Expression in *Escherichia coli*, purification and characterization of heparinase I from *Flavobacterium heparinum*

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The use of heparin for extracorporeal therapies has been problematical due to haemorrhagic complications; as a consequence, heparinase I from *Flavobacterium heparinum* is used for the determination of plasma heparin and for elimination of heparin from circulation. Here we report the expression of recombinant heparinase I in *Escherichia coli*, purification to homogeneity and characterization of the purified enzyme. Heparinase I was expressed with an N-terminal histidine tag. The enzyme was insoluble and inactive, but could be refolded, and was purified to homogeneity by nickel-chelate chromatography. The cumulative yield was 43%, and the recovery of purified heparinase I was 14.4 mg/l of culture. The N-terminal sequence and the molecular mass as analysed by matrix-assisted laser desorption MS were consistent with predictions from the heparinase I gene structure. The reverse-phase HPLC profile of the tryptic digest, the

Michaelis–Menten constant K_m (47 μ g/ml) and the specific activity (117 units/mg) of purified recombinant heparinase I were similar to those of the native enzyme. Degradation of heparin by heparinase I results in a characteristic product distribution, which is different from those obtained by degradation with heparinase II or III from *F. heparinum*. We developed a rapid anion-exchange HPLC method to separate the products of enzymic heparin degradation, using POROS perfusion chromatography media. Separation of characteristic di-, tetra- and hexasaccharide products is performed in 10 min. These methods for the expression, purification and analysis of recombinant heparinase I may facilitate further development of heparinase I-based medical therapies as well as further investigation of the structures of heparin and heparan sulphate and their role in the extracellular matrix.

INTRODUCTION

Heparin, a highly sulphated heterogeneous polysaccharide, has been used extensively as an anticoagulant drug for more than 50 years, both for thrombotic diseases [1] and for regional or systemic anticoagulation during extracorporeal therapies [2]. Neutralization of the anticoagulant effect of heparin and determination of plasma levels of heparin has been problematic [2,3], and the use of heparin during extracorporeal therapies can result in severe haemorrhagic complications [4–8]. Heparinase I (heparin lyase I; EC 4.2.2.7), a heparin-degrading lyase from *Flavobacterium heparinum*, has been proposed for clinical use to meet the challenges of heparin-based therapies. To achieve efficient deheparinization of patients after extracorporeal therapies, heparinase I has been immobilized in an extracorporeal reactor that can eliminate heparin from the blood [9–12] and, currently, injection of heparinase I for the same purpose is in phase I clinical trials [13,14]. Heparinase I has recently been approved by the Food and Drug Administration for a differential clotting time measurement to determine blood heparin levels [15,16].

Heparin lyases (heparinases I, II and III), purified from *F. heparinum*, have long been of interest as the only accessible source of heparin-degrading enzymes [17–22]. The disaccharide repeat unit of heparin and heparan sulphate comprises an N-acetylated glucosamine and a glucuronic acid connected through 1-4 linkages, and may be modified by 6-O-sulphation, 3-O-sulphation, 2-N-deacetylation and 2-N-sulphation of the glu-

cosamine, and by 2-O-sulphation and C-5 epimerization of the D-glucuronic acid to L-iduronic acid [23,24]. These modifications occur to varying degrees, and each of the heparinases is specific for certain sequences of modification along the heparin chain [22,25–27]. The susceptibility of a given oligosaccharide to heparinase degradation can be used to infer its content of cleavable linkages; as a consequence, heparinases have been useful in characterizing heparin-derived oligosaccharides [28–30].

In addition to its anticoagulant effect, heparin modulates the activity of proteins such as fibroblast growth factor, vascular endothelial growth factor, lipoprotein lipase and apolipoprotein E100 [31,32], with the specificity of interaction often being embedded in sequences of the disaccharide repeat units of heparin [33–37]. This involvement of heparin in intercellular signalling, through modulation of fibroblast growth factor and vascular endothelial growth factor, has motivated investigations of the effect of heparinases in angiogenesis and morphogenesis. Heparinases I and III are potent anti-angiogenic agents *in vitro* and *in vivo* [38], and heparinase I affects the embryonic development of model systems [39,40].

The use of heparinases has been impaired by difficulties in separating the three heparinases produced by *F. heparinum*. This problem has been further complicated by co-purification of *F. heparinum* sulphatases that are active towards heparin-derived oligosaccharides [41]. It is thus desirable to develop a purification scheme for each of the heparinases, such that they are entirely free from contamination by other heparinases and sulphatases. This, in addition to the clinical applications of heparinase I, has

Abbreviations used: r-heparinase I, recombinant heparinase I; IPTG, isopropyl β -D-thiogalactopyranoside; GdHCl, guanidine hydrochloride; MALDI-MS, matrix-assisted laser desorption MS. Heparin oligosaccharides are abbreviated as follows: I, α -L-idopyranosyluronic acid; G, β -D-glucopyranosyluronic acid; U, either α -L-idopyranosyluronic acid (IdoA) or β -D-glucopyranosyluronic acid (GlcA); Δ U, IdoA or GlcA with an unsaturated C-4–C-5 bond; H, α -D-2-deoxy-2-aminoglucopyranose. Subscripts 2S and 6S indicate 2-O- and 6-O-sulphation respectively, and NS indicates N-sulphation of the hexosamine.

