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Heterogeneous nucleation helps the search for initial crystallization conditions of γ -glutamyl transpeptidase from *Bacillus licheniformis*

Here, the crystallization and preliminary X-ray diffraction studies of *Bacillus licheniformis* γ -glutamyl transpeptidase (BIGT) are reported. The serendipitous finding of heterogeneous nucleants in the initial experiments provided the first crystallization conditions for the protein. Crystals were grown by hanging-drop vapour diffusion using a precipitant solution consisting of 20%(w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M Tris-HCl pH 8.2. The protein crystallized in the orthorhombic space group $P2_12_12_1$, with one heterodimer per asymmetric unit and unit-cell parameters $a = 60.90$, $b = 61.97$, $c = 148.24$ Å. The BIGT crystals diffracted to 2.95 Å resolution.

1. Introduction

γ -Glutamyl transpeptidases (γ -GTs) are enzymes that catalyse the transfer of the γ -glutamyl moiety of γ -glutamyl-derived peptides, such as glutathione (γ -Glu-Cys-Gly), to water, amino acids or dipeptides (Tate & Meister, 1981; Castellano & Merlino, 2012).

These proteins have attracted much interest because of their important roles in degrading glutathione, the most abundant antioxidant in the cell, and have various applications in the biomedical and biotechnological fields (Suzuki *et al.*, 2007; Castellano & Merlino, 2012).

γ -GTs belong to the N-terminal nucleophilic (Ntn) hydrolase superfamily. The members of this superfamily are encoded by a single gene and are translated as a unique polypeptide chain, which then undergoes autoproteolytic cleavage into a heavy and a light chain, which are usually called the large and the small subunit, respectively. Ntn hydrolases share common structural features: they have a conserved active-site architecture, tertiary and quaternary fold, although their pairwise sequence identities can be as low as 15%. The predominant elements of secondary structure in Ntn hydrolases include a long four-layer $\alpha\beta\beta\alpha$ structure, with two antiparallel β -sheets packed against each other and sandwiched between α -helical layers (Oinonen & Rouvinen, 2000). In the central β -sheet sandwich, the strands are arranged in an antiparallel fashion: one of the two central sheets is essentially flat, whereas the other one may be twisted at the end. Both the large and the small subunits contribute strands to the nearly flat β -sheets.

To date, γ -GTs from different sources have been studied (Ogawa *et al.*, 1991; Okada *et al.*, 2007; Lin *et al.*, 2006; Boanca *et al.*, 2007; Castellano *et al.*, 2010, 2011; Murty *et al.*, 2012; Castellano & Merlino, 2012; Rajput *et al.*, 2013), but only five members of the γ -GT family have been structurally characterized (Castellano & Merlino, 2012). These crystal structures include those of *Escherichia coli* γ -GT (EcGT; PDB entry 2dbu; Okada *et al.*, 2006), *Helicobacter pylori* γ -GT (HpGT; PDB entry 2nqo; Boanca *et al.*, 2007), *Bacillus subtilis* γ -GT (BsGT; PDB entry 2v36; B. Sharath, A. A. Prabhune, C. G. Suresh, A. J. Wilkinson & J. A. Brannigan, unpublished work), *B. halodurans* γ -GT (BhGT; PDB entry 2nlz; New York SGX Research Center for Structural Genomics, unpublished work) and *Thermoplasma acidophilum* γ -GT (TaGT; PDB entry 2i3o; New York SGX Research Center for Structural Genomics, unpublished work).

Bacillus licheniformis was selected as a nonpathogenic host strain for the isolation of a γ -GT as this bacterium has been extensively used

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for large-scale production of biotechnologically relevant enzymes such as subtilisin (Jacobs *et al.*, 1985), amylase (Morgan & Priest, 1981), the antibiotic bacitracin (Haddar *et al.*, 2007) and β -mannanase (Veith *et al.*, 2004).

The putative *B. licheniformis* γ -GT gene can be translated into a 61.3 kDa precursor consisting of a signal peptide of 25 residues, a large subunit of 374 residues and a small subunit of 187 residues (Lin *et al.*, 2006; Yang *et al.*, 2011). Also, the deduced amino-acid sequence (Swiss-Prot Q65KZ6) of BIGT shares 27–76% identity with those of BsGT (Swiss-Prot P54422), HpGT (Swiss-Prot O25743), EcGT (Swiss-Prot P18956), BhGT (Swiss-Prot Q9KEI5) and TaGT (Swiss-Prot Q9HJH4). Purified recombinant BIGT has been well characterized (Lin *et al.*, 2006). It is a heterodimer that efficiently cleaves L- γ -glutamyl-p-nitroanilide (L- γ -Glu-p-NA) with a k_{cat} of 105 s⁻¹ and a K_m of 21 μ M. BIGT is a moderately thermostable enzyme and is among the most stable characterized γ -GTs (Pica *et al.*, 2012, 2013), showing 80% of its activity at 328 K and a denaturation temperature of 333–338 K (Chang *et al.*, 2010; Yang *et al.*, 2011). Deletion analyses of the recombinant enzyme have demonstrated that both the N-terminal and the C-terminal sequences are crucial for the expression of its active form in host cells and for its thermal stability (Lin *et al.*, 2008; Chang *et al.*, 2010).

