Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

De novo purification scheme and crystallization conditions yield high-resolution structures of chitinase A and its complex with the inhibitor allosamidin

Yannis Papanikolau,[a] Giorgos Tavlas,[a] Constantinos E. Vorgias[b] and Kyriacos Petratos[a]*

[a]Institute of Molecular Biology and Biotechnology (IMBB)–FORTH, PO Box 1527, 71110 Heraklion, Greece, and [b]National and Kapodistrian University of Athens, Faculty of Biology, Department of Biochemistry and Molecular Biology, 15784 Athens, Greece

Correspondence e-mail: petratos@imbb.forth.gr

Received 1 July 2002
Accepted 26 November 2002

PDB References: chitinase A, 1edq, r1edqsf; chitinase A–allosamidin complex, 1ffq, r1ffqsf.

The purification scheme of chitinase A (ChiA) from *S. marcescens* has been extensively revised. The pure enzyme crystallizes readily under new crystallization conditions. The ChiA crystal structure has been refined to 1.55 Å resolution and the crystal structure of ChiA co-crystallized with the inhibitor allosamidin has been refined to 1.9 Å resolution. Allosamidin is located in the deep active-site tunnel of ChiA and interacts with three important residues: Glu315, the proton donor of the catalysis, Asp313, which adopts two conformations in the native structure but is oriented towards Glu315 in the inhibitor complex, and Tyr390, which lies opposite Glu315 in the active-site tunnel.

1. Introduction

Chitinase A (ChiA) is one of the chitinolytic enzymes from *Serratia marcescens*. It belongs to family 18 of the glycosyl hydrolase superfamily (Henrissat & Bairoch, 1996). The precursor consists of 563 residues and the secreted mature protein consists of 540 residues (MW 58 515 Da). ChiA has previously been reported to act as an exo-chitinase (Roberts & Cabib, 1982), excising diacetylchitobiose $(NAG)_2$ from the non-reducing end of the chitin polysaccharide chain. The crystal structure of ChiA was initially determined at 2.3 Å resolution (Perrakis *et al.*, 1994). Several crystal structures of family 18 enzymes have been published: chitinase B (ChiB) from *S. marcescens* (van Aalten *et al.*, 2000), endo-β-*N*-acetylglucosaminidases F_1 (van Roey *et al.*, 1994) and H (Rao *et al.*, 1995), chitinase A1 from *Bacillus circulans* (Matsumoto *et al.*, 1999), hevamine from the rubber tree *Hevea brasiliensis* (Terwisscha van Scheltinga *et al.*, 1994) and the chitinase from the fungal pathogen *Coccidioides immitis* (Hollis *et al.*, 2000). In the latter work, the authors provide a model for the binding of allosamidin. Recently, ChiB soaked with allosamidin provided a structure of the respective complex at 2.5 Å resolution (van Aalten *et al.*, 2001). It was recently shown (Uchiyama *et al.*, 2001) that ChiA is an exo-chitinase, cleaving $(NAG)_2$ in a processive manner from the reducing end of crystalline chitin. The latter finding agrees with the results of our structural studies of ChiA mutants co-crystallized with substrates (Papanikolau *et al.*, 2001). In order to investigate in detail the structure–function relationship of ChiA, we have focused on improvement of the precision of the native crystal structure. We have developed a novel purification scheme and applied new crystallization conditions that yield crystals diffracting to high resolution. Moreover, the structure of the ChiA complex with allosamidin, a potent natural inhibitor of bacterial family 18 chitinases (Spindler & Spindler-Barth, 1999), has been determined and refined at 1.9 Å resolution. Allosamidin is produced by *Streptomyces* sp. (Sakuda *et al.*, 1987) and consists of two β(1,4)-linked *N*-acetylallosamine rings and one allosamizoline, which is an oxazoline derivative. Our ChiA–allosamidin structure is compared with the hevamine–allosamidin complex (Terwisscha van Scheltinga *et al.*, 1995).

