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Cloning, recombinant production, crystallization and preliminary X-ray diffraction studies of a family 84 glycoside hydrolase from *Clostridium perfringens*

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Clostridium perfringens is a ubiquitous environmental organism that is capable of causing a variety of diseases in mammals, including gas gangrene and necrotic enteritis in humans. The activity of a secreted hyaluronidase, attributed to the NagH protein, contributes to the pathogenicity of this organism. The family 84 catalytic module of one of the three homologues of NagH found in *C. perfringens* (ATCC 13124) has been cloned. The 69 kDa catalytic module of NagJ, here called GH84C, was overproduced in *Escherichia coli* and purified by immobilized metal-affinity chromatography (IMAC). Crystals belonging to space group $I222$ or $I2_12_12_1$ with unit-cell parameters $a = 130.39$, $b = 150.05$, $c = 155.43$ Å were obtained that diffracted to 2.1 Å. Selenomethionyl crystals have also been produced, leading to the possibility of solving the phase problem by MAD using synchrotron radiation.

1. Introduction

Clostridium perfringens, a Gram-positive spore-forming anaerobic bacterium, is a causative agent of gas gangrene, necrotic enteritis and food poisoning in humans (Hatheway, 1990; McDonel, 1986; Rood & Cole, 1991). This organism is notable for the assortment of exotoxins that contribute to the pathogenicity of the organism (Petit *et al.*, 1999). Among these toxins is the μ-toxin (here called NagH), a glycoside hydrolase with hyaluronidase activity (EC 3.2.1.35) thought to be encoded by the *nagH* gene (Canard *et al.*, 1994). Analysis of the recently completed and annotated genomic DNA sequence of *C. perfringens* (strain 13; Shimizu *et al.*, 2002) reveals that in addition to the μ-toxin, this organism may produce numerous secreted glycoside hydrolases. The NagH toxin is thought to participate in the spread of *C. perfringens* deeper within a wound through the degradation of hyaluronic acid, a major carbohydrate component of connective tissue (Canard *et al.*, 1994). The additional complement of secreted glycoside hydrolases may also play a supporting role in tissue destruction and/or bacterial nutrition.

The NagH protein is a large and complex multi-modular protein containing 1628 amino acids. The putative catalytic module comprising the first ~660 amino acids places this protein in family 84 according to the CAZy glycoside hydrolase classification (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The genome of *C. perfringens* (strain 13) contains four additional ORFs which encode proteins having N-terminal family 84 catalytic modules. These putative proteins are also multi-modular and range in size from 1001 to 1297 amino acids. Currently, no three-dimensional structure is available for any family 84 glycoside hydrolase. In an effort to initiate structure–function studies of this family of enzymes, we have dissected the modular structure of NagJ, here called GH84C, the 1001-amino-acid four-module protein from *C. perfringens* (see Fig. 1

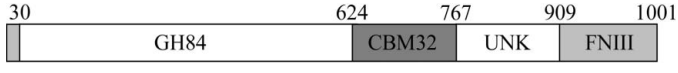
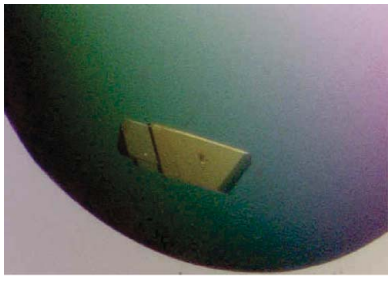


Figure 1
Modular structure of GH84C. Numbers indicate the amino acid at the putative module boundaries. Amino acids 1–30 represent the N-terminal secretion signal. Amino acids 31–624 represent the GH84C catalytic module. Amino acids 625–767 represent a family 32 carbohydrate-binding module. Amino acids 768–909 bound an unknown module. Amino acids 910–1001 share a distant sequence identity with fibronectin type III repeats.



for a schematic representation of the modular arrangement). The catalytic module consisting of 593 amino acids was recombinantly produced. In this communication, we report the cloning, recombinant overproduction, crystallization and preliminary X-ray diffraction experiments of the 69 kDa catalytic module in the absence of its accessory modules.