In the present study, crystallization and preliminary X-ray diffraction studies of BIGT are reported. The determination of the three-dimensional structure of the protein will help the understanding of the structure–function relationship in this enzyme and its application in biotechnological processes.

(a)

(b)

Figure 1

(a) Image of the first crystals of BIGT grown by vapour diffusion close to a contaminating fibre. (b) The best crystals of BIGT grew using 20% (w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate in 0.1 M Tris-HCl buffer pH 8.2.

Table 1

Data-collection statistics.

Space group	$P2_12_12_1$
Wavelength (Å)	1.54
Unit-cell parameters (Å)	$a = 60.90, b = 61.97, c = 148.24$
Resolution	50.0–2.95 (3.00–2.95)
No. of observed reflections	86331
No. of unique reflections	11668
Completeness (%)	98.8 (99.2)
Mosaicity	1.4
Multiplicity	7.4 (5.8)
$R_{\text{merge}}^{\dagger}$ (%)	12.8 (47.6)
$\langle I/\sigma(I) \rangle$	10.0 (5.1)
V_M (Å ³ Da ⁻¹)	2.22
No. of subunits per asymmetric unit	One heterodimer
Solvent content (%)	44.56

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

2. Materials and methods

2.1. Expression and purification of recombinant *B. licheniformis* γ -GT

BIGT was overexpressed in recombinant *E. coli* M15 cells and purified as described previously (Lin *et al.*, 2008). BIGT purity was verified by 10% SDS-PAGE; pooled fractions were dialysed against 50 mM Tris-HCl buffer pH 8.0 to remove imidazole and salt. The protein was lyophilized and the lyophilized protein was dissolved in 10 mM Tris-HCl buffer pH 7.4 and then concentrated to 40 mg ml⁻¹. Crystallization experiments were performed by diluting this stock solution.

2.2. Crystallization

Preliminary crystallization trials were performed at 293 K by sitting-drop vapour-diffusion methods. These experiments were carried out using a crystallization workstation (Hamilton Robotics) at the Istituto di Biostrutture e Bioimmagini. For each crystallization drop, 0.2 μ l enzyme solution (20 mg ml⁻¹ in 5 mM Tris-HCl buffer pH 7.4) was added to 0.2 μ l of each precipitant from the Crystal Screen, Crystal Screen 2 and Index kits (Hampton Research, Aliso Viejo, California, USA) and then incubated at 300 K. Crystalline precipitates were obtained in some conditions from the Index kit, namely conditions 32, 45, 85, which were used in further investigation of the crystallization conditions using the hanging-drop vapour-diffusion method in 24-well Linbro plates. Optimization of the crystallization conditions was performed by fine-tuning the protein and precipitant concentrations using drops consisting of 1 μ l protein solution and 1 μ l precipitant solution (or of 0.5 μ l protein solution and 0.5 μ l precipitant solution) equilibrated over 0.5, 0.6 or 1 ml of the respective precipitant.

The optimal crystals appeared in drops consisting of 20% (w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M Tris-HCl buffer pH 8.2 using a protein concentration of 8–10 mg ml⁻¹.

2.3. Data collection and processing

For data collection, crystals were flash-cooled in liquid nitrogen using a cryoprotectant solution [20% (v/v) glycerol, 20% (w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate in 50 mM Tris-HCl buffer pH 8.2], picked up in a nylon loop and quickly vitrified in a stream of nitrogen gas at 100 K. Diffraction images were collected using a Rigaku MicroMax-007 HF generator producing Cu K α radiation (45 kV and 60 mA) and equipped with a Saturn 944 CCD detector. The crystal-to-detector distance was set to 70 mm, with all frames collected at 100 K. Diffraction data were recorded over a 350°

Figure 2

Diffraction pattern of a *BIGT* crystal. Diffraction spots are detectable up to 2.95 Å resolution.

rotation of the crystal with a width of 0.5° per image. The data were processed using *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

BIGT with an N-terminally attached His₆ tag was expressed in *E. coli* as described previously (Lin *et al.*, 2006, 2008). The recombinant enzyme contains an exogenous sequence of MRGSHHHHHHGS instead of the 28 N-terminal residues of the precursor protein (Swiss-Prot Q65KZ6). Purification of the recombinant protein by Ni-NTA agarose affinity chromatography yielded 26.4 mg of highly purified protein per litre of bacterial culture. The expressed protein exhibited hydrolase and transpeptidase activity, as reported previously (Lin *et al.*, 2006). The first crystallization screening using commercially available solutions revealed initial conditions that allow the formation of crystalline precipitates. The first ordered microcrystals of *BIGT* originated from heterogeneous nucleation on a contaminating fibre using the hanging-drop vapour-diffusion method and appeared in 25% (w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate in 0.1 M Tris-HCl buffer pH 8.5 within 1 week of incubation (Fig. 1*a*). Fibres have already been shown to be effective in inducing crystallization of other proteins, some of which are notoriously difficult to crystallize (D'Arcy *et al.*, 2003; Georgieva *et al.*, 2007; Thakur *et al.*, 2007).

