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Expression, purification and crystallization of *Helicobacter pylori* L-asparaginase

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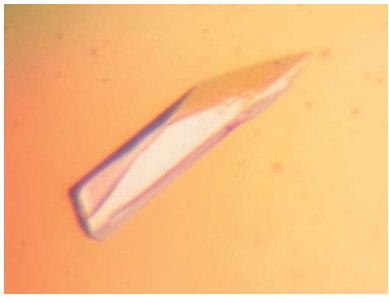
The L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* are effective drugs that have been used in the treatment of acute childhood lymphoblastic leukaemia for over 30 years. However, despite their therapeutic potential, they can cause serious side effects as a consequence of their intrinsic glutaminase activity, which leads to L-glutamine depletion in the blood. Consequently, new asparaginases with low glutaminase activity, fewer side effects and high activity towards L-asparagine are highly desirable as better alternatives in cancer therapy. L-Asparaginase from *Helicobacter pylori* was overexpressed in *E. coli* and purified for structural studies. The enzyme was crystallized at pH 7.0 in the presence of 16–19%(*w*/*v*) PEG 4000 and 0.1 *M* magnesium formate. Data were collected to 1.6 Å resolution at 100 K from a single crystal at a synchrotron-radiation source. The crystals belong to space group *I*222, with unit-cell parameters $a = 63.6$, $b = 94.9$, $c = 100.2$ Å and one molecule of L-asparaginase in the asymmetric unit. Elucidation of the crystal structure will provide insight into the active site of the enzyme and a better understanding of the structure–activity relationship in L-asparaginases.

1. Introduction

Bacterial L-asparaginases (EC 3.5.1.1) catalyze the conversion of L-asparagine to L-aspartic acid and ammonia. Two types of L-asparaginases, I and II, have been identified. Type II (periplasmic) L-asparaginases have been intensively studied for many years as a consequence of their wide use in the treatment of acute childhood lymphoblastic leukaemia since the early 1970s (Duval *et al.*, 2002; Avramis & Tiwari, 2006). The antineoplastic effect of type II L-asparaginases stems from their ability to deplete the L-asparagine circulating in the blood and tissues. Asparagine is a crucial amino acid for protein, DNA and RNA synthesis, and is a cell-cycle-specific requirement for the G1 phase of cell division (Stams *et al.*, 2005). In contrast to normal cells, leukaemic cells are characterized by a generally low expression of asparagine synthetase (Richards & Kilberg, 2006). As a result, they are unable to synthesize their own L-asparagine and rely on extracellular supplies of this amino acid for survival and growth (Verma *et al.*, 2007).

The L-asparaginases from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) are currently used in clinical practice. Recent statistics have shown that L-asparaginases in combination with other drugs, such as corticosteroids, have significantly improved the cure rates of leukaemia patients (Holcenberg, 2005; Apostolidou *et al.*, 2007). Despite their beneficial effects, EcAII and ErA can also cause serious side effects, including liver disorders, acute pancreatitis, hyperglycaemia, immunosuppression and other dysfunctions. Their toxicity is partially attributed to their intrinsic low glutaminase activity, which leads to a significant reduction in the L-glutamine concentration in the blood (Carlsson *et al.*, 1995). Indeed, the glutaminase activities of EcAII and ErA amount to 2% and 10% of their asparaginase activities, respectively. Hence, it is thought that L-asparaginases that have high activity against L-asparagine and low specificity towards L-glutamine should be more efficient and induce fewer side effects (Distasio *et al.*, 1982).



