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Crystallization and preliminary crystallographic studies of the recombinant dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114

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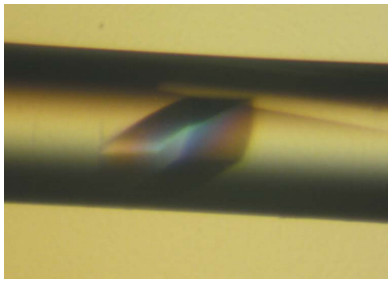


Dihydropyrimidinases are involved in the reductive pathway of pyrimidine degradation, catalysing the hydrolysis of 5,6-dihydrouracil and 5,6-dihydrothymine to the corresponding *N*-carbamoyl β-amino acids. This enzyme has often been referred to as hydantoinase owing to its industrial application in the production of optically pure amino acids starting from racemic mixtures of 5-monosubstituted hydantoins. Recombinant dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114 (SmelDhp) has been expressed, purified and crystallized. Crystallization was performed using the counter-diffusion method with capillaries of 0.3 mm inner diameter. Crystals of SmelDhp suitable for data collection and structure determination were grown in the presence of agarose at 0.1%(*w*/*v*) in order to ensure mass transport controlled by diffusion. X-ray data were collected to a resolution of 1.85 Å. The crystal belongs to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 124.89$, $b = 126.28$, $c = 196.10$ Å and two molecules in the asymmetric unit. A molecular-replacement solution has been determined and refinement is in progress.

1. Introduction

Dihydropyrimidinases (EC 3.5.2.2) are involved in the reductive pathway of pyrimidine degradation, catalysing the hydrolysis of 5,6-dihydrouracil and 5,6-dihydrothymine to the corresponding *N*-carbamoyl β-amino acids. They have long been known as hydantoinases owing to their application in the production of optically pure amino acids (Martinez-Rodriguez *et al.*, 2002; Wilms *et al.*, 2001) and several of these enzymes are known to have hydrolyzing activity towards six-membered and five-membered rings (Soong *et al.*, 1999; Moller *et al.*, 1988; Arcuri *et al.*, 2000; Clemente-Jimenez *et al.*, 2003). However, hydantoinases without dihydropyrimidinase activity have also been reported (Kim *et al.*, 2000; Runser & Meyer, 1993). Furthermore, Lohkamp and coworkers have recently described the dihydropyrimidinases from *Saccharomyces kluyveri* and *Dictyostelium discoideum* as being unable to hydrolyze hydantoin (Lohkamp *et al.*, 2006), thus supporting the proposal to use the name hydantoinase only for those enzymes hydrolyzing five-membered rings and the name dihydropyrimidine for those hydrolyzing both five- and six-membered rings (Syldatk *et al.*, 1999). Several hydantoinase/dihydropirimidinase structures have been reported from various sources (Abendroth *et al.*, 2002; Cheon *et al.*, 2002; Xu *et al.*, 2003), as well as a complex of a dihydropyrimidinase with a nonsubstituted dihydrouracil (Lohkamp *et al.*, 2006). However, the difference in substrate recognition has not been explained to date, nor has the structure determination been achieved of a complex with a substituted analogue of any substrate.

Dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114 (SmelDhp) has been shown to possess both hydantoinase and dihydropyrimidinase activities (work in preparation). In this paper, we describe the cloning, overexpression, purification, crystallization and preliminary crystallographic studies of this enzyme. Although initial crystallization conditions were determined using the vapour-diffusion technique, crystal quality and size were improved using the counter-diffusion technique with a three-layer configuration (Garcia-Ruiz, 2003). The crystal structure of this enzyme will provide an insight into

the substrate specificity of dihydropyrimidinase and hydantoinase enzymes and will help to establish the evolutionary relation between enzymes belonging to the cyclic amidohydrolase superfamily.

2. Materials and methods

2.1. Microbes and culture conditions

S. meliloti CECT 4114 was used as the donor of the dihydropyrimidinase gene (Smeldhp; the nucleotide sequence of the dihydropyrimidinase gene of *S. meliloti* CECT 4114 has been deposited in the GenBank database under accession No. DQ779921). It was grown at 303 K for 24 h on Luria–Bertani (LB) agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl pH 7.2, 1.5% agar). *Escherichia coli* BL21 was used to clone and express the dihydropyrimidinase gene.

