Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

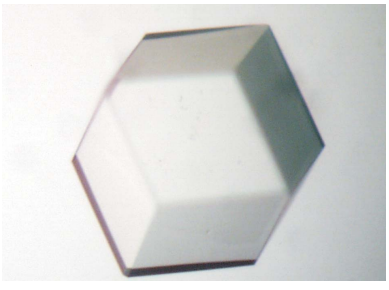
Crystallization and preliminary X-ray diffraction studies of trypsin-like proteases from the gastric fluid of the marine crab *Cancer pagurus*

Jan-Hendrik Hehemann,[a] Lars Redecke,[b] Markus Perbandt,[a] Reinhard Saborowski[c] and Christian Betzel[a]*

[a]Institute of Biochemistry and Molecular Biology, University of Hamburg, 20146 Hamburg, Germany, [b]Center of Experimental Medicine, Institute of Biochemistry and Molecular Biology I, University Hospital Hamburg-Eppendorf, c/o DESY, 22603 Hamburg, Germany, and [c]Biologische Anstalt Helgoland, Alfred Wegener Institute for Polar and Marine Research, 27483 Helgoland, Germany

Correspondence e-mail: betzel@unisgi1.desy.de

Received 6 December 2006
Accepted 20 February 2007



The digestive fluid of the marine crab *Cancer pagurus* (Decapoda, Brachyura) contains highly stable proteases which display enhanced activity in aqueous mixtures of organic solvents. Three trypsins were isolated from the gastric fluid and two of them, *C.p.*TryII and *C.p.*TryIII, were purified to homogeneity by anion-exchange chromatography and crystallized by hanging-drop vapour diffusion. Diffraction data were collected at a synchrotron to 0.97 and 3.2 Å resolution, respectively. The crystal of *C.p.*TryII belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 52.06$, $b = 62.00$, $c = 71.66$ Å. Based on the Matthews coefficient, one protein molecule per asymmetric unit is suggested. In contrast, crystals of *C.p.*TryIII, which belong to the cubic space group $P2_13$ with unit-cell parameters $a = b = c = 215.4$ Å, are assumed to contain 12 molecules per asymmetric unit.

1. Introduction

Proteolytic enzymes catalyze a variety of important metabolic reactions and facilitate the digestion of alimentary proteins. In industry, proteases have gained attention as valuable biocatalysts in many biotechnological processes (Ogrydziak, 1993; Rao *et al.*, 1998; Gupta *et al.*, 2002). Since the demand for specific enzymes is continuously increasing, many bacteria, fungi or plant species have been systematically screened in search of enzymes with unique properties.

Crustaceans occupy a large variety of ecological niches and cope with varying environmental conditions. In terms of food utilization, crustaceans show a large diversity of highly efficient digestive enzymes (Ceccaldi, 1988; Brunet *et al.*, 1994; Jones *et al.*, 1997; Johnston & Yellowlees, 1998; Le Vay *et al.*, 2001). Even at low temperatures, the gastric enzymes from the Antarctic krill (*Euphausia superba*), for instance, cause rapid postmortem autolysis (Ellingsen & Mohr, 1987). Owing to their high catalytic activity, proteases from marine crustaceans have already been successfully applied in medicine and dermatology, *e.g.* in wound debridement (Campbell *et al.*, 1987; Sakharov & Prieto, 2000) and in the removal of dental plaque (Hahn Berg *et al.*, 2001). Several proteases in the digestive fluid of marine crustaceans show trypsin-like characteristics.

Trypsins belong to the S1 family of serine endopeptidases, hydrolyzing the C-terminal peptide bonds of arginine and lysine residues. They are among the most extensively studied enzymes (Walsh, 1970), with significant potential for practical application in animal nutrition, the food industry, medical skin care (Gudmundsdóttir & Pálsdóttir, 2005) and protein-sequence analysis (Shevchenko *et al.*, 1996). The structure of numerous trypsins and trypsinogens from vertebrates have been analysed in detail by X-ray crystallography (*e.g.* Sweet *et al.*, 1974; Kossiakoff *et al.*, 1977). Although the amino-acid sequence of trypsins can vary significantly, the tertiary structure consisting of two β-barrel domains which harbour the catalytic triad is highly conserved.

