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Crystallization and preliminary X-ray analysis of the complex of porcine pancreatic elastase and a hybrid squash inhibitor

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A hybrid inhibitor consisting of the scaffold of a squash-type inhibitor and a specific inhibitory peptide optimized from the third domain of ovomucoid inhibitor from turkey against porcine pancreatic elastase was synthesized by peptide synthesis. The complex formed by this hybrid inhibitor and the porcine pancreatic elastase was crystallized using the hanging-drop method with citrate in the crystallization solution. The space group was determined to be $P2_12_12_1$, with unit-cell parameters a = 56.33, b = 56.44, c = 72.76 Å. A complete X-ray diffraction data set was collected under cryogenic conditions to 1.8 Å.

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1. Introduction

The design of potent inhibitors is of great importance for many human diseases such as coagulopathy, emphysema, several inflammatory conditions, cancer infiltration, some forms of arthritis and many bacterial, viral and parasitic infections (Helm *et al.*, 2000). In addition, the protease–inhibitor interaction is an interesting example and useful model system for studying the specificity of protein–protein interactions and molecular recognition.

As described previously, we have optimized the inhibitory properties of a peptide consisting of seven amino acids against porcine pancreatic elastase (PPE; Hilpert *et al.*, 2000). This peptide stems from the third domain of the turkey ovomucoid inhibitor (OMTKY3), which inhibits a broad spectrum of proteases (for a review, see Saxena & Tayyab, 1997). The optimized inhibitory peptide is highly specific for PPE. For increased stability of this peptide against proteolytic attack from PPE, the peptide sequence was built into the structure of a squash-type inhibitor (Otlewski & Krowarsch, 1996). For these experiments, the squash inhibitor from *Ecballium elaterium* (EETI II) was chosen. EETI II is a strong trypsin inhibitor (bovine pancreatic), with a K_d of $1.3 \times 10^{-12}\,M$ (Favel *et al.*, 1989). The optimized inhibitory peptide was introduced into the binding region of the squash inhibitor EETI II, while position P1 (for nomenclature, see Schechter & Berger, 1967) in the peptide and in the squash-type inhibitor was conserved. The hybrid squash inhibitor was named HEI-TOE I: H for hybrid, E for elastase, I for inhibitor, T for turkey, O for ovomucoid and E for *E. elaterium*. The hybrid inhibitor was synthesized and the inhibition of trypsin and elastase activity by HEI-TOE I was measured. The introduction of the inhibitory peptide into the squash-type inhibitor led to a major change in its specificity. It became a highly specific PPE inhibitor. The K_d of HEI-TOE I for porcine pancreatic elastase was determined to $9.8 \times 10^{-8}\,M$ (unpublished results). In fact, the specificity of HEI-TOE I is very similar to that of the inhibitory peptide (Hilpert *et al.*, 2000). Here, we describe the crystallization and data collection of the complex of the porcine pancreatic elastase and the hybrid squash inhibitor HEI-TOE I.

2. Experimental

2.1. Peptide sequence and synthesis

The peptide HEI-TOE I comprises 28 amino acids (MW = 2971 Da) with the sequence PC-TLEYMRCKQDSDCLAGCVCGPNGFCG.

The peptides were prepared in the laboratory of J. Schneider-Mergener according to standard Fmoc machine protocols using a multiple peptide synthesizer (Abimed, Langenfeld, Germany) and were analyzed by reverse-phase high-pressure liquid chromatography and MALDI-TOF mass spectrometry. During the peptide synthesis the SH group is protected in such a way that no measurable amount of oxidation occurs.

Following the synthesis of the hybrid squash inhibitor peptide, the peptide was purified by reverse-phase high-pressure liquid chromatography. The six cysteine residues were oxidized with charcoal as described previously (Volkmer-Engert *et al.*, 1998). The degree of cyclization was characterized by means of a determination of the amount of free cysteine with 5,5′-dithiobis(2-nitrobenzoic acid). No free SH groups were detectable after the oxidation step. The same procedure as described above was applied as an additional purification step.

The purity of the final product was greater than 98%. The amount of pure peptide per resin column used was about 1 mg.

