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Crystallization and preliminary X-ray analysis of native and recombinant human bile-salt dependent lipase: strategies for improvement of diffraction quality

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Human bile-salt dependent lipase (BSDL), secreted into both the digestive tract and human milk, is integral to the effective absorption of dietary lipids. In attempts to obtain crystals suitable for high-resolution X-ray crystallographic studies, various forms of the enzyme have been crystallized, including native and desialidated human milk BSDL and both intact recombinant BSDL and a truncated form lacking the heavily glycosylated C-terminal repeat region. Trigonal crystals of native BSDL, with unit-cell parameters $a = b = 90.0$, $c = 156.1$ Å, were obtained using 15–20%(*w*/*v*) PEG 8000 as precipitant. These crystals diffract to 3.5 Å along the unique axis, but to only 5–7 Å in orthogonal directions. Crystals of recombinant truncated BSDL grown from 15–20%(*w*/*v*) PEG 6000 are orthorhombic, space group $P2_12_12_1$, with unit-cell parameters $a = 59.2$, $b = 90.0$, $c = 107.7$ Å, and diffract to 2.6 Å resolution. These are suitable for structural analysis by X-ray crystallography.

1. Introduction

Bile-salt dependent lipase (BSDL) is a lipolytic enzyme that has attracted interest because of several unique structural and functional properties (Wang & Hartsuck, 1993). Chief amongst these is its activation mechanism, which depends on the binding of primary bile salts, and its functional role in the uptake of dietary cholesterol. The latter activity has given it the alternative name of cholesterol esterase.

In humans, BSDL is secreted by the pancreas into the digestive tract and by the mammary gland into human milk. In the newborn infant, it survives passage through the stomach and is then activated by bile salts in the intestine, where it plays an important role in lipid digestion (Atkinson *et al.*, 1981; Wang *et al.*, 1989). BSDL has a broad substrate specificity, being able to hydrolyse both long-chain and short-chain fatty-acid esters of glycerol, as well as long-chain phospholipids and esters of cholesterol and the fat-soluble vitamins A, D and E. Whereas many of these activities overlap with those of other lipases, hydrolysis of cholesteryl and vitamin esters appears to be an activity that is unique to BSDL (Rudd & Brockman, 1984).

Human BSDL comprises 722 amino-acid residues and is divided into an N-terminal catalytic domain and a C-terminal domain of unknown function. The structure of the homologous bovine enzyme (Wang *et al.*, 1997; Chen *et al.*, 1998) confirmed that the N-terminal domain has an α/β hydrolase fold (Ollis *et al.*, 1992; Schrag & Cygler, 1997) and belongs to what has been termed the 'large esterase family' (Cygler *et al.*, 1993; Heikinheimo *et al.*, 1999). Sequence data, coupled with site-directed mutagenesis experiments, had previously identified a classic Ser-His-Asp catalytic triad comprising Ser194, His435 and Asp320 (Wang & Hartsuck, 1993). Compared with other lipases, however, BSDL is characterized by a unique activation mechanism which requires the binding of bile salts. It has been proposed that bile salts interact with two sites on the protein (Bläckberg & Hernell, 1993). One site is specific for primary bile salts (those with a 7α hydroxyl group) and is associated with enzyme activation, whereas the second is less specific, able to bind both primary and secondary bile salts and is a prerequisite for binding of the enzyme to aggregated lipid surfaces.

The C-terminal domain of human BSDL comprises a series of 16 almost identical 11-residue repeats with the consensus sequence GAPPVPPTGDS. However, the C-terminal domain tandem repeats differ both in composition and number between species and are even missing altogether in the salmon enzyme. In humans, the number of repeats is normally about 16, but this number can vary between individuals (Strömqvist *et al.*, 1997). Both human milk and pancreatic BSDL are heavily glycosylated and most of the carbohydrate is associated with the C-terminal region, which has many potential O-glycosylation sites. This region is reminiscent of the repetitive domains found in mucous glycoproteins.

Table 1
Crystallization conditions for BSDL variants.