In addition to high activity, the trypsin-like enzymes from the gastric fluid of the edible marine crab *Cancer pagurus* display some unique properties such as long-term stability and enhanced activity in organic solvents (Saborowski *et al.*, 2004). *C. pagurus* inhabits wide areas of the northeast Atlantic. Owing to their weight, which can reach 1 kg, these crabs are highly suitable for harvesting large amounts of biotechnologically valuable enzymes. Comparative

structural analysis of vertebrate and crustacean trypsins would be useful in order to elucidate the molecular mechanisms of enhanced activity and stability. Only one crystal structure of a trypsin from a crustacean species, that from the freshwater crayfish *Pontastacus leptodactylus*, has been published to date (Fodor *et al.*, 2005). Here, we describe for the first time the purification, crystallization and preliminary crystallographic analysis of two trypsin-like proteases from the marine crustacean species *C. pagurus*.

2. Materials and methods

2.1. Origin of samples and enzyme purification

Adult crabs of 400–800 g weight were captured around the island of Helgoland, North Sea (lat 54°11′N, long 7°53′E). After starvation for 2 d in aquaria (288 K), the digestive fluid was drawn from the stomach of individuals through the oesophagus using a syringe and a flexible Teflon tube. The fluid was immediately transferred into reaction cups and centrifuged at 15 000*g* for 10 min. Using a NAP-10 Sephadex G25 column (Amersham Biosciences, NJ, USA), the supernatant was desalted and buffered in 10 m*M* imidazole pH 6.9 (buffer *A*). Subsequently, 500 µl of the processed extracts was loaded onto a UNO Q6 anion-exchange column (6 ml, BioRad, Hercules, USA) which was pre-equilibrated with buffer *A*. After washing the column, a linearly increasing salt gradient from 0 to 1 *M* NaCl was applied for protein elution. Fractions with trypsin activity (substrate L-BAPNA; Erlanger *et al.*, 1961) eluted at salt concentrations of 0.5, 0.6 and 0.7 *M* and were denoted *C.p.*TryI, *C.p.*TryII and *C.p.*TryIII, respectively. Following dialysis against buffer *A*, the monodispersity