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placed high demands on both yield and purity of heparinase I preparations, which can be met by a recombinant production route in a host that does not have endogenous heparinase activity. We have previously cloned the gene for heparinase I and showed that the recombinant enzyme (r-heparinase I) was active [42,43]. In the present paper we describe a process for the expression and purification of homogeneous heparinase I in high yield from *Escherichia coli* cultures, and show that the protein product is functionally similar to native heparinase I.

EXPERIMENTAL

Materials

Heparin, from porcine intestinal mucosa, was from Kabi Pharmacia, Franklin, OH, U.S.A. (154 USP units/mg) and from Hepar, Franklin, OH, U.S.A. (170 USP units/mg). Native heparinase I was purified from *F. heparinum* culture media by the method of Yang et al. [20] and further purified by reverse-phase HPLC [42]. Synthetic oligonucleotide primers and the basic peptide (Arg-Gly)₁₅ were synthesized by the Biopolymers Laboratory, Center for Cancer Research, MIT. Mops was from United States Biochemicals (Cleveland, OH, U.S.A.), iodoacetic acid from Calbiochem (La Jolla, CA, U.S.A.), urea and 2-mercaptoethanol from Bio-Rad (Hercules, CA, U.S.A.), ampicillin, kanamycin, guanidine hydrochloride (99%) and nickel sulphate from Sigma (St. Louis, MO, U.S.A.), dithiothreitol from Mallinckrodt Inc. (Chesterfield, MO, U.S.A.), Coomassie Brilliant Blue R-250 from Gibco (Gaithersburg, MD, U.S.A.), calcium acetate from MCB (Norwood, OH, U.S.A.), and imidazole from American Bioanalytical (Natick, MA, U.S.A.). All other reagents were from major commercial suppliers.

Construction of expression plasmids

A clone containing the heparinase I gene was isolated from a pUC18 genomic library, and two constructs were made by PCR, both flanked by *Nde*I and *Bam*HI restriction sites: one with the entire gene (384 amino acids) and one with the gene without its putative leader sequence, that read Met-Gln²²-Gln²³-Lys²⁴... (-L heparinase I; 364 amino acids) [42]. The -L heparinase I gene was cloned in four different expression vectors, pET-3a, pET-12a, pET-15b and pET-28a (Novagen, Madison, WI, U.S.A.), to give the constructs -L3a, -L12a, -L15b and -L28a respectively. For subcloning in pET-3a, pET-15b and pET-28a, the pUC18 plasmid containing the -L heparinase I gene insert was digested simultaneously with *Nde*I and *Bam*HI (New England Biolabs, Beverly, MA, U.S.A.), and the -L heparinase I gene was isolated using low-melt agarose (Gibco) gels. The vector was digested similarly, treated with alkaline phosphatase (Promega, Madison, WI, U.S.A.) for 30 min at 37 °C, heat-inactivated at 65 °C for 10 min and purified on a low-melt agarose gel. Vector and insert were ligated for 15 h at 16 °C using T4 ligase (New England Biolabs).

For subcloning of -L heparinase I in pET-12a, a *Sal*I restriction site was introduced at the N-terminus by PCR with degenerate primers. The promoter proximal primer was 5'-GCG TCG ACG CAG CAA AAA AAA TCC-3' and the promoter distal primer was 5'-AGG GGG ATC CCT ATC AGG CAG TTT CGC-3'. In a reaction volume of 25 µl, approx. 50 ng of the original heparinase I clone, amplified from the pUC18 library, was used as template for 12 cycles of PCR as described [42]. The PCR products from seven reactions were separated on a low-melt agarose gel, pooled and ligated into a T-vector. The T-vector, prepared by the method of Marchuk et al. [44], is a Bluescript plasmid (Stratagene, La Jolla, CA, U.S.A.) which has been

digested by *Eco*RV (New England Biolabs) and extended with deoxythymidine triphosphate (dTTP) using *Taq* polymerase (Perkin Elmer, Norwalk, CT, U.S.A.). The ligation mixture was transformed in DH5α competent *E. coli* (Gibco) and a plasmid with the -L heparinase I gene insert was isolated. The subcloned gene and pET-12a were digested sequentially, firstly with *Sal*I and then with *Bam* HI (New England Biolabs), the vector was treated with alkaline phosphatase as described above, and then vector and insert were isolated from low-melt agarose and ligated overnight. The translated sequence encoded by the new construct read: MRAKLLGIVLTTPIAISSFAST^{*QQK*}... (standard single-letter amino acid code), where the amino acids in italics are Gln²²-Gln²³-Lys²⁴ from the heparinase I gene and the first 22 amino acids constitute the OmpT leader sequence and a signal peptidase cleavage site. All plasmids containing the heparinase I gene insert were amplified in DH5α, and purified using Miniprep (Qiagen, Chatsworth, CA, U.S.A.); however, we have subsequently achieved better yields by amplifying in Novablue (Novagen).

DNA sequencing

The -L heparinase I gene in pUC18 was sequenced from denatured double-stranded plasmid using the Sequenase kit (United States Biochemical) and synthetic oligonucleotide primers designed for different regions of the gene in both directions. The sequence was identical to the region corresponding to amino acids 22–384 found on sequencing the clone isolated from the pUC18 genomic library [42].