2. Materials and methods

2.1. Expression, purification and crystallizations

The ChiA protein was overexpressed from *Escherichia coli* strain A5745 by thermoinduction. The original purification scheme (Vorgias *et al.*, 1992) did not reproducibly yield crystallizable protein. In order to obtain crystals that diffract to high resolution, we have developed a novel purification protocol. The culture medium containing the secreted protein was loaded onto a Sepharose CL-6B with 1.5 *M* ammonium sulfate, 20 m*M* Tris pH 7.5 and washed with 1.2 *M* ammonium sulfate. An ammonium sulfate gradient (1.2–0 *M*) was applied. Fractions containing ChiA were pooled and dialyzed against 20 m*M* sodium acetate pH 4.8 and PEG (polyethylene glycol) 6000 was added to a final concentration of 15%(*w*/*v*). The protein solution was loaded onto SP-Sepharose. A gradient of increasing NaCl (0–0.2 *M*) and decreasing PEG 6000 concentration [15–0%(*w*/*v*)] was applied. The ChiA-containing fractions were pooled and

Table 1
Data-collection and refinement parameters for ChiA and ChiA–allosamidin structures.

Values in parentheses refer to the highest resolution shell.

	ChiA	ChiA–allosamidin
Space group	$C222_1$	$C222_1$
Unit-cell parameters (Å)	a = 199.5, b = 131.8, c = 59.4	a = 199.1, b = 131.8, c = 59.5
X-ray source†	X11	Rotating anode
Wavelength (Å)	0.9116	1.5418
Resolution range (Å)	10–1.55 (1.61–1.55)	10–1.9 (1.97–1.9)
No. unique reflections	112857	60767
Data redundancy	5.0 (4.3)	3.05 (2.7)
Completeness (%)	99.8 (100.0)	98.6 (96.2)
Mean $I/\sigma(I)$	27.8 (6.8)	22.6 (4.1)
R_{merge}	0.032 (0.216)	0.056 (0.249)
No. of reflections with $I > \sigma(I)$	108205	56566
Refinement		
R_{factor}	0.187	0.189
R_{free}‡	0.215	0.232
No. of protein atoms	4220	4170
No. of ordered waters	909	660
No. of heteroatoms		43
R.m.s. deviations from ideal values		
Bond lengths (Å)	0.010	0.012
Bond angles (Å)	0.023	0.028
Dihedrals (°)	11.9	13.2
Average B values (Å^2)		
Main-chain atoms	19.6	25.3
Side-chain atoms	21.6	27.4
Inhibitor atoms		20.0
Solvent atoms	41.3	41.4

† X11: EMBL/DESY synchrotron beamline. Rotating anode: Rigaku RU-3HR in our laboratory in Heraklion. ‡ R_{free} is calculated from a 5% subset of the diffraction data.

diluted three times with water. Finally, the protein solution was loaded onto hydroxyapatite, washed with 0.5 m*M* NaCl and eluted with 200 m*M* ammonium sulfate. The pure enzyme (30 mg ml^{-1}) used in crystallization was stored in 2 m*M* Tris–HCl buffer pH 7.5.

ChiA was initially crystallized (Vorgias *et al.*, 1992) with ammonium sulfate. In order to improve these 'salting-out' crystallization conditions, we employed knowledge acquired using ionic strength-reducing agents (Papanikolau & Kokkinidis, 1997). According to this, a number of ionic strength reducers (methanol, ethanol, 2-propanol, glycerol, PEG 200, PEG 400 and PEG 6000) were initially screened in the presence of ammonium sulfate. The best results were obtained with sodium citrate as the precipitating agent and methanol as the ionic strength-reducing agent. Hanging drops consisting of 4 µl protein solution plus 2 µl reservoir solution [0.75 *M* sodium citrate pH 7.2 and 20%(*v*/*v*) methanol] were used for crystallization. Rod-shaped crystals (smallest dimension 0.3 mm) appeared 3–4 d later at 291 K. More details on the development of the purification protocol and the search for crystallization conditions are presented elsewhere (Papanikolau & Petratos, 2002). The crystals of the allosamidin complex were obtained by co-crystallization. Solid allosamidin was added to the protein solution until no activity could be assayed. The crystallizations proceeded as described above.

2.2. Data collection, structure solution and refinement

The crystals were briefly washed with cryoprotectant solution [30%(*v*/*v*) glycerol and 0.6 *M* sodium citrate pH 7.2]. They were then plunged into liquid nitrogen and mounted in a cold nitrogen stream (100 K). X-ray data for native ChiA were collected on beamline X11 at the EMBL/DESY synchrotron and data for the ChiA–inhibitor complex were collected on a Rigaku rotating-anode (RU-3HR) source in our laboratory in Heraklion. The data were processed and scaled with the *HKL* suite (Otwinowski & Minor, 1997). Table 1 summarizes the results of the data collection and processing.