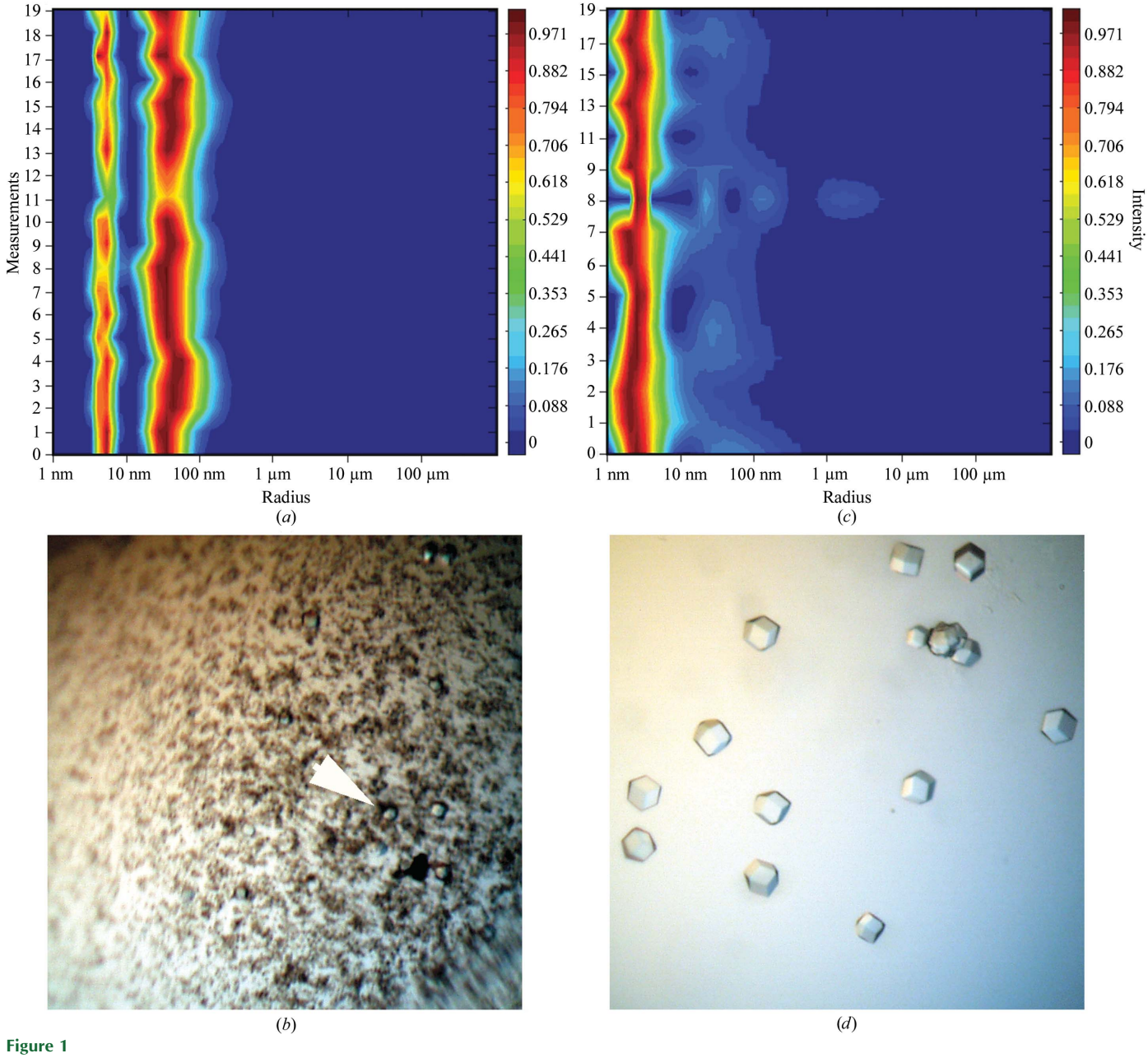


Figure 1
Dynamic light-scattering (DLS) analysis and initial crystallization of *C.p.*TryIII at various purification stages. After the first chromatographic purification step using a UNO Q6-R column, a bimodal distribution of *C.p.*TryIII was observed by DLS analysis (*a*), resulting in the growth of small crystals (*b*, white arrow) accompanied by large amounts of precipitate. A second purification step using Mono Q anion-exchange chromatography resulted in a unimodal distribution of *C.p.*TryIII (*c*) and enhanced the quality of the crystals significantly (*d*).

Table 1
Crystallographic data and data-collection statistics of *C.p.*TryII and *C.p.*TryIII.

Values in parentheses are for the highest resolution shell.

	*C.p.*TryII	*C.p.*TryIII
X-ray source	X13, HASYLAB/DESY	X13, HASYLAB/DESY
Wavelength (Å)	0.808	0.802
Space group	$P2_12_12_1$	$P2_13$
Unit-cell parameters (Å)	a = 52.06, b = 62.00, c = 71.66	$a = b = c$ = 215.4
Resolution range (Å)	20–0.97 (0.99–0.97)	20–3.17 (3.23–3.17)
Mosaicity (°)	0.4	0.3
Total reflections	2207025	1254696
Unique reflections	137462 (6584)	66751 (3270)
$R_{\rm merge}$† (%)	5 (34)	10 (36)
Completeness (%)	99.7 (96.7)	99.5 (100)
Mean $I/\sigma(I)$	31.1 (2.8)	15.0 (5.4)
Redundancy	7.3 (4.1)	5.1 (5.1)

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $I_{h,i}$ is the intensity of the ith measurement of reflection h.

