Becton–Dickinson, Sparks, MD) at 25 °C for 2 days. Bacteria was collected from 1.5 ml of culture by centrifugation at 5000 rpm for 2 min and bacterial genomic DNA extracted by modification of a previously described method [20]. Briefly, the bacterial pellet was re-suspended in 567 μl TE buffer with addition of 30 μl 10% SDS and 3 μl of 20 mg/ml proteinase K. After incubation at 37 °C for 1 h, 100 μl of 5 M NaCl was added and mixed thoroughly, and then 80 μl CTAB/NaCl solution was added followed by incubation at 65 °C for 10 min. DNA was extracted with an equal volume of chloroform/isoamyl alcohol and then phenol/chloroform/isoamyl alcohol. After centrifugation, DNA in the aqueous phase was precipitated with 0.6 volumes of isopropanol, washed with 70% ethanol, and re-suspended in 100 μl TE buffer.

Flavobacterium heparinum was cultured in medium comprised of 1% tryptone, 0.2% soya peptone, 0.5% NaCl and 0.03% heparin sodium salt in phosphate buffer containing additional 1 mM L-histidine. Bacteria were collected and DNA prepared as described above for *B. thetaiotaomicron*.

Construction of recombinant plasmids and expression of GST-heparinase I in E. coli

DNA encoding *B. Thetaiotaomicron* heparinase I (cDNA) was prepared using the PCR with primers 5′-GGGGATCCATGCTGACTGCT CAGACT-3′ and 5′-GGGAATTCTTATCTTTCCGAATATCC-3′ and purified genomic DNA from *B. thetaiotaomicron* as template. The PCR product was digested and ligated into pGEX-2T vector at *Bam*HI and *Eco*RI sites in frame with coding sequence for GST at the 5′ end. A thrombin cut site was introduced between the GST and heparinase I. Sequence of the heparinase I cDNA insert was confirmed. *F. heparinum* heparinase I cDNA was cloned by the same approach using primers 5′-GCGGATCCC AGCAAAAAAAATCCGGT-3′, 5′-GCGAATTCCTATCTGGCAATT

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