Protein	Protein solution	Well solution	Crystals
Native full-length BSDL	10–30 mg ml^{-1} in water	0.4 *M* MOPS/NH_4OH pH 6.8, 15–20%(*w*/*v*) PEG 8000	Bipyramidal, up to 0.8 mm
Desialidated native BSDL	8 mg ml^{-1} in water	0.2 *M* bis–tris propane/HCl pH 6.7, 14% PEG 6000	Very thin small rods
Recombinant BSDL	12–15 mg ml^{-1} in 20 m*M* MOPS/NH_4OH pH 7.0	0.2 *M* HEPES/KOH pH 7.3, 16%(*w*/*v*) PEG-MME 5000	Thin needles or plates, 0.15 × <0.02 × <0.02 mm; reproduced by seeding
Truncated recombinant BSDL	0.5–0.8 mg ml^{-1} in 20 m*M* MOPS/NH_4OH pH 3.0, 0.1 *M* NaCl, 0.8 m*M* CHAPS	0.2 *M* PIPES/KOH pH 6.7, 15–25% PEG 6000, 2% glycerol	Rod shaped, ~0.2 × 0.06 × 0.02 mm; macroseeding needed

The biological significance of the C-terminal domain is not clear. Expression of recombinant BSDL variants which lack this domain has shown that it is not essential for catalytic activity or bile-salt activation (Hansson *et al.*, 1993; Loomes, 1995). It could, however, help to protect the enzyme from proteolysis during passage through the gut, since the truncated version lacking the C-terminal tail is more susceptible to proteolytic attack (Loomes, 1995). It may also have a role in maintaining the solubility of the enzyme or in intermolecular association.

We undertook crystallographic studies on human BSDL in order to understand its mechanism of activation, its mode of association with the lipid phase and its bile-salt-, cholesterol- and vitamin-binding properties at a molecular level. Recently determined three-dimensional structures for bovine BSDL (Wang *et al.*, 1997; Chen *et al.*, 1998) now also provide an opportunity for comparisons that will further address the complexities of this enzyme. Here, we describe a series of approaches aimed at obtaining crystals of human BSDL that are of sufficient quality for three-dimensional structure analysis by X-ray crystallography.

2. Materials and methods

2.1. Protein preparation

Native BSDL was purified from human milk as previously described (Bläckberg & Hernell, 1981) and was used unchanged in crystallization trials. For removal of the terminal sialic acid groups, this protein (25 mg ml^{-1} in 100 m*M* potassium acetate pH 5.5, 10 m*M* Na_2EDTA) was incubated with sialidase (*Clostridium perfringens*, Boehringer Mannheim) at a level of 0.04 mg sialidase per milligram of BSDL with an incubation time of 24 h. The progress of desialidation was monitored using precast isoelectric focusing gels (Pharmacia). Prior to incubation, the protein did not focus as a single band, indicating charge heterogeneity; after incubation, it focused as a discrete band at an isoelectric point of 3.4 and was then further purified by gel filtration using a Superdex 75 column (Pharmacia).

Both the full-length and truncated recombinant human BSDL proteins were expressed in baby hamster kidney (BHK) cells as previously described (Loomes, 1995). Yields of purified protein were approximately 10 mg and 1 mg per litre of culture medium, respectively, for the two proteins. For the truncated molecule, the C-terminus (Phe518) corresponded to a site identified by proteolysis experiments on the full-length molecule. This lies close to the transition to the tandem-repeat region identifiable from the sequence. The purification protocol for this truncated molecule was modified from that published to give improved yields. Briefly, the centrifuged cell-culture medium was loaded onto a heparin-Sepharose column (Hep-Pac cartridge, Pharmacia) and the protein was eluted with an NaCl gradient in 10 m*M* sodium phosphate pH 7.6. In some preparations, fractions containing the truncated recombinant BSDL were passed through concanavalin A Sepharose (Pharmacia) to remove any N-glycosylated protein. Following this step, CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 0.8 m*M* final concentration} was added to prevent absorption losses during concentration. The protein was further purified using gel filtration (Superose-12; Pharmacia) with 20 m*M* MOPS/NH_4OH pH 7.0, 0.1 *M* NaCl, 0.8 m*M* CHAPS. Despite its low concentration (~1 mg ml^{-1}), the protein solution was close to saturation and was used at this concentration in crystallization trials.

2.2. Crystallization

In all cases, a systematic search for initial crystallization conditions used screens based on orthogonal arrays (Kingston *et al.*, 1994). Crystallization trials used the hanging-drop method, with 1 or 2 µl protein drops mixed with equal volumes of well solution. The best results were obtained at room temperature. The initial crystallization conditions were refined with fine variations of pH, ionic strength and precipitant concentration.

3. Results and discussion

The search for conditions that would support the growth of well ordered crystals of human BSDL suitable for X-ray structural analysis has involved the use of both native and recombinant proteins and modified versions of each. Different problems were encountered for each protein. In the case of native BSDL, the difficulties were probably a consequence of the heavily glycosylated C-terminal repeat region. The glycan chains are a source of chemical and structural heterogeneity, and the amino-acid sequence of the C-terminal domain suggests that it may not have a unique defined structure. For the truncated recombinant form (see below), the problems arose principally from its low solubility.