It was reported previously (Perrakis *et al.*, 1997) that the N-terminal domain of ChiA under cryoconditions rotates about 5° with respect to the catalytic domain of the molecule. For this reason, the ChiA model (1ctn; Perrakis *et al.*, 1994) was divided into N-terminal (residues 24–130) and C-terminal (residues 131–561) substructures. These were used as search models in *AMoRe* (Navaza, 1994). In the molecular-replace-

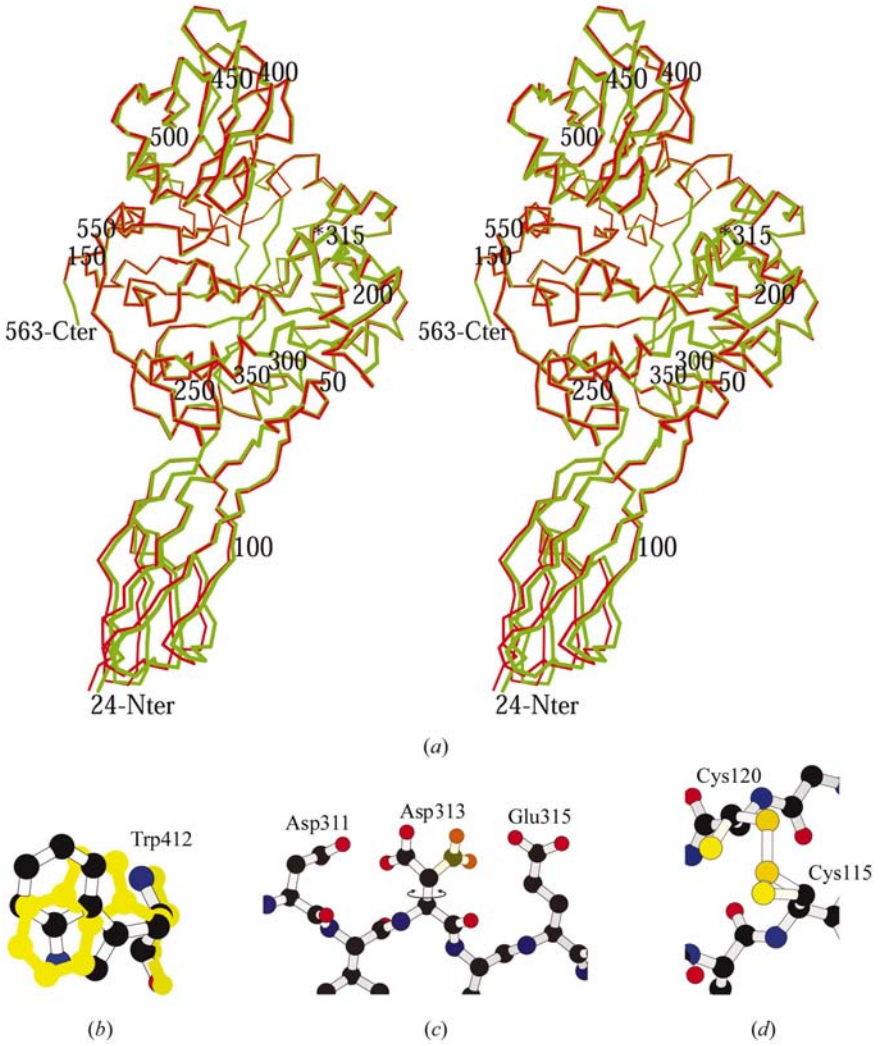

Figure 1
Refined *versus* initial ChiA structure. (*a*) Stereo drawing of the C$^{\alpha}$ atoms of the initial ChiA model (PDB code 1ctn), shown in red, superimposed on the final refined structure (PDB code 1edq), shown in green. The C-terminal domains of the models were used for the superposition. The N-terminal domain of the refined structure rotates by 2°. (*b*) Residue Trp412 in the initial (yellow) and final model (atom coloured). (*c*) Double conformation of Asp313 in the refined model. (*d*) Oxidized and reduced conformations of residues Cys115 and Cys120.

ment solution, the N-terminal domain rotates by 2° with respect to the C-terminal domain of the original structure. This solution was further refined with *REFMAC* (Murshudov *et al.*, 1997) and *ARP* (Lamzin & Wilson, 1993). All computations involved programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Model building was performed with *O* (Jones *et al.*, 1991). The allosamidin model was initially modelled in a weighted difference Fourier map. Table 1 summarizes the refinement statistics of both structures. The figures were produced with *BOBSCRIPT* (Esnouf, 1997) and *GRASP* (Nicholls *et al.*, 1991).