Fermentation and harvesting

For each of the four expression constructs, 1 ng of plasmid was transformed in BL21(DE3) (Novagen), and a single colony was grown overnight and diluted to an absorbance of 0.06 unit in 50–400 ml of LB and 250 µg/ml ampicillin or 30 µg/ml kanamycin. (For -L28a, equally good expression was found when the colony was grown for 4–5 h to an absorbance of 2.5 units, concentrated 2-fold in medium and frozen with 15% glycerol at –70 °C in aliquots of 1.3 ml, which could then be used directly as inoculum.) The culture was grown to an absorbance of 0.5 unit, induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 2 h, harvested by centrifugation (4 °C; 3500 g; 10 min), washed in cold PBS and resuspended in 0.05 vol. of 50 mM Tris, 2 mM EDTA, pH 8.4, or (for -L15b and -L28a) in 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9 (the binding buffer for nickel-chelate chromatography). The resuspended culture was transferred to a borosilicate glass tube, sonicated for 2 min on ice using a Branson (Danbury, CT, U.S.A.) model 450 sonicator (power 3, 50% pulse), and centrifuged at 4 °C and 15000 g for 30 min (for the insoluble expression in -L12a and -L28a, centrifugation was at 4000 g for 20 min). The supernatant constituted the soluble fraction and the pellet the insoluble fraction of the crude lysate.

Solubilization and refolding

The insoluble fraction of the cell lysate was resuspended in 4 M guanidine hydrochloride (GdHCl), 20 mM Tris, 500 mM NaCl, 5 mM imidazole, 0.5% 2-mercaptoethanol, pH 7.9, kept for 20 min at 4 °C and centrifuged at 12000 g for 20 min. The supernatant contained the solubilized inclusion bodies and was refolded by dilution in 20 vol. of binding buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9) for 2 h. Precipitates were separated by centrifugation, and the supernatant was filtered

on a 0.45 μm sterile filter (Millipore, Bedford, MA, U.S.A.) before further purification.

Purification

The refolded r-heparinase I (from expression in -L28a) was purified by nickel-chelate chromatography using a column of 1.5 ml of Sepharose 6B Fast Flow resin covalently linked to nitrilotriacetic acid (Novagen) and an FPLC system (Pharmacia, Piscataway, NJ, U.S.A.). Briefly, the resin was charged with 8 ml of 200 mM NiSO_4 and equilibrated with 5 ml of binding buffer. Then, for the 'primary nickel chromatography' step, 50 ml of refolded solubilized inclusion bodies (containing 20 mg of total protein) was applied, followed sequentially by 8 ml of binding buffer, 8 ml of 15 % (v/v) elution buffer (20 mM Tris, 500 mM NaCl, 200 mM imidazole, pH 7.9), and 8 ml of 100 % elution buffer, all at 0.5 ml/min. r-Heparinase I was recovered in 6 ml of the 100 % elution buffer step, and the buffer was exchanged with thrombin cleavage buffer (20 mM Tris, 150 mM NaCl, 2.5 mM CaCl_2 , pH 8.4) using two PD10 size-exclusion columns (Pharmacia) following the instructions of the manufacturer. Thrombin (3 units; 1 unit degrades 1 mg of target protein in 16 h at 23 °C; from Novagen) was added, and the mixture was incubated overnight at 4 °C. After reaction with thrombin, the r-heparinase I mixture (7 ml) was applied to a recharged nickel-chelate column for the 'secondary nickel chromatography' step, and cleaved r-heparinase I was collected in the flow-through fraction. At each step, a fraction of the batch volume was retained for characterization. The yield of a step is expressed as the absolute enzymic activity of the recovered material divided by the absolute activity of the starting material. The absolute activity of the starting material of a step i is equal to the absolute activity of the recovered material of step $i-1$ multiplied by θ_{i-1}/θ_i , where θ_i is the fraction of batch volume remaining after step i .

For soluble r-heparinase I with a histidine tag (from expression using -L15b), the soluble lysate (15 ml) was used for the two-step nickel chromatography purification method, which was performed as described above, except that the thrombin reaction mixture and the flow-through fraction from the secondary nickel chromatography step were spin-concentrated with 10 kDa molecular mass cut-off membranes (Centricon p10; Amicon, Beverly, MA, U.S.A.).

Total protein and enzyme assays

Total protein was measured by the method of Bradford [45] using reagent from Bio-Rad and BSA (Sigma) as standard in the range 0.2–1.0 mg/ml.

Heparinase I activity was measured by adding 10–100 μl of sample (containing about 1 μg of heparinase I) to a cuvette with 3 ml of 2 mg/ml heparin (Kabi Pharmacia) in 100 mM Mops, 5 mM calcium acetate at pH 7.0 and 30 °C, and directly measuring the increase in absorbance at 232 nm as a function of time using an HP 8452A diode array spectrophotometer (Hewlett Packard). Activity is expressed as units (μmol of product formed/min) using a molar absorption coefficient for the unsaturated C-4–C-5 bond of uronic acids of $3800 \text{ cm}^{-1} \cdot \text{M}^{-1}$ [21]. To obtain the initial rate as a function of substrate concentration, solutions of 100 mM Mops, 5 mM calcium acetate, pH 7.0, with heparin concentrations of 0.015, 0.03, 0.053, 0.1, 0.25, 0.4, 1.0 and 5.0 mg/ml were prepared from stocks at 0 and 5 mg/ml. The reaction rate was measured as above, using only the initial 20–25 s of reaction. The data were fitted to the Michaelis–Menten equation $\{V = V_{\text{max}} \cdot [\text{heparin}] / (K_m + [\text{heparin}])\}$ using non-linear least-squares regression, inherent to the data analysis program DeltaGraph© Pro 3.5 (DeltaPoint, Inc., Monterey, CA, U.S.A.).