The crystal structures of bacterial L-asparaginases from *E. coli* and *Er. chrysanthemi* (Swain *et al.*, 1993; Aghaiypour *et al.*, 2001; Sanches *et al.*, 2003; Lubkowski *et al.*, 2003; Kozak *et al.*, 2002) and those of L-glutaminase-asparaginases from *Pseudomonas* 7a, *Acinetobacter glutaminasificans* and *Wolinella succinogenes* (Lubkowski, Wlodawer, Ammon *et al.*, 1994; Lubkowski, Wlodawer, Housset *et al.*, 1994; Lubkowski *et al.*, 1996) have been elucidated. The majority of L-asparaginases crystallize as homotetramers with 222 symmetry and exhibit high similarity in their tertiary and quaternary structures. The enzyme monomer consists of ∼330 residues arranged in two domains: a larger domain at the N-terminus and a smaller domain at the C-terminus of the enzyme. The active site is located between the N- and C-terminal domains of two adjacent monomers. The residues responsible for ligand binding form the rigid part of the active site. The flexible part of the active site controls access to the binding pocket and carries a Thr residue which is highly conserved in all L-asparaginases as the catalytic nucleophile (Aghaiypour *et al.*, 2001). In contrast to type II L-asparaginases, type I (cytoplasmic) L-asparaginases display allosteric behaviour and reduced affinity for L-asparagine despite the overall structural similarity between the two types of enzyme (Yun *et al.*, 2007). The recently identified plant L-asparaginases comprise a structurally and evolutionarily distinct family of L-asparaginases that differ from the bacterial enzymes in their substrate-specificity and affinity profiles (Borek *et al.*, 2004).

In an effort to identify and characterize new L-asparaginases with better therapeutic properties, we have initiated structural and biochemical studies on *Helicobacter pylori* type II L-asparaginase (HpA) and report here on its cloning, overexpression and crystallization. The expressed enzyme contains 333 amino acids with a calculated molecular weight of 35 686 Da based on its amino-acid sequence and exhibits ∼50% sequence identity to the periplasmic *W. succinogenes* L-asparaginase. The availability of the pure enzyme will allow full biochemical characterization, including studies on its oligomerization state and assessment of its potential in therapeutic settings. Preliminary kinetic data have shown a significantly low L-glutaminase:L-asparaginase ratio (data not shown).

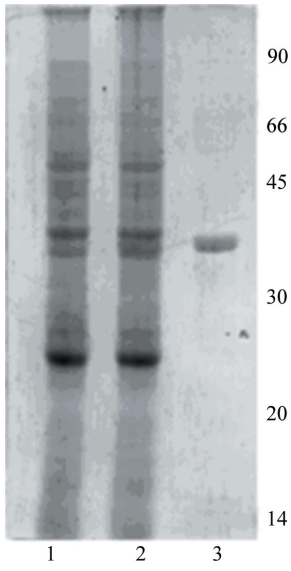


Figure 1
Purity of *H. pylori* L-asparaginase in 12% SDS–PAGE. Lanes 1 and 2, crude lysate of *E. coli* BL21 (DE3) cells harbouring the pET22b/HpA plasmid before (lane 1) and after (lane 2) 16 h induction with 0.5 m*M* IPTG; lane 3, purified HpA. Molecular-weight standards are shown on the right (in kDa).

2. Materials and methods

2.1. Expression and purification

A DNA fragment encoding the mature form of *H. pylori* J99 L-asparaginase (Gene ID 890242) was amplified from genomic DNA with *Pfu* polymerase (Fermentas) using 3′-CAG**CCATGG**CTC-AAAATTTACCCACCATTGC-5′ and 3′-CT**GCGGCCG**CTTAA-TACTCTTCAAACATTTC-5′ as the forward and reverse primers, respectively. *Nco*I and *Not*I restriction sites (bold) were included in the 3′-ends of the oligonucleotides, respectively. The resulting PCR product was digested with *Nco*I and *Not*I restriction enzymes and cloned into a pET22b vector (Novagen) immediately after the *E. coli* signal peptide sequence. The fidelity of the resulting construct was verified by DNA sequencing. The resultant plasmid was named pET22b/HpA and was transformed into BL21 (DE3) *E. coli* cells. Cell cultures were grown at 310 K in LB medium containing 100 µg ml^{-1} ampicillin until the optical density at 600 nm reached 0.8. HpA expression was induced with 0.5 m*M* isopropyl *β*-D-1-thio-galactopyranoside (IPTG) for a further 16 h at 310 K. Cells from a 2 l culture were harvested by centrifugation at 4000*g* (277 K) for 15 min, suspended in 70 ml 20 m*M* potassium phosphate buffer pH 6.0 and sonicated. Cell debris was removed by centrifugation at 15 000*g* (277 K) for 30 min and the soluble fraction of the cell-free extract was loaded onto an SP-Sepharose column (1 × 5 cm) equilibrated with 20 m*M* potassium phosphate buffer pH 6.0. The resin was washed with five volumes of column buffer to remove nonbound proteins. Bound HpA was eluted with a 0–0.6 *M* KCl linear gradient in column buffer. L-Asparaginase samples were desalted with PD-10 prepacked columns (Amersham Pharmacia Biotech). The purity of the resultant enzyme solution was confirmed by 12% SDS–PAGE after staining with Coomassie Brilliant Blue R-250. A single major band corresponding to a molecular weight of approximately 35 kDa was found (Fig. 1). The final yield is approximately 6 mg from 1 l of bacterial culture.