3. Results and discussion

3.1. Refined ChiA

The refined structure of the enzyme at 1.55 Å resolution (PDB code 1edq) retains the overall topology and folding determined previously (Fig. 1*a*). The r.m.s. deviation for the C^{α} atoms is 0.31 Å and the r.m.s. deviation for all atoms is 0.75 Å (the maximum deviation is 6.5 Å). In these calculations, the N- and C-terminal domains are superimposed separately owing to the 2° rotation of the N-terminal domain with respect to the catalytic domain. Although residues Tyr170 and Glu147 are found in generously allowed regions of the Ramachandran plot, they are unambiguously modelled in electron density. Residues Ile79, Ile226 and Thr351 are remodelled as Val79, Val226 and Val351, respectively. These three changes are in agreement with the latest sequence AB015996 deposited in GENBANK. Residue 475 remains modelled as glycine. This is in agreement with our sequencing data but in disagreement with sequence AB015996, where residue 475 is arginine.

A detailed comparison of the refined and initial structures shows that 49 residues assume quite different conformations, *e.g.* Trp412 (Fig. 1*b*). Moreover, 28 residues, which are mainly on the surface of the molecule, are modelled with multiple conformations. One of these is the conserved residue Asp313 (Fig. 1*c*). In addition, residues Cys115 and Cys120 adopt two conformations (Fig. 1*d*). In one conformation the two cysteines are oxidized and form a disulfide bridge, while in the other they are reduced.