Gel electrophoresis

SDS/PAGE was carried out by the method of Laemmli [46] using precast 12 % gels, Mini-protein II apparatus and a Silver Stain Plus staining kit (all from Bio-Rad). Alternatively, the gels were stained with Coomassie Blue essentially as described [47]. Molecular mass markers were from Pharmacia and contained phosphorylase B (94.0 kDa), BSA (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Tryptic digest

Approx. 42 μg (1 nmol) of native or r-heparinase I was denatured in 50 μl of a solution of 8 M urea and 0.4 M ammonium bicarbonate, subsequently reduced with 5 mM dithiothreitol at 65 °C for 2 min, cooled to room temperature and alkylated with 10 mM iodoacetamide for 15 min. The total reaction volume was brought to 200 μl with water, 1.5 μg of bovine pancreatic trypsin (Boehringer Mannheim) was added, and the digestion was carried out at 37 °C for 24 h. A 80 μl sample of the digest was separated by reverse-phase HPLC using an HP1090 system and a C4 column (2.1 mm $\times$ 30 cm; Vydac, Hesperia, CA, U.S.A.), with elution from 2 to 80 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 150 $\mu\text{l}/\text{min}$, and monitoring at 210 and 277 nm. The elution schedule was 10 min isocratic at 2 % acetonitrile, 2–30 % acetonitrile gradient in 60 min, 30–60 % in 30 min and 60–80 % in 15 min.

Peptide sequencing and MS of purified heparinase I

The purified heparinase I was sequenced using an Applied Biosystems Sequencer model 477, with an on-line model 120 phenylthiohydantoin amino acid analyser (Biopolymers Laboratory, Center for Cancer Research, MIT). The molecular mass of r-heparinase I was determined by matrix-assisted laser desorption MS (MALDI-MS) on a Voyager Elite (PerSeptive Biosystems, Framingham, MA, U.S.A.) operated in the linear mode. Protein solution (1 μl ; 10 pmol) was mixed with 9 μl of a solution of 10 mg/ml sinnapinic acid (the matrix) dissolved in water/acetonitrile (1:1, v/v), then 1 μl of the resulting mixture was deposited on the target and allowed to dry before ionization.

Separation and characterization of heparin degradation products

Heparin digestion was carried out with both native and r-heparinase in 5 mM calcium acetate, 100 mM Mops buffer, pH 7.0, overnight. The reaction mixture was injected into a POROS Q/M (4.6 mm $\times$ 100 mm) anion-exchange column connected to a BioCAD system (PerSeptive Biosystems). The saccharides were eluted using a linear gradient of 0.2 M NaCl/10 mM Tris, pH 7.0, to 1.4 M NaCl/10 mM Tris, pH 7.0, in 35 ml and monitored at 232 nm. For the analytical separations the flow rate was fixed at 5 ml/min and 0.5 mg of digested heparin was loaded on to the column. Preparative separation of heparin fragments (generated by heparinase I digestion at an enzyme:substrate ratio of 0.02 $\mu\text{g}/\text{mg}$ for 96 h) was carried out using a similar gradient. A 2 mg sample of heparin was loaded on to the column and the flow rate used was 7 ml/min. The individual saccharide peaks were collected in 50 ml Falcon tubes and the peaks from multiple runs were pooled. The pooled fractions were desalted using a Bio-Gel P-2 fine (Bio-Rad) size-exclusion column (3 cm $\times$ 100 cm) with 10 mM ammonium bicarbonate as the running buffer, and subsequently lyophilized. The lyophilized saccharides were reconstituted in water and analysed using anion-exchange chromatography. Purified heparin disaccharides

(I-S and III-S from Sigma) were used as external standards. The purity and chemical composition of the collected tetrasaccharides were analysed using non-covalent complexes with (Arg-Gly)₁₅ by MALDI-MS [48,49]. A 1 µl sample of basic peptide (0.1 mg/ml) was mixed with 8 µl of caffeic acid [10 mg/ml in 1:1 (v/v) water/acetonitrile]. To this was added 1 µl of saccharide solutions (0.1 mg/ml), and 1 µl of the final mixture was deposited on the target and allowed to dry before analysis with a Vestec 2000 linear time-of-flight mass spectrometer (Vestec, Houston, TX, U.S.A.).

RESULTS

Expression

Initially, the heparinase I gene without the putative leader sequence (-L heparinase I) was expressed as a sequence that reads Met-Glu²²-Glu²³... using the pBR322-derived pET-3a expression plasmid [42]. The expression level was low, with the induced band being barely discernible in an SDS/PAGE gel (Figure 1), and the specific activity was around 0.8 unit/mg of protein, whereas the specific activity obtained for purified native heparinase I is 130 units/mg [21].