2.2. Cloning and sequence analysis of Smeldhp

A single-colony isolate of *S. meliloti* CECT 4114 was chosen for DNA extraction using a slightly modified version of the method described by Van Eys *et al.* (1989). Using a sterile inoculating loop, the bacterial colony was transferred from the LB plate to 50 µl double-distilled water. The cells were lysed by boiling at 373 K for 10 min followed by immediate chilling on ice. After cooling for 5 min, cell debris was removed by centrifugation (Van Eys *et al.*, 1989). A sample of the supernatant containing genomic DNA (about 5 µl) was used to amplify the gene encoding the dihydropyrimidinase by PCR. The primers used were designed based on GenBank sequence accession No. NC003047 (Capela *et al.*, 2001; Galibert *et al.*, 2001). These were SmelDht5 (5′-AATCTAGAGTGACAGGAAAAACG-CCATGAGCACTGTCATCAAGGG-3′) and SmelDht3 (5′-AAA-AGCTTTTAATGATGATGATGATGATGGACGCCGCTTGCG-GGAATGCC-3′). The latter included a polyhistidine tag (His_6 tag) before the stop codon. The *Xba*I- and *Hin*dIII-digested fragment was purified from agarose gel using QIAquick (Qiagen) and ligated into pBluescript II SK(+) plasmid (pBSK, Stratagene Cloning Systems) cut with the same enzymes to create plasmid pSER38.

Once the fragment had been cloned, it was sequenced at least twice using standard T3 and T7 primers, using the dye dideoxy nucleotide-sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems). The sequence was aligned and compared with all of the amino-acid sequence databases available from the internet using *Basic Local Alignment Search Tool* (*BLAST*; Altschul *et al.*, 1990). *ClustalW* (Jeanmougin *et al.*, 1998) was used to compare the sequences used for the initial model and similarity percentages were calculated with *BLAST* and *SEAVIEW* (Galtier *et al.*, 1996).

2.3. Expression of Smeldhp

The BL21 strain containing pSER38 was grown in LB medium supplemented with 100 µg ml^{-1} ampicillin. A single colony was transferred into 10 ml LB medium with ampicillin at the above-mentioned concentration in a 100 ml flask. This culture was incubated overnight at 310 K with shaking. 500 ml of LB with the appropriate concentration of ampicillin in a 2 l flask was inoculated with 5 ml of the overnight culture. After 2 h of incubation at 310 K with vigorous shaking, the OD_{600} of the resulting culture was 0.3–0.5. For induction of expression of the Smeldhp gene, isopropyl β-thio-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 m*M* and the culture was continued at 310 K for a further 4 h. The cells were collected by centrifugation (Beckman JA2-21, 7000*g*, 277 K, 10 min) and stored at 253 K until use.

2.4. Purification of SmelDhp

BL21 pSER38 cells were resuspended in 50 ml wash buffer (300 m*M* NaCl, 0.02% NaN_3, 50 m*M* sodium phosphate pH 7). The cell walls were disrupted on ice by sonication using a UP 200 S Ultrasonic Processor (Dr Hielscher GmbH, Germany) for six periods of 30 s, with pulse mode 0.5 and sonic power 60%. The cell debris was centrifuged (Beckman JA2-21, 10 000*g*, 277 K, 20 min) and discarded. The supernatant was applied onto a column containing Talon metal-affinity resin (Clontech Laboratories Inc.) and washed three or four times with wash buffer. After washing, SmelDhp enzyme was eluted with elution buffer (100 m*M* NaCl, 0.02% NaN_3, 50 m*M* imidazole, 2 m*M* Tris pH 8). Protein purity was determined at different stages of the purification by SDS–PAGE (Fig. 1*a*). An additional gel-filtration chromatography step was carried out using a Superdex 200 gel-filtration column (Amersham Biosciences) to eliminate any DNA co-eluting with the protein (Fig. 1*b*). The purified enzyme was concentrated using an Amicon ultrafiltration system with Amicon YM-3 membranes and dialyzed against 20 m*M* Tris–HCl buffer pH 8.

