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Expression, purification, crystallization and preliminary X-ray diffraction analysis of conjugated bile salt hydrolase from *Bifidobacterium longum*

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Conjugated bile salt hydrolase (BSH) catalyses the hydrolysis of the amide bond that conjugates bile acids to glycine and to taurine. The BSH enzyme from *Bifidobacterium longum* was overexpressed in *Escherichia coli* BL21(DE3), purified and crystallized. Crystallization conditions were screened using the hanging-drop vapour-diffusion method. Crystal growth, with two distinct morphologies, was optimal in experiments carried out at 303 K. The crystals belong to the hexagonal system, space group $P622$ with unit-cell parameters $a = b = 124.86$, $c = 219.03$ Å, and the trigonal space group $P321$, with unit-cell parameters $a = b = 125.24$, $c = 117.03$ Å. The crystals diffracted X-rays to 2.5 Å spacing. Structure determination using the multiple isomorphous replacement method is in progress.

1. Introduction

Bile acids normally exist in a conjugated form bonded to glycine or taurine by a carboxyl side chain and effect the digestion and absorption of lipids. Circulation of bile acids occurs in the bowel, with most of the bile acids discharged into the intestine being absorbed through the ileum to be recovered by the liver (Russell, 2003). Stagnation of this circulation leads to diseases of the hepatic/bile duct and digestive tract. In recent years, increased attention has been given to the possibility of using bile-salt conjugation carried out by lactic acid bacteria to lower serum cholesterol levels in hypercholestraemic humans or to prevent hypercholesterolaemia in individuals with normal cholesterol levels (Drasar *et al.*, 1966; Larsen *et al.*, 2000). Bile acids are also the natural ligands for the farnesoid-X nuclear receptor. Therefore, bile acids may be important regulators of gene expression in the liver and intestines (Torchia *et al.*, 2001).

The hydrolysis of conjugated bile salts is a common microbe-assisted biotransformation. As *Bifidobacteria* are one of the main bacterial groups in the gut, they play an important role in the transformation of bile salts in the intestine (Grill *et al.*, 1995). Members of the genus *Bifidobacterium* have the ability to deconjugate bile salts; six different salts are deconjugated by all strains of the organism. Bile salt hydrolase (BSH; cholylglycine hydrolase; EC 3.5.1.24) catalyses the hydrolysis of glycine- and taurine-conjugated bile salts into the corresponding amino-acid residues and the deconjugated bile salts. On the basis of sequence similarity with BSH enzymes of other bacteria, as well as with penicillin V acylase (PVA) from *Bacillus sphaericus*, the crystal structure of which has been elucidated (Suresh *et al.*, 1999), BSH from *B. longum* can be classified as belonging to the N-terminal nucleophile hydrolase family (Brannigan *et al.*, 1995), with Cys as the N-terminal catalytic residue. The majority of the amino acids identified in the catalytic centre and oxyanion hole of PVA are conserved in the sequence of BSH (Tanaka *et al.*, 2000). It will be interesting to study the mechanism of action of BSH and its specificity in comparison with PVA *via* structural analysis. With this in mind, we have crystallized BSH from *B. longum* and carried out preliminary crystallographic analysis.

2. Materials and methods

2.1. Construction of the overexpression plasmid pET26/bsh

The gene encoding *B. longum* BSH was amplified from the plasmid pBH1351 (Tanaka *et al.*, 2000) by PCR using the oligonucleotide primers 5′-GGAGTC**ATTAAT**GTGCACTG-GTGTCCGTTTC-3′ and 5′-GGAA**GAATTC**ATCGGGCGACGCTGATGAG-3′, which incorporate the restriction endonuclease sites *Ase*I and *Eco*RI (highlighted in bold), respectively. Digested PCR product was cloned into the T7 promotor-based pET26b(+) expression vector (Novagen) digested at *Nde*I and *Eco*RI restriction sites and transformed into the *Escherichia coli* BL21(DE3) host strain for overexpression. The sequence of the coding region was confirmed by DNA sequencing.

2.2. Overexpression and purification

20 ml of stationary phase (BL21/pET26/bsh) culture was inoculated into 2 l of Luria–Bertani medium containing 35 μg ml^{-1} kanamycin. The culture was grown at 310 K to an

OD_{600} value of 0.6, followed by induction with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation after 5 h and the cell pellet was resuspended in 50 m*M* potassium phosphate buffer pH 6.5 containing 10 m*M* DTT prior to storage at 203 K.