2.2. Crystallization and data collection

The purified protein was concentrated by ultrafiltration using Amicon YM-10 filters and stored in 10 m*M* HEPES–NaOH pH 7.0 after buffer exchange. The final protein concentration was 3.7 mg ml^{-1} as measured by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. Initial crystallization trials were performed with Index Screen (Hampton Research) using the sitting-drop vapour-diffusion method at 289 K in a 96-well Corning

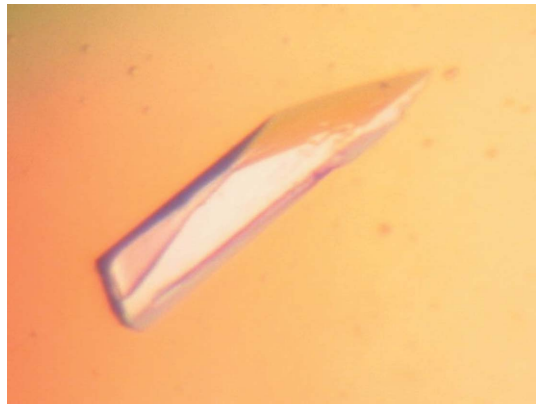


Figure 2
Crystal of *H. pylori* L-asparaginase. Typical dimensions are 0.35 × 0.10 × 0.10 mm.

Table 1
Crystal parameters and data-processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	*I*222
Unit-cell parameters (Å)	a = 63.6, b = 94.9, c = 100.2
Resolution range (Å)	99–1.6 (1.63–1.60)
Wavelength (Å)	0.8081
Temperature (K)	100
Total reflections	1153231 (57700)
Unique reflections	40100 (1973)
Completeness (%)	98.9 (97.8)
Mosaic spread (°)	0.6
$R_{\rm merge}$† (%)	9.0 (36.7)
$\langle I/\sigma(I)\rangle$	31.4 (5.3)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

plate. The drops contained 1.0 µl protein solution and an equal volume of precipitant solution. Crystals were obtained after 2 d in condition No. 92, which consisted of 15%(*w*/*v*) PEG 4000 and 0.1 *M* magnesium formate. Optimization of the crystallization conditions was performed with the hanging-drop vapour-diffusion method. Crystals suitable for X-ray crystallographic analysis (Fig. 2) were grown at 289 K by mixing 2.5 µl of a protein stock solution with an equal volume of a reservoir solution containing 16–19%(*w*/*v*) PEG 4000, 0.1 *M* magnesium formate, 0.1 *M* HEPES–NaOH pH 7.0. The hanging drops were equilibrated against 800 µl reservoir solution and crystals grew in about 4 d. Data were collected to 1.6 Å resolution on the X13 beamline at EMBL Hamburg (c/o DESY) from a single crystal soaked for a few seconds in a crystallization solution containing 20%(*v*/*v*) glycerol as a cryoprotectant and flash-cooled to 100 K in a nitrogen-gas cold stream. A total of 400 images were collected with 0.5° rotation per image at a wavelength of 0.8081 Å using a MAR CCD detector, a crystal-to-detector distance of 140 mm and an average exposure time of 10 s per image. Data were processed with the *HKL* package (Otwinowski & Minor, 1997) and the final statistics are summarized in Table 1.