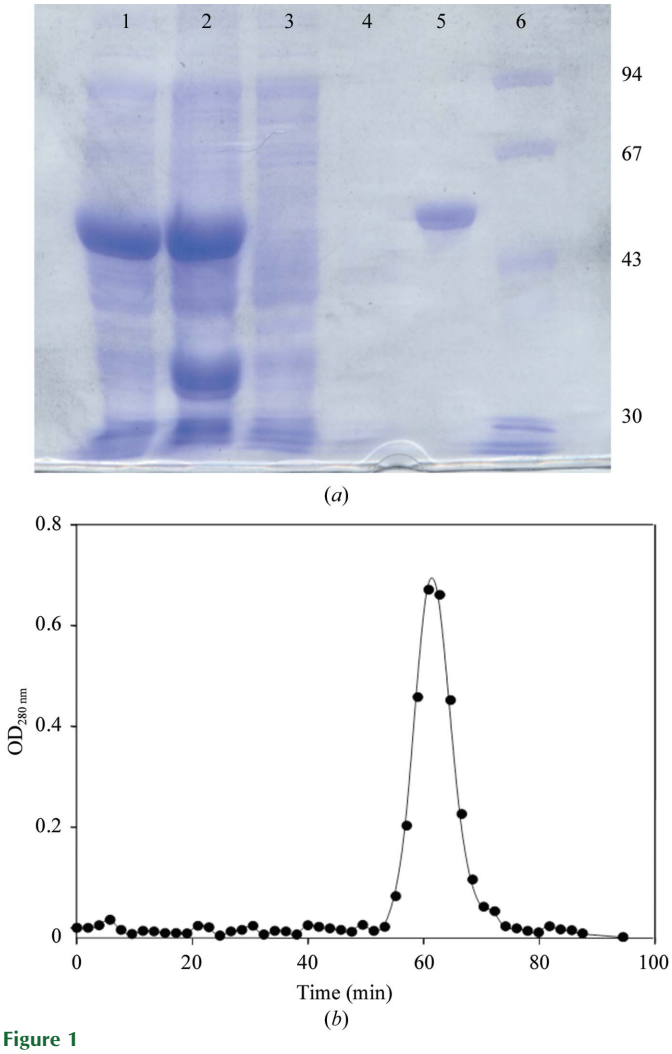


Figure 1
(*a*) SDS–PAGE analysis of each purification step of the dihydropyrimidinase from *S. meliloti* CECT4114. Lanes 1 and 2, supernatant and pellet of the resuspended crude extract after cell sonication; lane 3, eluate after adding the sonicated supernatant to the metal-affinity column; lane 4, flowthrough after washing the metal-affinity column with buffer; lane 5, purified SmelDhp (4.25 µg); lane 6, low-molecular-weight markers (kDa). (*b*) Size-exclusion chromatography of the purified SmelDhp. The enzyme showed a homotetrameric native structure.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell (1.90–1.85 Å).

Wavelength (Å)	1.54
Space group	$C222_1$
Unit-cell parameters (Å)	a = 124.89, b = 126.28, c = 196.10
Resolution range (Å)	39.9–1.85
No. of observations	445300
No. of unique reflections	129593
Data completeness (%)	98.4 (89.1)
R_{sym} (%)	6.2 (31.1)
Average $I/\sigma(I)$	13.0 (3.1)
Redundancy	3.47 (1.83)
Molecules per ASU	2
Matthews coefficient (Å^3 Da^{-1})	3.6
Solvent content (%)	65.37

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the ith measurement of reflection h and $\langle I(h)\rangle$ is the weighted mean of all measurements of h.

Further dialysis in the same buffer supplemented with 0.5 m*M* $ZnCl_2$ was carried out at 277 K for 24 h prior to crystallization trials. The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

2.5. Crystallization

Recombinant His_6-tagged SmelDhp at a concentration of 18 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 0.5 m*M* $ZnCl_2$ was used to perform initial crystallization screening with Hampton Research Crystal Screen I (based on the sparse-matrix method; Jancarik & Kim, 1991) at 293 K. The hanging-drop vapour-diffusion method was used, with drops made by mixing equal volumes (2 µl) of 18 mg ml^{-1} enzyme solution and reservoir solution, which were suspended over 1.0 ml reservoir. Crystal improvement was achieved by the counter-diffusion technique, with the protein in the same buffer and at the same concentration as for the hanging-drop experiments, using capillaries of 0.3 mm inner diameter with 4.0 *M* sodium formate, 0.1 *M* sodium acetate pH 4.6 as precipitant in the presence of agarose at 0.1%(*w*/*v*). Each capillary was set up by mixing 6 µl of protein in 20 m*M* Tris–HCl pH 8.0 and 0.5 m*M* $ZnCl_2$. This solution was mixed with 0.7 µl warmed (313–318 K) 1.0%(*w*/*v*) agarose in 20 m*M* Tris–HCl pH 8.0. The mixture was introduced into the capillary from the narrow end by capillary force and sealed with wax. The second layer was produced by pouring 2.0 µl 0.5%(*w*/*v*) buffered agarose sol on top of the protein layer from the upper part of the capillary and letting it gel. Finally, 30 µl of the precipitant (third layer) was poured on top of the agarose physical buffer and sealed with wax. Crystals suitable for data collection and structure determination grew within one week.