Fine-tuning of crystallization conditions was performed by varying the pH (and also using bis-tris and HEPES buffers) and the PEG concentration (and also varying the PEG molecular weight and using MPEGs) using hanging-drop vapour-diffusion methods. The best crystals, which were suitable for X-ray diffraction studies, appeared in drops consisting of 20% (w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate in 0.1 M Tris-HCl buffer pH 8.2 (Fig. 1*b*). Diffraction data (Fig. 2) were collected from these crystals. All data sets were indexed and processed with the *HKL-2000* suite of programs (Otwinowski & Minor, 1997). Statistics of data processing are reported in Table 1. The crystals belonged to space group *P*2₁2₁ and diffracted to 2.95 Å resolution. The calculated Matthews coefficient

(V_M) of 2.22 Å³ Da⁻¹ suggests that there is one heterodimer in the asymmetric unit, with a solvent content of 44.6% (Matthews, 1968).

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References

- Boanca, G., Sand, A., Okada, T., Suzuki, H., Kumagai, H., Fukuyama, K. & Barycki, J. J. (2007). *J. Biol. Chem.* **282**, 534–541.
- Castellano, I., Di Salle, A., Merlino, A., Rossi, M. & La Cara, F. (2011). *Extremophiles*, **15**, 259–270.
- Castellano, I. & Merlino, A. (2012). *Cell. Mol. Life Sci.* **69**, 3381–3394.
- Castellano, I., Merlino, A., Rossi, M. & La Cara, F. (2010). *Biochimie*, **92**, 464–474.
- Chang, H.-P., Liang, W.-C., Lyu, R.-C., Chi, M.-C., Wang, T.-F., Su, K.-L., Hung, H.-C. & Lin, L.-L. (2010). *Biochemistry*, **75**, 919–929.
- D'Arcy, A., Mac Sweeney, A. & Haber, A. (2003). *Acta Cryst.* **D59**, 1343–1346.
- Georgieva, D. G., Kuil, M. E., Oosterkamp, T. H., Zandbergen, H. W. & Abrahams, J. P. (2007). *Acta Cryst.* **D63**, 564–570.
- Haddar, H. O., Aziz, G. M. & Al-Gelawi, M. H. (2007). *Pak. J. Biol. Sci.* **10**, 972–976.
- Jacobs, M., Eliasson, M., Uhlén, M. & Flock, J. I. (1985). *Nucleic Acids Res.* **13**, 8913–8926.
- Lin, L.-L., Chou, P.-R., Hua, Y.-W. & Hsu, W.-H. (2006). *Appl. Microbiol. Biotechnol.* **73**, 103–112.
- Lin, L.-L., Yang, L.-Y., Hu, H.-Y. & Lo, H.-F. (2008). *Curr. Microbiol.* **57**, 603–608.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Morgan, F. J. & Priest, F. G. (1981). *J. Appl. Microbiol.* **50**, 107–114.
- Murty, N. A., Tiwary, E., Sharma, R., Nair, N. & Gupta, R. (2012). *Enzyme Microb. Technol.* **50**, 159–164.
- Ogawa, Y., Hosoyama, H., Hamano, M. & Motai, H. (1991). *Agric. Biol. Chem.* **55**, 2971–2977.
- Oinonen, C. & Rouvinen, J. (2000). *Protein Sci.* **9**, 2329–2337.
- Okada, T., Suzuki, H., Wada, K., Kumagai, H. & Fukuyama, K. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 6471–6476.
- Okada, T., Suzuki, H., Wada, K., Kumagai, H. & Fukuyama, K. (2007). *J. Biol. Chem.* **282**, 2433–2439.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Pica, A., Russo Krauss, I., Castellano, I., La Cara, F., Graziano, G., Sica, F. & Merlino, A. (2013). *Biochim. Biophys. Acta*, **1834**, 149–157.

- Pica, A., Russo Krauss, I., Castellano, I., Rossi, M., La Cara, F., Graziano, G., Sica, F. & Merlino, A. (2012). *Biochim. Biophys. Acta*, **1824**, 571–577.
- Rajput, R., Verma, V. V., Chaudhary, V. & Gupta, R. (2013). *Extremophiles*, **17**, 29–41.
- Suzuki, H., Yamada, C. & Kato, K. (2007). *Amino Acids*, **32**, 333–340.
- Tate, S. S. & Meister, A. (1981). *Mol. Cell. Biochem.* **39**, 357–368.
- Thakur, A. S., Robin, G., Guncar, G., Saunders, N. F., Newman, J., Martin, J. L. & Kobe, B. (2007). *PLoS One*, **2**, e1091.
- Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K. H., Ehrenreich, P., Bäumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A. & Gottschalk, G. (2004). *J. Mol. Microbiol. Biotechnol.* **7**, 204–211.
- Yang, J.-C., Liang, W.-C., Chen, Y.-Y., Chi, M.-C., Lo, H.-F., Chen, H.-L. & Lin, L.-L. (2011). *Int. J. Biol. Macromol.* **48**, 414–422.