3. Results and discussion

The crystals of HpA belonged to space group *I*222, with unit-cell parameters a = 63.6, b = 94.9, c = 100.2 Å. Assuming the presence of one molecule of the enzyme in the asymmetric unit and a molecular weight of 35 686 Da, the Matthews coefficient $V_{\rm M}$ (Matthews, 1968) is 2.27 Å^3 Da^{-1}, corresponding to a solvent content of ∼46%. The presence of one molecule in the asymmetric unit is in contrast to most other bacterial type II L-asparaginases, which crystallize as non-crystallographic homotetramers. Similarly to HpA, L-asparaginase-glutaminase from *A. glutaminasificans* also crystallizes with a monomer in the asymmetric unit. In this case, the active homotetramer could be generated by crystal symmetry operations (Lubkowski, Wlodawer, Housset *et al.*, 1994). Structure determination of HpA will be pursued by molecular replacement using the coordinates of the monomer from the homotetrameric *W. succinogenes* L-asparaginase (PDB code 1wsa; Lubkowski *et al.*, 1996). In combination with biochemical and mutagenesis data, the crystal structure of HpA will provide new clues for protein-engineering efforts to improve the therapeutic potential of L-asparaginases.

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References

Aghaiypour, K., Wlodawer, A. & Lubkowski, J. (2001). *Biochemistry*, **40**, 5655–5664.
Apostolidou, E., Swords, R., Alvarado, Y. & Giles, F. J. (2007). *Drugs*, **67**, 2153–2171.
Avramis, V. I. & Tiwari, P. N. (2006). *Int. J. Nanomedicine*, **1**, 241–254.
Borek, D., Michalska, K., Brzezinski, K., Kisiel, A., Podkowinski, J., Bonthron, D. T., Krowarsch, D., Otlewski, J. & Jaskólski, M. (2004). *Eur. J. Biochem.* **271**, 3215–3226.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Carlsson, H., Stockelberg, D., Tengborn, L., Braide, I., Carneskog, J. & Kutti, J. (1995). *Eur. J. Haematol.* **55**, 289–293.
Distasio, J. A., Salazar, A. M., Nadji, M. & Durden, D. L. (1982). *Int. J. Cancer*, **30**, 343–347.
Duval, M., Suciu, S., Ferster, A., Rialland, X., Nelken, B., Lutz, P., Benoit, Y., Robert, A., Marel, A.-M., Vilmer, E., Otten, J. & Philippe, N. (2002). *Blood*, **99**, 2734–2739.
Holcenberg, J. (2005). *J. Pediatr. Hematol. Oncol.* **27**, 246–247.
Kozak, M., Borek, D., Janowski, R. & Jaskólski, M. (2002). *Acta Cryst.* D**58**, 130–132.
Lubkowski, J., Dauter, M., Aghaiypour, K., Wlodawer, A. & Dauter, Z. (2003). *Acta Cryst.* D**59**, 84–92.
Lubkowski, J., Palm, G. J., Gilliland, G. L., Derst, C., Rohm, K. H. & Wlodawer, A. (1996). *Eur. J. Biochem.* **241**, 201–207.
Lubkowski, J., Wlodawer, A., Ammon, H. L., Copeland, T. D. & Swain, A. L. (1994). *Biochemistry*, **33**, 10257–10265.
Lubkowski, J., Wlodawer, A., Housset, D., Weber, I. T., Ammon, H. L., Murphy, K. C. & Swain, A. L. (1994). *Acta Cryst.* D**50**, 826–832.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 2283–2287.
Sanches, M., Barbosa, J. A. R. G., de Oliveira, R. T., Abrahão Neto, J. A. & Polikarpov, I. (2003). *Acta Cryst.* D**59**, 416–422.
Stams, W. A., den Boer, M. L., Beverloo, H. B., van Wering, E. R. & Pieters, R. (2005). *Leukemia*, **19**, 318–319.
Swain, A. L., Jaskólski, M., Housset, D., Rao, J. K. M. & Wlodawer, A. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 1474–1478.
Richards, N. G. & Kilberg, M. S. (2006). *Annu. Rev. Biochem.* **75**, 629–654.
Verma, N., Kumar, K., Kaur, G. & Anand, S. (2007). *Crit. Rev. Biotechnol.* **27**, 45–62.
Yun, M.-K, Nourse, A., White, S. W., Rock, C. O. & Heath, R. J. (2007). *J. Mol. Biol.* **369**, 794–811.