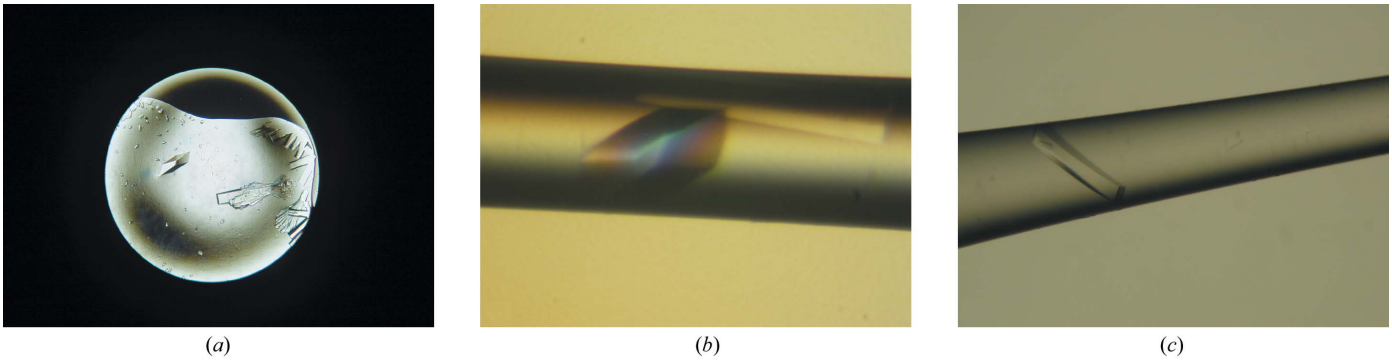
2.6. Data collection and refinement

The target crystal was identified under a microscope using polarized light. The contents of the capillary were extracted over a glass plate by increasing the pressure at the upper end. The presence of agarose fibres prevents the crystal from sticking to the capillary walls and facilitates its extraction. The selected SmelDhp crystal was fished out of the drop with a loop and transferred to a 5 µl drop of mother solution containing 20%(*v*/*v*) glycerol as a cryoprotectant. After soaking for less than 60 s, the crystal was placed in a cool nitrogen stream (100 K) produced by a Krioflex liquid-nitrogen device. X-ray diffraction data were recorded on a Bruker Smart6000 CCD detector with Kappa configuration (X8 Proteum) using Cu $K\alpha$ radiation from a Bruker Microstar micro-focus rotating-anode generator operating at 45 kV and 60 mA. A total of 539 frames were collected with a crystal-to-detector distance of 70 mm, 0.5° oscillation angle and 70 s exposure per frame. Data were integrated with *SAINT* and scaled and corrected for adsorption with *SADABS* from the *PROTEUM* software suite (Bruker AXS Inc.). Statistics of data collection are summarized in Table 1.

The initial model was calculated with the *SWISS-MODEL* server using *ProMod*II for modelling and *GROMOS*96 for energy minimization (Schwede *et al.*, 2000). As input files, we used the sequence of SmelDhp (without the His_6 tag) and the coordinates of D-hydantoinase from *Bacillus* sp. AR9 (PDB code 1yny; Radha Kishan *et al.*, 2005) and dihydropyrimidinase (PDB code 1kcx; Deo *et al.*, 2004) and D-hydantoinase from *B. stearothermophilus* SD1 (PDB code 1k1d; Cheon *et al.*, 2002), which showed sequence similarities of 46, 44 and 45%, respectively. The resulting model, comprising 457 of the 484 residues present in the sequence, served as the search model for molecular replacement using *MOLREP* (Vagin & Teplyakov, 1997). Refinement is ongoing using *REFMAC*5 (Murshudov *et al.*, 1997) from the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994) and *Coot* (Emsley & Cowtan, 2004) for visualization and manual fitting.