The cell suspension was thawed and lysed by sonication. Cell debris was removed by centrifugation at 18 000*g* for 20 min at 277 K. To remove residual DNA, 1% streptomycin sulfate was added and the suspension was stirred for 20 min prior to centrifugation for 10 min at 18 000*g* and 277 K. The cell extract was subjected to ammonium sulfate (AS) protein precipitation at 80% saturation, resuspended and dialysed against potassium phosphate buffer pH 6.5. The dialysate was treated with 24% AS and applied to a 200 ml octyl-Sepharose column (Sigma) equilibrated with the same concentration of AS in potassium phosphate buffer pH 6.5 and eluted with a linear gradient of AS. Fractions were analysed by SDS–PAGE and those containing BSH were pooled and dialysed against Tris–HCl buffer pH 8.0 and applied to a MonoQ column (Amersham Pharmacia Biotech) equilibrated with the same buffer and eluted with a 0–1 *M* NaCl linear gradient. The enzyme fractions were pooled and concentrated in a Centriprep concentrator (Amicon). The concentrated preparation was buffer-exchanged into 10 m*M* potassium phosphate buffer pH 6.5 containing 10 m*M* DTT using PD-10 desalting columns (Amersham Pharmacia Biotech) and finally concentrated to 16 mg ml^{-1} protein before storage at 203 K. Protein concentration was determined in accordance with the method of Lowry *et al.* (1951), with BSA as a standard.

2.3. Dynamic light scattering

Dynamic light-scattering experiments were performed on a DynaPro instrument (Protein Solutions). Measurements were made at 291 K using the purified protein at 0.1–0.5 mg ml^{-1} in buffer solution containing 10 m*M* potassium phosphate pH 6.5 and 10 m*M* DTT.

2.4. Crystallization

The initial screening of crystallization conditions was carried out by the sparse-matrix method using Crystal Screen kits (Hampton Research, USA) and Clear Strategy Screens I and II. All crystallization screens were performed at 296 K employing the hanging-drop vapour-diffusion method in 24-well Linbro plates (ICN Biomedicals). Crystallization conditions were refined by variation of precipitant [15–35%(*w*/*v*) PEG 400; 30% AS saturation and above], pH (5.0–8.5), protein concentration (10–30 mg ml^{-1}), additives (ethylene glycol, glycerol, methanol, ethanol, acetone, β-octyl glucoside, β-ME, dioxane, sucrose and maltose) and temperature (277, 293 and 303 K). In each trial, a hanging drop of 1 µl protein solution mixed with 1 µl reservoir solution was equilibrated against a reservoir of 500 µl precipitant solution.

2.5. Data collection

Preliminary diffraction data were collected in-house using an R-AXIS IV^{++} image-plate detector mounted on a Rigaku RU-2000 rotating-anode X-ray generator running at 50 kV and 100 mA producing Cu $K\alpha$ (λ = 1.5418 Å) radiation. The beam was focused using Osmic confocal mirrors. Crystals were immersed in a cryoprotectant solution consisting of mother liquor supplemented with 30% glycerol. The crystals were mounted in a loop and flash-cooled in a stream of nitrogen gas cooled to 100 K. All intensity data were indexed, integrated and scaled using the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The exposure time was 8 min per frame, the crystal-to-detector distance was 200 mm and the oscillation range was 0.5°.

3. Results and discussion

The preparation of recombinant protein has provided a reliable supply of pure protein for crystallization. A final yield of 7.5 mg of pure protein per litre of cell culture was obtained. The purity of the protein was confirmed using SDS–PAGE, native PAGE and dynamic light-scattering analysis, the latter indicating the presence of tetramers in solution. The SDS–PAGE (12%) analysis using medium-molecular-weight markers (Sigma) gave a single band at 36.5 kDa (Fig. 1), whereas the molecular weight estimated using gel chromatography with standard proteins (aprotinin, carbonic anhydrase, BSA and alcohol dehydrogenase; Sigma) was 146 kDa, confirming the homotetrameric form of BSH in solution. The specific activity of the purified enzyme used for the crystallization was 30 IU mg^{-1} using glycocholic acid (Sigma) as substrate at pH 6.0 and 315 K.