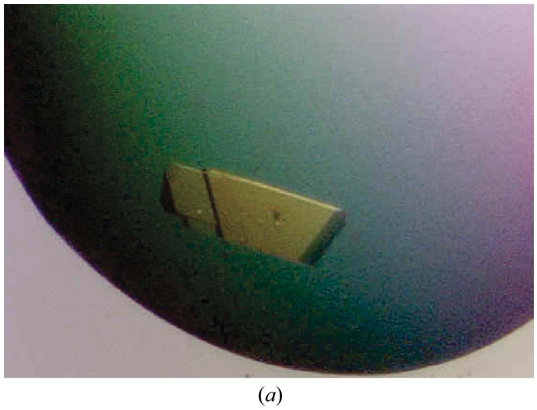
2. Materials and methods

2.1. Cloning, production and purification of GH84C

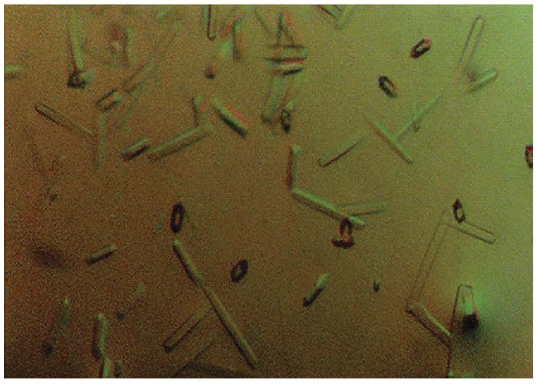
The DNA fragment encoding the GH84C catalytic module was amplified from *C. perfringens* genomic DNA (strain ATCC 13124; purchased from Sigma, St Louis, MO, USA) by PCR using the oligonucleotide primers 5′-CAT ATG GCT AGC GTA GGA CCT AAA ACT GGG-3′ and 5′-GAA TTC GGA TCC TTA TAT TAA TGT TAA ATC AAA ACT TAA AGC-3′ (Qiagen, Mississauga, ON, Canada). The PCR product was obtained using Platimum Pfx polymerase (Invitrogen, Carlsbad, CA, USA) with an initial 5 min 367 K hot start followed by 30 thermal cycles of 367 K for 45 s, 317 K for 45 s and 341 K for 2 min and a final 5 min extension time at 341 K. This allowed amplification of nucleotides 91–1872 of the *gh84C* gene, which encode amino acids 31–624 of GH84C. The amplified DNA fragment was cloned into pET-28a(+) (Novagen, Mississauga, ON, Canada) *via* the introduced *Nhe*I and *Bam*HI restriction endonuclease sites to produce pET-GH84Ccat. The encoded polypeptide comprises an N-terminal six-histidine tag separated from the GH84 module by a thrombin-cleavage sequence.

The GH84C catalytic module was overproduced in *Escherichia coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) in Luria–Bertani media containing 50 µg ml^{-1} kanamycin (Sigma). Centrifugation at 3354*g* was used to harvest the cells. Following resuspension in binding buffer (20 m*M* Tris pH 8.0 containing 500 m*M* NaCl), the cells were lysed with a French pressure cell and the cell debris was pelleted by centrifugation at 17 608*g*. The supernatant was then loaded onto Sigma His-Select resin followed by elution with a step gradient of binding buffer supplemented with increasing concentrations of imidazole up to 500 m*M*. Fractions were electrophoresed by SDS–PAGE and those fractions containing protein judged to be greater than 95% pure were pooled, concentrated and buffer-exchanged into 20 m*M* Tris pH 8.0 using a stirred ultrafiltration cell (Amicon) with a 3.5 kDa cutoff membrane (Fisher, Nepean, ON, Canada). This gave a final yield of ∼76 mg per litre of culture.

Selenomethionine-labelled GH84C was produced in the methionine-auxotroph *E. coli* strain B834 (DE3) (Novagen). Minimal media was prepared as per the instructions of the manufacturer (Athena Enzyme Systems, Baltimore, MD, USA). Kanamycin was added to a final concentration of 50 µg ml^{-1}. Cultures were grown to an OD_{600} of 1.0 and protein production was induced with 0.5 m*M* isopropyl *β*-D-thiogalactoside (IPTG). Cell lysis, purification and protein concentration were performed as above. The overall yields were approximately 2 mg per litre of culture.



(*a*)



(*b*)

Figure 2
(*a*) Crystals of native GH84C catalytic module. (*b*) Crystals of selenomethionine-derivatized GH84C catalytic module.