3.2. Structure of the ChiA–allosamidin complex

The overall refined structure of the ChiA–allosamidin complex (PDB code 1ffq) is

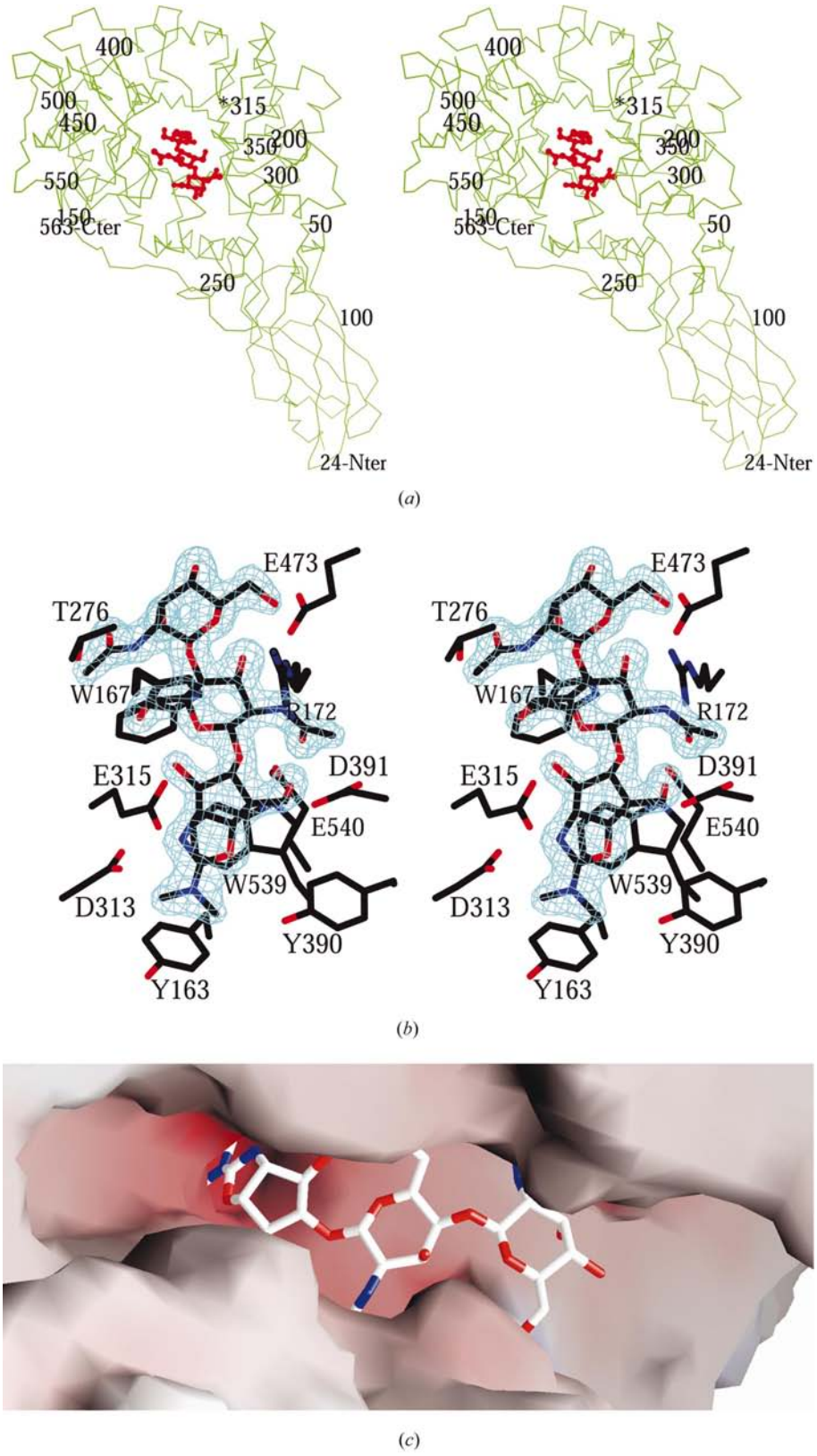

Figure 2
ChiA–allosamidin complex. (*a*) Stereoview of the C^{α}-atom trace of the enzyme molecule (green) with the inhibitor (red) bound to the active-site tunnel (PDB code 1ffq). (*b*) Allosamidin in the electron density of the weighted $2F_o - F_c$ map contoured at 2σ (blue) and the neighbouring residues of ChiA. (*c*) Close-up view of allosamidin bound to the enzyme's active-site tunnel at subsites −1 to −3. The allosamizoline moiety fits into a local well.

shown in Fig. 2(*a*). Close-up views of the active-site region are shown in Figs. 2(*b*) and 2(*c*). The inhibitor binds to the −1, −2 and −3 subsites (Davies *et al.*, 1997) of ChiA. Overall, allosamidin binds to ChiA and hevamine (Terwisscha van Scheltinga *et al.*, 1995) in a similar manner. The structures of both allosamidin complexes demonstrate the role of a number of amino-acid residues. In particular, Trp539 of ChiA and its hevamine counterpart Trp255 act as a docking surface for the sugars at subsite −1. These tryptophan residues in both enzymes participate in *cis*-peptide bonds. Furthermore, residue Tyr163 of ChiA and its hevamine counterpart Tyr6 form a pocket in which the allosamizoline moiety of the inhibitor is docked. Some of the most important interactions of allosamidin comprise residues Asp313, Glu315 and Tyr390, which are directly involved in the catalytic mechanism (Papanikolau *et al.*, 2001).

3.3. Summary

The most striking feature revealed from the refined structure of ChiA is the double conformation of Asp313. This residue is adjacent to the proposed proton donor Glu315. One conformation of Asp313 points towards Glu315 and the other points towards Asp311 and away from Glu315. Although Asp313 is shown to flip-flop between Glu315 and Asp311 in the native ChiA structure, it is oriented towards Glu315 in the enzyme–inhibitor complex.

In view of recent findings (Papanikolau *et al.*, 2001; Uchiyama *et al.*, 2001), ChiA is an exo-chitinase that cleaves $(NAG)_2$ units in a processive manner from the reducing end of chitin. Allosamidin binds to ChiA in a way that hinders the entrance of substrate to the active-site tunnel. The inhibitor binds tightly to the active site of the enzyme *via* several polar and hydrophobic interactions. The most significant of these are mediated *via* the three critical residues of the catalytic mechanism (Papanikolau *et al.*, 2001), namely Asp313, Glu315 and Tyr390. Comparisons of the allosamidin complex of ChiA with the corresponding complexes of hevamine and ChiB strengthen the above results.