The native enzyme isolated from human milk (Bläckberg & Hernell, 1981) was crystallized using the conditions given in Table 1. Large bipyramidal crystals (Fig. 1) up to 0.8 mm in length were obtained. The crystals were trigonal, space-group $P3_121$ or $P3_221$,

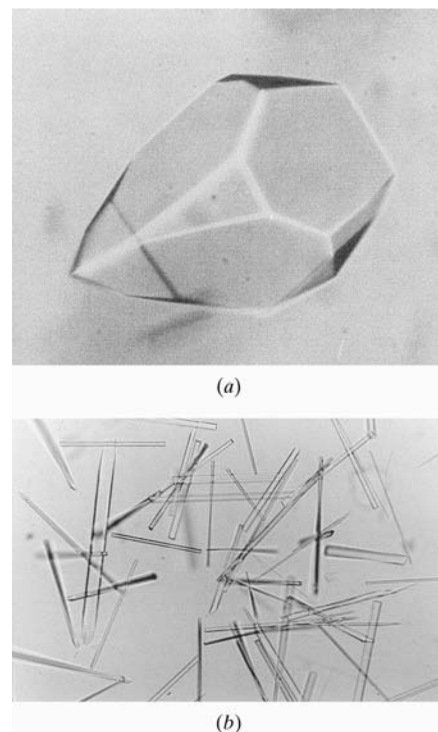

Figure 1
(*a*) Trigonal crystal of native full-length human BSDL and (*b*) orthorhombic crystals of truncated recombinant human BSDL.

with unit-cell parameters $a = b = 90.0$, $c = 156.1$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Diffraction was highly anisotropic, however, reaching ~3.5 Å resolution along c^*, but only ~5–7 Å resolution in orthogonal directions. Consequently these crystals, which have a similar cell to that described by Wang *et al.* (1997) for bovine BSDL, were not suitable for high-resolution X-ray analysis. To reduce heterogeneity, the terminal sialic acid residues of the glycan chains were removed with neuraminidase. This desialidated protein was indeed electrophoretically purer and gave crystals of a different morphology (thin small rods); see Table 1. The crystals have not yet been characterized by X-ray diffraction, however, owing to their small size.

The recombinant BSDL used in our crystallization trials was produced by expression in BHK cells (Loomes, 1995). The use of a mammalian cell-culture system produced a protein that was glycosylated but with non-native glycan chains, since glycosylation is cell-line specific. This full-length glycosylated recombinant BSDL gave a new crystal form superior to the native crystals. The diffraction data indicated a primitive monoclinic lattice with approximate unit-cell parameters $a = 90.6$, $b = 146.2$, $c = 115.7$ Å, $\beta = 102.9°$, space group $P2$ or $P2_1$. Diffraction extended isotropically to ~3.5 Å resolution.

The best crystals were eventually obtained using a truncated form of the enzyme comprising residues 1–518. The construct used for expression of the truncated form was designed on the basis of a cleavage site identified by limited proteolysis (Loomes, 1995). This truncated form lacked the glycosylated C-terminal tail and was expected to give a more compact and homogenous molecule. Difficulties were experienced because of its low solubility; for most preparations, concentrations in excess of 1.0 mg ml^{-1} could not be achieved without irreversible precipitation. Even at this low concentration, however, well formed rod-like crystals were obtained (Fig. 1). Although extremely small, they could be increased to a useable size (~0.2 × 0.05 × 0.02 mm) by several rounds of macroseeding.

The crystals of truncated human BSDL are orthorhombic, $a = 59.3$, $b = 90.0$, $c = 107.7$ Å, space group $P2_12_12_1$, with a solvent content of 49% and one molecule in the asymmetric unit. Native data were collected with an R-AXIS IIC image-plate detector on a Rigaku RU-200 rotating-anode generator using a single crystal flash-frozen at 113 K. For such thin crystals, a fine collimator (0.1 mm diameter) was found to be important for maximizing the signal-to-background ratio. The resulting data set was 89% complete to 2.6 Å resolution (66% complete in the outer shell, 2.7–2.6 Å), with a redundancy of 3.1 (outer shell, 2.5) and mean $I/\sigma(I)$ of 6.3 (outer shell, 2.0). The data set comprised 15 650 unique reflections with a merging R factor of 10.4% (outer shell, 34.9%); structure determination is in progress. These results illustrate the utility of chemical modification as an aid to crystallization, either enzymatically (desialidation or deglycosylation) or by protein engineering.

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