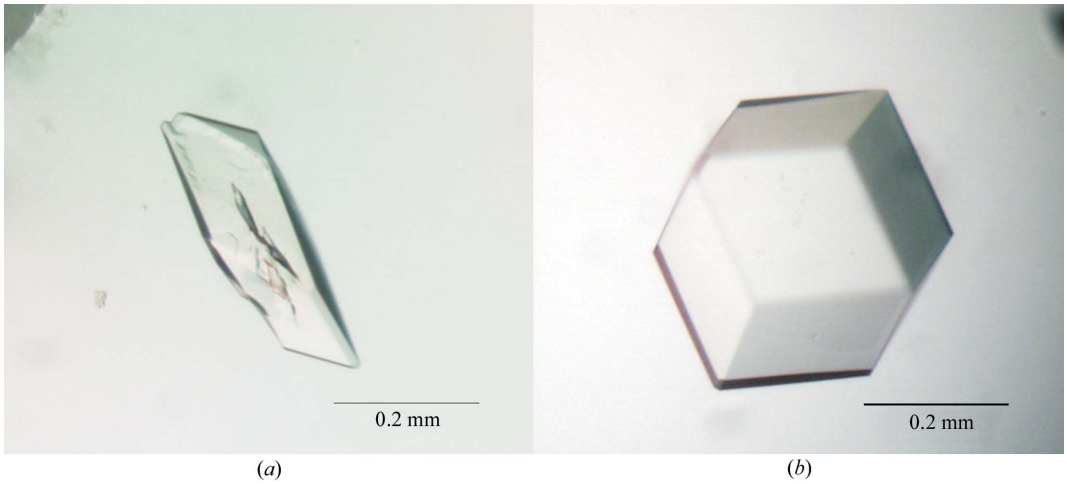
and purity of the isolated trypsins was analyzed by dynamic light-scattering (DLS) measurements in a Spectroscatterer 201 (RiNA GmbH, Berlin, Germany) and by SDS–PAGE analysis followed by Coomassie Brilliant Blue staining, respectively. At this purification stage, only *C.p.*TryII exhibited monodisperse behaviour and sufficient purity (>98%). Therefore, *C.p.*TryII was dialysed against 10 m*M* Tris–HCl pH 7.5, concentrated to 10 mg ml^{-1} (Vivaspin 15 R concentrator, 10 000 MWCO, Sartorius, Germany) and used for crystallization experiments. The *C.p.*TryI sample and a sizeable fraction of the *C.p.*TryIII sample were separately applied onto a Mono Q anion-exchange column (Amersham Biosciences, NJ, USA) for final purification using the buffer conditions described above. The pooled fractions of the eluted trypsin peaks were again analyzed by DLS and SDS–PAGE. Since *C.p.*TryI still showed insufficient purity, it was not used for crystallization experiments. In contrast, monodisperse and pure (>98%) *C.p.*TryIII was dialysed against 10 m*M* Tris–HCl pH 7.5, concentrated to 4.5 mg ml^{-1} and used for crystallization.

2.2. Dynamic light-scattering (DLS) analysis and crystallization

Crystallization experiments were started with *C.p.*TryIII since it showed the highest purity and yield. Initial crystallization screening parameters were deduced from conditions documented in the Protein Data Bank (PDB; Berman *et al.*, 2000; http://www.rcsb.org) and the Biological Macromolecule Crystallization Database (BMCD; Gilliland *et al.*, 1994; http://www.xpdb.nist.gov:8060/BMCD4/) for successful crystallization of trypsins from various species. These conditions consistently pointed to ammonium sulfate as the most promising precipitant. Therefore, the ammonium sulfate concentration (0.8–3.2 *M*) and the pH (4–9) of the reservoir solution were initially screened using the hanging-drop vapour-diffusion method at 291 K.