To improve expression levels and optimize the purification scheme, the -L heparinase I gene was cloned in three other

Figure 1 SDS/PAGE analysis of the expression level obtained with various expression plasmids

For each of the cultures, one sample was taken immediately before induction ($t = 0$) and one was taken at harvest, 2 h after induction ($t = 2$). The samples were spun down and resuspended in SDS loading buffer to a concentration of about 0.5 mg dry cell weight/ml. Then 45 µl of each sample was loaded on a precast 12% polyacrylamide gel, which was electrophoresed and stained with Coomassie Blue. The samples are: -L3a [lanes 1 ($t = 0$) and 2 ($t = 2$)], -L12a [lanes 3 ($t = 0$) and 4 ($t = 2$)], -L15b [lanes 5 ($t = 0$) and 6 ($t = 2$)] and -L28a [lanes 7 ($t = 0$) and 8 ($t = 2$)]. Molecular mass markers (kDa) are shown in lane 9.

expression systems: pET-12a, which contains a 21-amino-acid OmpT leader peptide, and pET-15b and pET-28a, both of which contain a 21-amino-acid histidine tag leader sequence with six consecutive histidine residues. pET-28a has kanamycin resistance, whereas the other three plasmids have ampicillin resistance. An overview of the constructs and the expression levels obtained from them is given in Table 1. -L15b gave the same low expression level of soluble protein as -L3a, e.g. 0.9 unit/mg, corresponding to less than 1% of the soluble protein being active heparinase I. -L12a and -L28a, on the other hand, gave a high level of expression of insoluble material which could be solubilized in 4 M GdHCl and refolded by dilution to give specific activities of 6 and 19 units/mg respectively. Figure 1 shows SDS/PAGE analysis of the cellular proteins before and after induction for each of the four constructs. Expression of -L3a and -L15b in BL21(DE3) pLysS, which carries the pLysS plasmid encoding T7 lysozyme, yielded similar results as expression in BL21(DE3).

We investigated the possible causes of the observed differences in expression levels among the four plasmids using metabolic pulse labelling with ³⁵S in the presence of rifampicin [50,51] and an activity assay for β -lactamase [52]. We found that, for the constructs that expressed heparinase I poorly (-L15b and -L3a), β -lactamase was expressed at high levels, controlled by the T7 promoter and apparently translated from the same mRNA as heparinase I (results not shown). This was not the case for the constructs that expressed heparinase I at high levels (-L12a and -L28a). The T7 termination sequence of the pET plasmids should serve to terminate transcription between the heparinase I and β -lactamase genes; DNA sequencing of -L15b and -L12a downstream of the heparinase I gene revealed no differences between these two plasmids in their T7 terminator regions (results not shown).

Purification

Cultures of *E. coli* harbouring the expression plasmid -L28a were induced at an A_{600} of 0.8–1.0, and continued to grow to an A_{600} of around 3.5 at the time of harvest. Continued growth after induction was not observed for plasmids -L3a and -L15b, which produced soluble, active r-heparinase I; this suggests that active heparinase I may be toxic to the cells.

The concentrated cell culture was sonicated on ice; this released approx. 0.2 mg of protein/mg dry cell weight. Of the total amount of protein, 68% was soluble in Tris buffer, whereas the activity of soluble r-heparinase I was less than 1% of the activity that could be obtained from refolding of the inclusion bodies. Thus isolation of the inclusion bodies alone resulted in elimination of two-thirds of the contaminating proteins without significant loss of r-heparinase I. During purification of native

Table 1 Expression constructs

For each of the expression constructs used in this study, the heparinase I expression level is expressed as units of activity recovered per mg of total cellular protein. For -L3a and -L15b, which gave soluble expression, both measurements were done in the soluble fraction of the crude lysate. For -L12a and -L28a, which gave insoluble expression, the total protein and heparinase I activities measured in the soluble fraction and in the insoluble fraction after refolding were added together. The β -lactamase activity was analysed in the crude lysate of cultures harvested at $A_{600} = 0.8$ –1.0 after a 2 h induction. N.D., not determined.

Construct	Leader sequence	Antibiotic resistance	β -Lactamase expression (unit/ml)	Heparinase I expression (units/mg of protein)
-L3a	—	Ampicillin	N.D.	0.8
-L12a	OmpT	Ampicillin	< 0.001	6.0
-L15b	Histidine tag	Ampicillin	0.10	0.9
-L28a	Histidine tag	Kanamycin	N.D.	19.0

Figure 2 SDS/PAGE analysis of purification from insoluble and soluble expression of heparinase I

Whole-cell samples, corresponding to 20 μ l of the fermentation broth, were electrophoresed on precast 12% polyacrylamide gels, after resuspension in SDS/PAGE loading buffer. Both gels were silver stained. The left-hand gel shows samples from three stages of purification from inclusion bodies (-L28a): after refolding of the isolated inclusion bodies (lane A1), the eluate from the primary nickel column (lane A2) and the flow-through from the secondary nickel column (lane A3); molecular mass markers at 97, 67, 43, 30, 20.1 and 14.4 kDa are shown in lane A4. The right-hand gel shows the same molecular mass markers (lane B1) and the purified r-heparinase I from soluble expression in -L15b (lane B2).

heparinase I from *F. heparinum*, precipitation with protamine sulphate increased the absolute heparinase I activity of the crude cell lysate by 42-fold, supposedly due to removal of nucleic acids that may inhibit heparinase I [21]. We did not observe any purification by precipitation with protamine sulphate, and did not include this step in cell extract processing. Denaturation of the inclusion bodies was completed after 15 min in 4 M GdHCl. Addition of 2-mercaptoethanol in the denaturing buffer to a concentration of 0.5% (v/v) increased the recovery of active r-heparinase I after refolding by 40%, possibly by preventing non-native disulphide bridge formation between the two cysteines in processed protein, i.e. Cys-135 and Cys-297 [42]. Refolding was approx. 95% complete after 1 h, as judged by the recovery of activity. Both SDS/PAGE (Figure 2) and the specific activity of 58 units/mg after renaturation, compared with 130 units/mg obtained for purified native heparinase I [21], suggested that the refolded inclusion bodies contained at least 45% r-heparinase I.