3. Results and discussion

An 18 mg ml^{-1} protein sample buffered in 20 m*M* Tris–HCl pH 8.0 and 0.5 m*M* $ZnCl_2$ was used to determine crystallization conditions against Hampton Research Crystal Screen I using the vapour-diffusion method. Crystals were obtained from drops consisting of



(*a*) (*b*) (*c*)

Figure 2
(*a*) Crystals of recombinant SmelDhp grown by the vapour-diffusion method. (*b*) and (*c*) Crystals of recombinant SmelDhp grown in 0.3 mm inner diameter capillaries in the presence of 0.1%(*w*/*v*) agarose by the counter-diffusion technique.

2 µl protein solution and 2 µl 4.0 *M* sodium formate in 0.1 *M* sodium acetate pH 4.6 at 293 K. Fig. 2(*a*) shows an example of the crystallization obtained by the hanging-drop method; only a single isolated crystal was obtained, together with clusters that could not be separated. In order to obtain larger crystals of higher quality, crystallization experiments were set up using the hanging-drop method, varying the protein or precipitant concentration. At the same time, a three-chamber configuration of the counter-diffusion technique in capillaries of 0.3 mm inner diameter was set up. With capillaries of 0.2 mm inner diameter or larger, low agarose concentration is mandatory to ensures a scenario where mass transport is controlled by diffusion (Garcia-Ruiz *et al.*, 2001) and it can also help to improve crystal quality (Gavira *et al.*, 2006). Using this configuration, well faceted crystals with maximum dimensions of 0.6 × 0.2 × 0.15 mm suitable for X-ray diffraction experiments appeared within one week (Figs. 2*b* and 2*c*). However, no improvement was achieved using the hanging-drop variations.

The crystal belongs to the orthorhombic space group $C222_1$, with unit-cell parameters a = 124.89, b = 126.28, c = 196.10 Å. The SmelDhp crystal diffracted X-rays to a maximum resolution of 1.85 Å, with an overall $R_{\rm sym}$ of 6.2% and an $R_{\rm sym}$ of 31.1% for the 1.90–1.85 Å high-resolution shell (Table 1). The asymmetric unit contains two molecules of SmelDhp, with a corresponding crystal volume per protein weight ($V_{\rm M}$) of 3.56 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 65.4% by volume (Westbrook, 1985). During the SEC-HPLC experiments, which were used to eliminate possible co-eluted DNA, the SmelDhp peak appears at 200 kDa (Fig. 2), showing that the enzyme is a tetramer under the experimental conditions. The biochemical characterization of the enzyme and aggregation studies as a function of pH are in preparation for publication elsewhere.

Only two major peaks were obtained for the rotation–translation functions and these were independently refined as rigid bodies prior to positional refinement with *REFMAC*5 (Murshudov *et al.*, 1997) in the resolution range 40.0–2.5 Å. After the first 40 cycles of restrained positional refinement, the R and $R_{\rm free}$ (10% of the data) values were 0.32 and 0.38, respectively. Inspection of the $|F_{\rm o} - F_{\rm c}|$ electron-density maps clearly shows two major peaks at the active site. We have assumed that those peaks must be two Zn atoms as previously described for other hydantoinases (Cheon *et al.*, 2002; Xu *et al.*, 2003) and as this is the only metal present in the crystallization mix. One of the atoms is localized in the environment of the well described motif Asp-Xaa1-His-Xaa2-His for the dinuclear metal centres (Kim & Kim, 1998), corresponding to Asp54-Pro55-His56-Thr57-His58 in SmelDhp. The Zn atom is at a binding distance from both histidines and also seems to interact with residue Asp313. The second Zn atom interacts with His180 and His236 in a similar way and residue Lys147 acts as a bridge between both metal atoms. Refinement is in progress with manual rebuilding of the side chain and the location of residues at the C-termini is clearly visible in both $|2F_{\rm o} - F_{\rm c}|$ and $|F_{\rm o} - F_{\rm c}|$ electron-density maps. At the current stage of refinement, the R and $R_{\rm free}$ values are 0.28 and 0.30, respectively.