For *C.p.*TryIII, the homogeneity of the purified trypsin as analyzed by DLS measurements prior to crystallization proved to be crucial for optimal crystal growth. Parallel trials were performed with two isolates of *C.p.*TryIII (4.5 mg ml^{-1}), one purified by one-step liquid chromatography (UNO Q6) and the other by two-step liquid chromatography (UNO Q6 and Mono Q). Crystals of *C.p.*TryIII appeared within 5 d in droplets containing a reservoir solution consisting of 1.6 *M* ammonium sulfate and 1 *M* MES buffer pH 6. The bimodal size distribution (Fig. 1*a*) of *C.p.*TryIII purified by only a single chromatographic step (UNO Q6) was associated with the formation of large amounts of precipitate and several small crystals (Fig. 1*b*). In contrast, the monodisperse protein solution with an expected hydrodynamic radius of 2.4 nm obtained from *C.p.*TryIII samples subjected to both purification steps (Fig. 1*c*) yielded larger well ordered hexagonal crystals without any precipitate (Fig. 1*d*). Consequently, the latter *C.p.*TryIII fraction was used for optimization attempts. Well diffracting crystals were obtained from drops (4 µl) containing equal volumes of protein and reservoir (1.4 *M* ammonium sulfate, 0.1 *M* MES buffer pH 6.0) solutions equilibrated over a reservoir of 500 µl and grew to final dimensions of 0.3 × 0.3 × 0.2 mm (Fig. 2*b*).

For the crystallization of *C.p.*TryII, only monodisperse fractions that were purified by one-step liquid chromatography (UNO Q6) were used. Initial crystallization trials applying the hanging-drop vapour-diffusion method at 291 K and using ammonium sulfate as precipitant did not yield crystals. Therefore, a robot-assisted screening was performed using Crystal Screens I, II and Cryo and Index Screen (Hampton Research) as sitting-drop vapour-diffusion experiments in 288-well trays. Within two months, one single orthorhombic crystal was grown in a drop (300 nl) containing equal volumes of protein (10 mg ml^{-1}) and precipitant [70% 2-methyl-2,4-pentanediol (MPD), 0.1 *M* MES buffer pH 6.5] solutions equilibrated against 45 µl precipitant. The maximum dimensions of this crystal



(*a*) (*b*)

Figure 2
(*a*) The crystal of *C.p.*TryII grown by the sitting-drop vapour-diffusion method using 70% MPD as precipitant. The dimensions of this crystal have been determined as 0.4 × 0.15 × 0.1 mm. (*b*) Representative crystal of *C.p.*TryIII grown by the hanging-drop vapour-diffusion method using 1.4 *M* ammonium sulfate as precipitant. The dimensions of this crystal were 0.3 × 0.3 × 0.2 mm.

were 0.4 × 0.15 × 0.1 mm (Fig. 2*a*). Since the crystal diffracted well, no further optimization attempts were made.

2.3. Data collection and processing

Diffraction data were collected using synchrotron radiation on beamline X13 at HASYLAB/DESY (Hamburg, Germany) with a MAR Research CCD detector. Single native crystals were mounted on nylon loops and flash-cooled in a nitrogen-gas stream at 100 K. Prior to flash-cooling, the crystal of *C.p.*TryIII was briefly soaked in reservoir solution supplemented with 20%(*v*/*v*) glycerol as a cryoprotectant. In contrast, the crystal of *C.p.*TryII was grown in 70% MPD as precipitant, which acts as a cryoprotectant. Data were indexed, integrated and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 package (Otwinowski & Minor, 1997). Table 1 summarizes the data-collection and processing statistics.

3. Results and discussion

Following purification by anion-exchange chromatography, two trypsins from the gastric fluid of *C. pagurus* were successfully crystallized. Crystals of *C.p.*TryII grew in 70%(*v*/*v*) MPD and 0.1 *M* MES buffer pH 6.5 and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 52.06$, $b = 62.00$, $c = 71.66$ Å. A complete X-ray diffraction data set was collected to 0.97 Å resolution. Based on the estimated molecular weight (26 kDa) and the volume of the asymmetric unit, the presence of one *C.p.*TryII molecule in the asymmetric unit was suggested, corresponding to a Matthews coefficient V_M of 2.2 Å^3 Da^{-1} and a solvent content of 43.2% (Matthews, 1968). For *C.p.*TryIII, crystals grown in 1.4 *M* ammonium sulfate and 0.1 *M* MES buffer pH 6.0 were indexed in the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 215.4$ Å. For the *C.p.*TryIII crystals, DLS measurements clearly showed that sample homogeneity was a key factor for success in crystallization trials. Because these crystals were very fragile and radiation-sensitive, several attempts were required before suitable flash-cooling conditions were found. A complete data set was collected to 3.2 Å resolution. A molecular weight of 26 kDa suggests the presence of approximately 12 *C.p.*TryIII molecules in the asymmetric unit. This corresponds to a Matthews coefficient V_M of 2.6 Å^3 Da^{-1} and a solvent content of 53.0%.