Purification of r-heparinase I to homogeneity was achieved using two affinity-chromatography steps, with the high-affinity

histidine tag on r-heparinase I being removed between the steps. The key results from the purification process are summarized in Table 2. The refolded proteins were applied to a 1.5 ml nickel-chelate column and eluted at 200 mM imidazole after two wash steps. The loss of activity in this primary nickel affinity chromatography step was 53%, of which only a few percent could be recovered in the washes. After elution, the complexed nickel sulphate is stripped off the column with EDTA. By assaying the strip solution by SDS/PAGE, we found that an estimated 10% of the total heparinase I did not elute at 200 mM imidazole. Introduction of a 1 M wash eluted this material, but no additional activity was recovered, indicating that this material may bind strongly, perhaps as multivalent, inactive, aggregates. The two subsequent chromatography steps, desalting and non-interacting nickel chromatography, showed high recoveries of 91 and 78% respectively. On overnight incubation with thrombin the activity increased by 43%, while the activity of desalted material without thrombin cleavage increased by only 14%. The increase seems to be a combined effect of allowing heparinase I to refold further overnight after complete removal of GdHCl by chromatography, and of cleaving the leader sequence, which may allow a sub-population of misfolded species to refold. After the secondary nickel chromatography there were no side bands visible on a silver-stained SDS/PAGE gel. The specific activity was 117 units/mg, which is comparable with the highest specific activity, 130 units/mg, that has been obtained for purification of native heparinase I from *F. heparinum* [21]. The cumulative yield for the entire process was 43%, and the net recovery of homogeneous r-heparinase I was 14.4 mg/l of culture, or 12.4 mg/g dry cell weight.

Purification of soluble r-heparinase I expressed with the plasmid -L15b was much less efficient, as seen from the results summarized in Table 3. The low expression level necessitated two concentration steps, which introduced significant losses. The combined yield for the process was 8.3%, and the purified heparinase I was homogeneous, as evaluated by silver-stained SDS/PAGE analysis (Figure 2).

Characterization

The purified r-heparinase I was analysed by MALDI-MS (Figure 3). Heparinase I was the only detectable protein, and appeared with a single charge at a molecular mass of 41 743 Da and with a double charge at an m/z of 20905 Da. This result is consistent with the calculated molecular mass of the translated sequence of 41 723 Da.

Native and r-heparinase I were digested with trypsin after denaturation in urea, reduction with dithiothreitol and alkylation with dithiothreitol. The reverse-phase HPLC profile of the digests

Table 2 Purification from insoluble expression using the -L28a construct

A total of 300 ml of culture was harvested at an A_{600} of 3.4, after a 2 h induction with IPTG. N.D., not determined. For calculation of specific activity, it is assumed that thrombin cleavage does not change the total protein measurement.

Step	Volume (ml)	Fraction of batch volume	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Soluble lysate	6	1.00	42.5	10.0	0.2	—
Refolded inclusion bodies	50	1.00	20.3	1177	58	—
Primary nickel chromatography	5.9	0.96	7.1	478	67	42
Desalting	7	0.81	5.0	367	73	91
Thrombin cleavage	7	0.81	N.D.	525	105	143
Secondary nickel chromatography	7.8	0.72	3.1	362	117	78

Table 3 Purification from soluble expression using the -L15b construct

A total of 750 ml of culture was harvested at an A_{600} of 1.5, after a 2 h induction with IPTG. N.D., not determined.

Step	Volume (ml)	Fraction of batch volume	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude lysate	15	1.00	82.5	73.4	0.9	—
Primary nickel chromatography	4	0.93	0.8	36.1	45.1	53
Desalting	7	0.89	N.D.	29.1	—	84
Thrombin cleavage	6.8	0.86	N.D.	29.2	—	104
Spin concentration	0.31	0.81	N.D.	16.9	—	61
Secondary nickel chromatography	3	0.69	N.D.	9.8	—	68
Spin concentration	0.27	0.46	N.D.	2.8	—	43

Figure 3 MS profile for purified recombinant heparinase I

The count frequency of the detector (y -axis) is plotted against the mass-to-charge ratio (m/z) calculated from the time of flight.

Figure 5 Kinetic analysis of heparin degradation

The initial reaction rates are plotted against substrate concentration for native ($\diamond$) and recombinant (+) heparinase I. The non-linear least-squares fit to the Michaelis–Menten equation is plotted in each case: curve A, native heparinase I; curve B, recombinant heparinase I. mAU, milli-absorbance units.