After structural alignment of the catalytic domains of ChiA and ChiB, we find that the chitin-binding domain of ChiA lies towards the non-reducing end of the sugar, while the chitin-binding domain of ChiB lies towards the reducing end. Provided that ChiB cleaves $(NAG)_2$ units from the non-reducing end (Brurberg *et al.*, 1996; van Aalten *et al.*, 2001), we propose that ChiB functions with a mode similar to that of ChiA but in the opposite direction. Thus, ChiB is an exo-chitinase that cleaves $(NAG)_2$ units in a processive manner from the non-reducing end of chitin.

We thank Professor Klaus-Dieter Spindler for providing allosamidin and the EMBL staff in Hamburg for their assistance. The present work was supported by the European Union (BIOTECH program contract No. BIO4-CT-960670). EMBL/DESY visits were supported by the European Union (program contract No. HPRI-CT-1999-00017).

References

Aalten, D. M. F. van, Komander, D., Synstad, B., Gåseidnes, S., Peter, M. G. & Eijsink, V. G. H. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 8979–8984.

Aalten, D. M. F. van, Synstad, B., Brurberg, M. B., Hough, E., Riize, B. W., Eijsink, V. G. H. & Wierenga, R. K. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 5842–5847.

Brurberg, M. B., Nes, I. F. & Eijsink, V. G. H. (1996). *Microbiology*, **142**, 1581–1589.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Davies, G. J., Wilson, K. S. & Henrissat, B. (1997). *Biochem. J.* **321**, 557–559.

Esnouf, R. M. (1997). *J. Mol. Graph. Model.* **15**, 132–134.

Henrissat, B. & Bairoch, A. (1996). *Biochem. J.* **316**, 695–696.

Hollis, T., Monzingo, A. F., Bortone, K., Ernst, S., Cox, R. & Robertus, J. D. (2000). *Protein Sci.* **9**, 544–551.

Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.

Lamzin, V. S. & Wilson, K. S. (1993). *Acta Cryst.* D**49**, 129–147.

Matsumoto, T., Nonaka, T., Hashimoto, M., Watanabe, T. & Mitsui, Y. (1999). *Proc. Jpn Acad. B*, **75**, 269–274.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Nicholls, A., Sharp, K. A. & Honig, B. (1991). *Proteins*, **11**, 281–296.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Papanikolau, Y. & Kokkinidis, M. (1997). *Protein Eng.* **10**, 847–850.

Papanikolau, Y. & Petratos, K. (2002). *Acta Cryst.* D**58**, 1593–1596.

Papanikolau, Y., Prag, G., Tavlas, G., Vorgias, C. E., Oppenheim, A. B. & Petratos, K. (2001). *Biochemistry*, **40**, 11338–11343.

Perrakis, A., Ouzounis, C. & Wilson, K. S. (1997) *Folding Des.* **2**, 291–294.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. & Vorgias, C. E. (1994). *Structure*, **2**, 1169–1180.

Rao, V. H., Guan, C. & van Roey, P. (1995). *Structure*, **3**, 449–457.

Roberts, R. L. & Cabib, E. (1982). *Anal. Biochem.* **127**, 402–412.

Roey, P. van, Rao, V., Plummer, T. H. Jr & Tarentino, A. L. (1994). *Biochemistry*, **33**, 13989–13996.

Sakuda, S., Isogai, A., Matsumoto, S. & Suzuki, A. (1987). *J. Antibiot. (Tokyo)*, **40**, 296–300.

Spindler, K.-D. & Spindler-Barth, M. (1999). *Chitin and Chitinases*, edited by P. Jollès & R. A. A. Muzzarelli, pp. 201–209. Basel, Switzerland: Birkhäuser Verlag.

Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B. & Dijkstra, B. W. (1995). *Biochemistry*, **34**, 15619–15623.

Terwisscha van Scheltinga, A. C., Kalk, K. H., Beintema, J. J. & Dijkstra, B. W. (1994). *Structure*, **2**, 1181–1189.

Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J. & Watanabe, T. (2001). *J. Biol. Chem.* **276**, 41343–41349.

Vorgias, C. E., Kingswell, A. J. & Dauter, Z. (1992). *J. Mol. Biol.* **226**, 897–898.