2.2. Crystallization and X-ray data collection

Crystals were grown in 0.1 *M* sodium cacodylate pH 6.5, 15% PEG 6000 (Fluka) and 0.15 *M* KSCN by the hanging-drop vapour-diffusion method using GH84C at 20 mg ml^{-1}. Crystals took approximately five months at 291 K to grow to a suitable size (Fig. 2*a*). SeMet-labeled GH84C catalytic module (at 13 mg ml^{-1}) crystallized under the same conditions after approximately two months. In both cases, removal of the six-histidine tag was unnecessary for crystallization. Mother liquor supplemented with 15% MPD (Hampton Research) was used as a cryoprotectant: crystals were quickly soaked in this mixture then flash-frozen at 113.15 K. Diffraction experiments were performed on a system comprising an R-AXIS IV^{++} area detector, a Micromax-002 X-ray generator, Osmic Blue Optics and an Oxford Cryo 700 System. Images were collected every 0.5° with an exposure time of 4 min. All data processing was performed with the *CrystalClear*/*d*TREK* (Pflugrath, 1999) package included with the instrument.

3. Results and discussion

Although we successfully cloned the catalytic module of NagH from *C. perfringens* (ATCC 13124), we were entirely unsuccessful at producing a recombinant form in *E. coli*. In contrast, the catalytic module of GH84C (NagJ) was efficiently overproduced in *E. coli* and could be easily purified to homogeneity in a single chromatographic step by IMAC. Simple enzymatic endpoint assays of protein in 20 m*M* Tris pH 8.0 using standard substrates yielding colorimetric products indicated that this enzyme was active on the synthetic substrate *p*-nitrophenyl *N*-acetyl *β*-D-glucosaminide but not on *o*-nitrophenyl *N*-acetyl *β*-D-galactosamide, *p*-nitrophenyl *β*-D-glucopyranoside, *p*-nitrophenyl *β*-D-galactopyranoside or *p*-nitrophenyl *α*-D-galactopyranoside (all from Sigma), thus confirming that the catalytic module of GH84C does indeed possess glycoside hydrolase activity and is active as a hexosaminidase. Its activity on *β*-linked *N*-acetyl glucosamine suggests that this enzyme may also possess hyaluronidase activity, but we have not yet been able to confirm this.

The recombinant GH84C catalytic module appeared to be relatively stable and yielded diffraction-quality crystals (Fig. 2*a*). These

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution bin.

Wavelength (Å)	1.5418
Space group	$I222$ or $I2_12_12_1$
Unit-cell parameters (Å, °)	a = 130.39, b = 150.05, c = 155.43, α = 90.00, β = 90.00, γ = 90.00
Resolution range (Å)	20.00–2.10 (2.17–2.10)
Total No. of reflections	1384042
No. of unique reflections	171588
Average redundancy	16.14 (15.7)
Completeness (%)	99.9 (100.0)
$R_{\rm merge}$† (%)	10.3 (39.7)
$\langle I/\sigma(I)\rangle$	12.4 (4.8)

† $R_{\rm merge} = \sum |I(k) - \langle I\rangle| / \sum I(k)$.

crystals diffracted to a $d_{\rm min}$ of 2.1 Å and belonged to one of the orthorhombic space groups $I222$ or $I2_12_12_1$ (Table 1). The unit-cell parameters are a = 130.39, b = 150.05, c = 155.43 Å, $\alpha = \beta = \gamma = 90.00$. Given that the expected molecular weight of the module is 69.1 kDa, the asymmetric unit is anticipated to contain two molecules, with a solvent content of roughly 55%. The SeMet-labelled version of the GH84C catalytic module crystallized under the same conditions as the native; however, the crystals were somewhat smaller (Fig. 2*b*) and did not diffract beyond a $d_{\rm min}$ of ∼8 Å on our home equipment. Nevertheless, the SeMet-labelled protein is a good candidate for MAD using tunable synchrotron radiation to solve its structure.

Family 84 glycoside hydrolases are an important family of enzymes. They are found as toxins in flesh-eating bacteria but also play important roles in mammals. The human homologue (*i.e.* meningioma-expressed antigen 5 or O-GlcNAcase) is involved in regulating the levels of *O*-linked *N*-acetylglucosamine residues on nuclear proteins, which in turn has implications in type II diabetes (Lehman *et al.*, 2005). Recently, this enzyme's catalytic mechanism was shown to be one of substrate-assisted catalysis in which the acetamido group of *N*-acetylglucosamine is a key participant in the hydrolysis reaction (Macauley *et al.*, 2005). Determination of the structure of the catalytic module of GH84C will provide a basis for structural studies into the catalytic mechanism used by the family 84 glycoside hydrolase enzymes.

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