Figure 4 Reverse-phase HPLC separation of tryptic digests of native (A) and recombinant (B) heparinase I

Approx. 400 pmol of digested heparinase I was injected on to a C4 column and the peptides were eluted from 2 to 80% acetonitrile in 0.1% trifluoroacetic acid. Tryptic peptides were detected at 210 nm (upper line) and 277 nm (lower line). mAU, milli-absorbance units.

is shown in Figure 4. The overall appearances of the profiles are similar, with no significant differences. In particular, the peaks at 38 and 84 min, corresponding to the peptides td04 and td43, which were used for creating a screening probe for cloning [42] and which contain the active Cys-135 and the Cys-297 respectively [53], are clearly distinguishable.

The purified heparinase I was sequenced and the N-terminus was Gly-Ser-His-Met-Gln²²-Gln²³-Lys²⁴-Lys²⁵-Ser²⁶-Gly²⁷, with no other amino acids detectable in any of the 10 cycles. The first four amino acids are part of the thrombin cleavage site of the translated sequence and the *Nde*I site of the gene which was introduced for subcloning into expression vectors [42]. The next six residues are consistent with sequencing of the heparinase I gene, and the numbers refer to the open reading frame of the native heparinase I sequence [42]. Due to post-translational modifications, the N-terminal amino acid sequence of purified native heparinase I cannot be determined [42].

Kinetic analysis of native and recombinant heparinase I

The initial rate of increase in absorbance at 232 nm was determined with heparin concentrations ranging from 0.015 to 5 mg/ml (Figure 5). The data were fitted to the Michaelis–Menten expression $V = V_{\max} \cdot [\text{heparin}] / (K_m + [\text{heparin}])$, to give $K_m = 33 \mu\text{g/ml}$ and $47 \mu\text{g/ml}$, and $V_{\max} = 2.29$ and 2.37 milli-

Figure 6 Analysis of heparin degradation products

(A) Anion-exchange profiles of the products from the degradation of heparin with recombinant (upper profile) and native (lower profile) heparinase I. A 0.5 mg sample of digested heparin was injected on to a POROS column and oligosaccharides were eluted with a gradient of 0.2–1.4 M NaCl. (B) A representative MALDI-mass spectrum for the isolated tetrasaccharide peak 5. The two peaks are the peptide–oligosaccharide complex at a mass-to-charge ratio (m/z) of 4371.8 Da and the peptide at 3217.5 Da, from which the mass of the oligosaccharide was calculated by subtraction to be 1154.2 Da.

absorbance units/s for native and recombinant heparinase I respectively, with regression coefficients (r^2) of 0.981 and 0.983. Thus the kinetic constants for the recombinant and native heparinase I are essentially identical.

Anion-exchange separation of degradation products

Figure 6 shows the anion-exchange separation of the fragments following heparin digestion; the profiles with native heparinase and with recombinant heparinase are identical. Peak 1 and peak 2 are $\Delta U_{2S}-H_{NS}$ and $\Delta U_{2S}-H_{NS,6S}$ respectively, as determined from the elution time of purified standards. MS analysis showed that peaks 3, 4 and 5 had masses of 1075, 1075 and 1154 Da, corresponding to the structures $\Delta U_{2S}-H_{NS}-I_{2S}-H_{NS,6S}$, $\Delta U_{2S}-H_{NS,6S}-G-H_{NS,6S}$ and $\Delta U_{2S}-H_{NS,6S}-I_{2S}-H_{NS,6S}$ respectively, previously identified by Rice and Linhardt [54]. [Heparin oligosaccharides are abbreviated as follows: I is α -L-idopyranosyluronic acid, G is β -D-glucopyranosyluronic acid, U is either I or G, ΔU is I or G with an unsaturated C-4–C-5 bond, and H is α -D-2-deoxy-2-aminoglucopyranose. Subscripts 2S and 6S indicate 2-O- and 6-O-sulphation respectively, and NS indicates N-sulphation of

the hexosamine.] Peak 6 could not be analysed by MS, but the late elution indicates a hexasaccharide product [55].

DISCUSSION

Heparinases have been prepared from *F. heparinum*, induced by heparin, by at least five different groups of workers during the past 25 years. The challenges have been to isolate the three different heparinases from the co-induced chondroitinase [56] and sulphatase [57] activities, and to separate heparinases I, II and III from each other. Previously, investigators have used purification by hydroxyapatite and cellulose phosphate chromatography [17,19], agarose electrophoresis [57], immunoaffinity chromatography [58], and a combination of batch hydroxyapatite and anion-exchange chromatography [20] followed by hydroxyapatite HPLC [21]. In the present paper we have investigated the recombinant expression of the heparinase I gene, previously cloned by us [42], in *E. coli* using a variety of vectors. Expression of r-heparinase I proved to be highly sensitive to the choice of expression vector. Even in the case of -L15b and -L28a, for which the vectors have identical leader sequences and regulatory regions up to 300 bases upstream of the *Nde*I cloning site, and for which the -L heparinase I gene was inserted at identical subcloning sites, there was a 20-fold difference in expression level. This difference could be due in part to the simultaneous high-level expression of β -lactamase from the T7 promoter in -L15b, which might limit the availability of precursors for heparinase I synthesis or eliminate the effect of ampicillin in the medium and thus affect plasmid stability. Measurement of β -lactamase activity in the crude lysate turned out to be a rapid way to detect this problem and led us to use a vector selected for by kanamycin (pET-28a), which does not carry the β -lactamase gene. Expression from -L28a, followed by refolding of insoluble heparinase I and purification by nickel-chelate chromatography, yielded 14.4 mg of purified r-heparinase I per litre of culture. The structural and functional characterization presented in this study (Figures 4–6) shows that the recombinant and native enzymes are degraded similarly by trypsin and have comparable kinetic constants, and that the product profiles on separation by POROS anion-exchange chromatography are similar. There is, however, a difference between the molecular masses of native heparinase I (42 575 Da) [38] and of r-heparinase I (41 743 Da, including four non-native amino acids at the N-terminus). Enzymic degradation and MS showed that native heparinase I is O-glycosylated at serine-39 and its N-terminus is pyroglutamate (from glutamine-22) [59a], consistent with our interpretation of the sequencing and chemical modification data [38,42,53]. These modifications, which do not take place in *E. coli*, account for the difference in molecular mass between native and recombinant heparinase I. Further, the glycosylation consensus sequence and the Man-(1-O)-serine linkage of heparinase I [59a] is similar to those of two glycosylated enzymes from *F. meningosepticum* [59b,59c].