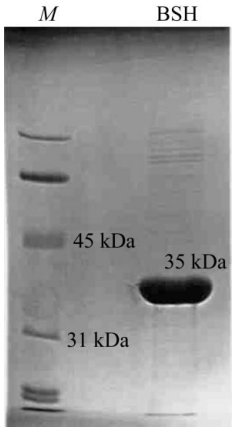
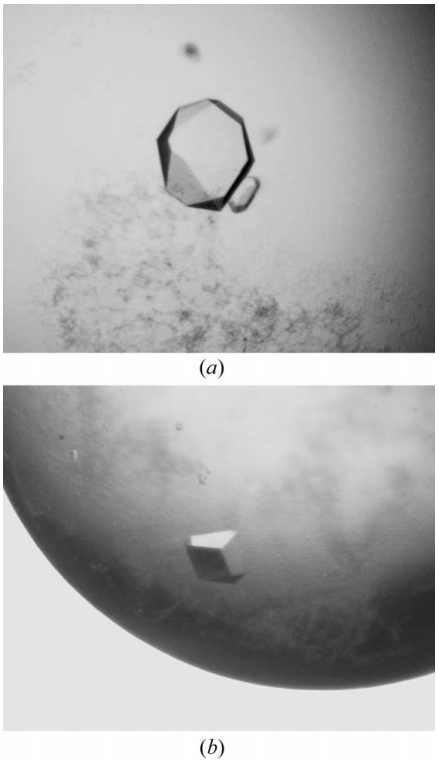


Figure 1
SDS–PAGE analysis of purified BSH. Lane *M* contains standard medium-range molecular-weight markers (Sigma). BSH indicates the lane containing recombinant bile salt hydrolase.

The sparse-matrix screens (Crystal Screens I and II from Hampton Research and Clear Strategy Screens I and II) showed that the protein crystallized with different morphologies under a variety of conditions. Initially, most of these crystals were not suitable for X-ray measurements owing to their small size and growth defects. The best crystals were obtained (i) with 30%(*w*/*v*) PEG 400, 100 m*M* HEPES pH 7.2 and 0.2 *M* $MgCl_2$ (hexagonal form only) and (ii) with ammonium sulfate (30% saturation), 0.5 *M* sodium formate, 0.2 *M* sucrose and 0.04%(*w*/*v*) β-octylglucoside at pH 6.7 (both hexagonal and trigonal forms). Crystals



(*a*)

(*b*)

Figure 2
(*a*) Hexagonal crystal form of BSH from *B. longum* grown from conditions using PEG 400 as the precipitant. (*b*) Trigonal crystal form of BSH from *B. longum* grown from conditions using ammonium sulfate as the precipitant.

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

	Hexagonal	Trigonal
Detector and wavelength (Å)	R-AXIS IV^{++}, 1.5418	R-AXIS IV^{++}, 1.5418
Space group	*P*622	*P*321
Unit-cell parameters		
$a = b$ (Å)	124.86	125.24
c (Å)	219.03	117.03
Resolution range (Å)	20–2.50 (2.54–2.50)	20–2.50 (2.55–2.50)
Total observations	473577	154427
Unique reflections	35692	37170
Average $I/\sigma(I)$	21.9 (5.5)	18.8 (3.6)
$R_{\rm merge}$† (%)	9.0 (48.0)	6.0 (39.0)
Completeness (%)	99.7 (100)	99.5 (100)

† $R_{\rm merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

appeared after 4 d from each of the conditions using 15 mg ml^{-1} protein solution in 10 m*M* sodium phosphate buffer pH 6.5 containing 10 m*M* DTT. The crystallization conditions were optimized for non-ionic detergent concentration and incubation temperature to improve the quality of crystals. The crystals grew to approximate dimensions of 0.4 × 0.2 × 0.2 mm in both forms (Fig. 2).

The best quality crystals, grown at 303 K, diffracted X-rays to 2.5 Å spacing and were found to belong to space groups *P*622, with unit-cell parameters $a = b = 87.66$, $c = 160.28$ Å, and *P*321, with unit-cell parameters $a = b = 125.24$, $c = 117.02$ Å. Detailed data-collection statistics are given in Table 1. The values of the Matthews coefficient $V_{\rm M}$ (Matthews, 1968), determined assuming dimers in the asymmetric unit in each case, were 3.5 and 3.8 Å^3 Da^{-1}, respectively, for the hexagonal and trigonal forms. The corresponding values for solvent content were 65.1 and 67.5%, respectively. The possibility of the presence of a tetramer in the asymmetric unit, although close to the extreme limit ($V_{\rm M}$ for the hexagonal and trigonal forms being 1.7 and 1.8 Å^3 Da^{-1} with solvent contents of 27 and 32%, respectively), cannot be ruled out. Despite sequence homology (30% identity) and shared tetrameric quaternary structure with PVA (Suresh *et al.*, 1999), preliminary attempts at molecular replacement have not been successful. Attempts to obtain crystals with heavy-atom derivatives for multiple isomorphous replacement methods are under way.

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