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References

Abendroth, J., Niefind, K. & Schomburg, D. (2002). *J. Mol. Biol.* **320**, 143–156.
Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). *J. Mol. Biol.* **215**, 403–410.
Arcuri, M. B., Antunes, O. A., Sabino, S. J., Pinto, G. F. & Oestreicher, E. G. (2000). *Amino Acids*, **19**, 477–482.
Capela, D. *et al.* (2001). *Proc. Natl Acad. Sci. USA*, **98**, 9877–9882.
Cheon, Y. H., Kim, H. S., Han, K. H., Abendroth, J., Niefind, K., Schomburg, D., Wang, J. & Kim, Y. (2002). *Biochemistry*, **41**, 9410–9417.
Clemente-Jimenez, J. M., Martinez-Rogriguez, S., Mingorance-Cazorla, L., De La Escalera-Hueso, S., Las Heras-Vazquez, F. J. & Rodriguez-Vico, F. (2003). *Biotechnol. Lett.* **25**, 1067–1073.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Deo, R. C., Schmidt, E. F., Elhabazi, A., Togashi, H., Burley, S. K. & Strittmatter, S. M. (2004). *EMBO J.* **23**, 9–22.
Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.
Galibert, F. *et al.* (2001). *Science*, **293**, 668–672.
Galtier, N., Gouy, M. & Gautier, C. (1996). *Comput. Appl. Biosci.* **12**, 543–548.
Garcia-Ruiz, J. M. (2003). *Methods Enzymol.* **368**, 130–154.
Garcia-Ruiz, J. M., Novella, M. L., Moreno, R. & Gavira, J. A. (2001). *J. Cryst. Growth*, **232**, 165–172.
Gavira, J. A., de Jesus, W., Camara-Artigas, A., Lopez-Garriga, J. & Garcia-Ruiz, J. M. (2006). *Acta Cryst.* F**62**, 196–199.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G. & Gibson, T. J. (1998). *Trends Biochem. Sci.* **23**, 403–405.
Kim, G. J. & Kim, H. S. (1998). *Biochem. J.* **330**, 295–302.
Kim, G. J., Lee, D. E. & Kim, H. S. (2000). *J. Bacteriol.* **182**, 7021–7028.
Lohkamp, B., Andersen, B., Piskur, J. & Dobritzsch, D. (2006). *J. Biol. Chem.* **281**, 13762–13776.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
Martinez-Rodriguez, S., Las Heras-Vazquez, F. J., Clemente-Jimenez, J. M., Mingorance-Cazorla, L. & Rodriguez-Vico, F. (2002). *Biotechnol. Prog.* **18**, 1201–1206.
Matthews, B. (1968). *J. Mol. Biol.* **33**, 491–497.
Moller, A., Syldatk, C., Schulze, M. & Wagner, F. (1988). *Enzyme. Microb. Technol.* **10**, 618–625.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Radha Kishan, K. V., Vohra, R. M., Ganesan, K., Agrawal, V., Sharma, V. M. & Sharma, R. (2005). *J. Mol. Biol.* **347**, 95–105.
Runser, S. M. & Meyer, P. C. (1993). *Eur. J. Biochem.* **213**, 1315–1324.
Schwede, T., Diemand, A., Guex, N. & Peitsch, M. C. (2000). *Res. Microbiol.* **151**, 107–112.
Soong, C. L., Ogawa, J., Honda, M. & Shimizu, S. (1999). *Appl. Environ. Microbiol.* **65**, 1459–1462.
Syldatk, C., May, O., Altenbuchner, J., Mattes, R. & Siemann, M. (1999). *Appl. Environ. Microbiol.* **51**, 293–309.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
Van Eys, G. J., Gravekamp, C., Gerritsen, M. J., Quint, W., Cornelissen, M. T., Schegget, J. T. & Terpstra, W. J. (1989). *J. Clin. Microbiol.* **27**, 2258–2262.
Westbrook, E. M. (1985). *Methods Enzymol.* **114**, 187–196.
Wilms, B., Wiese, A., Syldatk, C., Mattes, R. & Altenbuchner, J. (2001). *J. Biotechnol.* **86**, 19–30.
Xu, Z., Liu, Y., Yang, Y., Jiang, W., Arnold, E. & Ding, J. (2003). *J. Bacteriol.* **185**, 4038–4049.