Currently, work is in progress to analyse the amino-acid sequences of the trypsins isolated from the gastric fluid of *C. pagurus* in detail. Moreover, attempts to determine the molecular structures of *C.p.*TryII and *C.p.*TryIII by the molecular-replacement (MR) method are planned using the structure of a trypsin from the crayfish *P. leptodactylus* as a starting model (PDB code 2f91; Fodor *et al.*, 2005). Based on similarity searches including the available structures of trypsins from different species, a typical trypsin-like fold containing two β-barrel domains is expected for the structures of both *C. pagurus* trypsins. Thus, it is specific sequence modifications and smaller structure adjustments that must explain the extraordinary properties of crab trypsins (Saborowski *et al.*, 2004). In terms of *C.p.*TryIII, the expected 12 molecules per asymmetric unit will complicate the determination of the molecular structure. In order to avoid this problem, we are continuing to screen crystallization conditions, looking for crystals in other cells and other space groups that give higher resolution data.

References

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
Brunet, M., Arnaud, J. & Mazza, J. (1994). *Oceanogr. Mar. Biol. Annu. Rev.* **32**, 335–367.
Campbell, D., Hellgren, L., Karlstam, B. & Vincent, J. (1987). *Experientia*, **43**, 578–579.
Ceccaldi, H. J. (1988). *Rev. Fish. Sci.* **6**, 13–39.
Ellingsen, T. E. & Mohr, V. (1987). *Biochem. J.* **246**, 295–305.
Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961). *Arch. Biochem. Biophys.* **95**, 271–278.
Fodor, K., Harmat, V., Hetényi, C., Kardos, J., Antal, J., Perczel, A., Patthy, A., Katona, G. & Gráf, L. (2005). *J. Mol. Biol.* **350**, 156–169.
Gilliland, G. L., Tung, M., Blakeslee, D. M. & Ladner, J. E. (1994). *Acta Cryst.* D**50**, 408–413.
Gudmundsdóttir, A. & Pálsdóttir, H. M. (2005). *Mar. Biotechnol.* **7**, 77–88.
Gupta, R., Beg, Q. K. & Lorenz, P. (2002). *Appl. Microbiol. Biotechnol.* **59**, 15–32.
Hahn Berg, I. C., Kalfas, S., Malmsten, M. & Arnebrant, T. (2001). *Eur. J. Oral Sci.* **109**, 316–324.
Johnston, D. J. & Yellowlees, D. (1998). *J. Crust. Biol.* **18**, 656–665.
Jones, D. A., Kumlu, M., Le Vay, L. & Fletcher, D. J. (1997). *Aquaculture*, **155**, 285–295.
Kossiakoff, A. A., Chambers, J. L., Kay, L. M. & Stroud, R. M. (1977). *Biochemistry*, **16**, 654–664.
Le Vay, L., Jones, D. A., Puello-Cruz, A. C., Sangha, R. S. & Ngamphongsai, C. (2001). *Comput. Biochem. Physiol. A*, **128**, 623–630.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Ogrydziak, D. M. (1993). *Crit. Rev. Biotechnol.* **13**, 1–55.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rao, M. B., Tanksale, A. M., Ghatge, M. S. & Deshpande, V. V. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 597–635.
Saborowski, R., Sahling, G., Navarrete del Toro, M. A., Walter, I. & Gracía-Carreño, F. L. (2004). *J. Mol. Catal. B Enzym.* **30**, 109–118.
Sakharov, I. & Prieto, G. A. (2000). *Mar. Biotechnol.* **2**, 259–266.
Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996). *Anal. Chem.* **68**, 850–858.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H. & Blow, D. M. (1974). *Biochemistry*, **13**, 4212–4228.
Walsh, K. A. (1970). *Methods Enzymol.* **19**, 41–63.