The efficiency of recombinant expression is compared in Table 4 with the two most efficient methods for the expression and purification of native heparinase I presented by Lohse and Linhardt [21] and by Zimmerman et al. [13], and with the widely cited method of Yang et al. [20]. The specific activity of purified heparinase I obtained by recombinant expression is comparable with that achieved on expression of the native enzyme, with a net recovery that is 30–170 times higher. Another measure of the economic efficiency of a purification process is the required chromatography bed volume, since capital and operating costs associated with chromatography steps often dominate the cost of a fermentation process. The bed volume of the process presented here is 0.0033 ml of settled bed/unit of purified enzyme, compared

Table 4 Comparison of the efficiencies of expression and purification of native heparinase I from *F. heparinum* and of r-heparinase I in *E. coli*

Enzyme	Reference	Culture volume (litres)	Recovery of purified heparinase I (units/litre of culture)	Yield (%)	Specific activity (units/mg)
Native	[20]	10	0.08	0.8	26.6
Native	[13]	100	9.8	11.8	75
Native	[21]	5	62.4	10	130
Recombinant	This work	0.3	1676	43	117

with 0.86 ml/unit [21] and 0.57 ml/unit [13] for the two most efficient processes for the purification of native heparinase I.

Heparin digestion by heparinases I, II and III gives different product profiles due to the differences in the substrate specificities of the three enzymes: heparinase I cleaves linkages of the type $H_{NS,6X}-I_{2S}$ or $H_{NS,6S}-I_{2X}$; heparinase II cleaves $H_{NY,6X}-U_{2X}$; and heparinase III cleaves $H_{NY}-G$ or $H_{NAC,6S}-I$, where X is either sulphated or unsubstituted, Y is either sulphated or acetylated and U is either glucuronic or iduronic acid [26]. As a consequence of this molecular specificity, the three different heparinases may serve as tools to elucidate the primary structure of heparin and heparan sulphates from various proteoglycans, cells or tissues, in analogous ways to the use of glycosidases to study glycoproteins [60,61], provided that the composition of degradation products can be characterized rapidly and reproducibly. In the present paper we report the development and validation of a new, rapid technique for the separation of heparin degradation products using POROS anion-exchange chromatography. Reproducible product profiles could be obtained in less than 10 min, compared with more than 100 min for previous anion-exchange-based separations [54].

Efficient expression systems for wild-type and mutant heparinases will facilitate elucidation of the molecular mechanism of specificity in interactions between heparinases and their substrates. The methods for the expression and purification of r-heparinase I presented in this paper may be extended to expression of recombinant heparinase II or III or to mutants of the heparinases. In fact, the low level of expression of soluble heparinase I using pET-15b proved sufficient for the initial characterization of the properties of mutants of heparinase I [53,62]. It was found that substituting cysteine-135 with alanine completely abolished activity, while substituting cysteine-135 with serine decreased the specific activity to 2% of that of wild-type heparinase I [53], indicating that this residue is part of the active site of heparinase I.

Heparin and heparan sulphate take part in a wide range of biological functions in the extracellular matrix and in the circulation. Some of these can be controlled clinically with the use of heparinase I [9,16], while others have only recently been discovered [32]. The production and characterization of purified recombinant heparinase I presented here may contribute to providing a fundamental insight into the role of heparin in extracellular matrix processes, as well as to the development of new medical technologies.

We thank Dr. Richard Cook (Biopolymers Laboratory, MIT) for peptide sequencing and oligonucleotide synthesis, Dr. Noubar Afeyan (PerSeptive Biosystems) for the BioCAD instrument, and Dr. Oscar Garro and Veena Kulkarni for assistance. MS data were provided by the MIT Mass Spectrometry Facility, which is supported by National Institutes of Health grant no. RR 00317 (to K. Biemann); we thank Professor Klaus Biemann and Andrew Rhomberg for their help. We thank Professor Phil Robbins, Center for Cancer Research, MIT (supported by National Institutes of Health Grant no. GM31318). This work was supported by the National Institutes of Health

(GM 25810) and by the National Science Foundation through the Biotechnology Process Engineering Center, MIT.

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