2.2. Crystallization

Porcine pancreatic elastase (EC 3.4.21.36; MW = 25.9 kDa) was purchased from Serva (Heidelberg) and was used without any further purification. Crystallization was achieved by using the hanging-drop method with 24- well tissue-culture plates (Hampton Research). Crystals were grown under three slightly different conditions at room temperature as described in §3. 500 µl of 0.02 *M* citrate buffer pH 6.0 was always used as reservoir solution. The crystals grew within 3 d to dimensions of 0.1 × 0.05 × 0.6 mm. The crystals were soaked in paraffin oil as a cryoprotectant and flash-frozen with liquid nitrogen.

2.3. Data collection and structure determination

Diffraction data (Table 1) were collected to 1.8 Å resolution at synchrotron beamline X11, EMBL outstation, Hamburg (Germany) at 100 K on a MarCCD detector system. The synchrotron X-ray wavelength was 0.907 Å. The X-ray data were processed and merged with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Unit-cell parameters and space group were determined by the autoindexing routine in *DENZO*.

3. Results and discussion

As a first step, we started by optimizing crystallization conditions for porcine pancreatic elastase. The idea was to probe these conditions for the crystallization of the complex with the inhibitor and the elastase, as the amount of available inhibitor was very small. Crystals grown in a citrate buffer showed X-ray diffraction to high resolution. The growth of the crystals strongly depends on the citrate and protein concentrations. Crystals can be obtained at citrate buffer concentrations of between 0.4 and 17 m*M* in the hanging drop. An citrate buffer with twice the concentration of that in the hanging drop was used in the reservoir. The concentration of PPE must be above 0.4 m*M*. Under these conditions, crystals grow at pH values of 4–7.5 and at temperatures between 277 and 298 K. Interestingly, the first visible crystals grow within 10 min. For the co-crystallization experiments, it may be disadvantageous to the quality of the crystals if they grow too fast. Therefore, various compounds [NaCl (2 *M*), ethanol (96%), DMSO (99.6%), sodium sulfate (1 *M*), ammonium acetate (0.6 *M*) and PEG 4000 (30%)] were tested to increase the crystallization time of porcine pancreatic elastase. 0.2, 0.5 and 1 µl of the compounds were added to a 4.4 µl droplet containing 0.7 m*M* PPE and 1 m*M* citrate pH 6.0. PEG 4000 and ethanol had no influence on the crystallization rate. Addition of 0.2 µl DMSO or 0.2 and 0.5 µl sodium sulfate had a small dilatory effect on the crystallization speed. NaCl, 0.5 and 1 µl DMSO and 1 µl sodium sulfate did not lead to any crystals at all. The addition of 0.5 and 1 µl ammonium acetate slows down the crystallization process to a period of up to 2 d. With these optimized conditions the experiments for the co-crystallization were started.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 56.33, b = 56.44, c = 72.76
Contents of asymmetric unit	1 elastase–inhibitor complex
Resolution limits (Å)	14.0–1.8 (1.86–1.80)
No. of observations	138798
No. of unique reflections	22112 (1941)
Completeness (%)	98.3 (87.8)
$R_{\rm merge}$† (%)	3.7 (11.6)
$I/\sigma(I)$	21.0 (4.5)

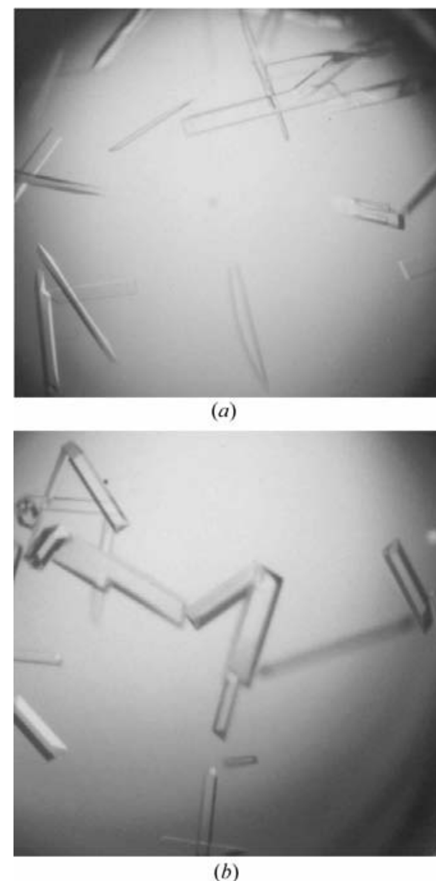
† $R_{\rm merge} = \sum_{hkl}\sum_{j=1}|I_{hkl} - I_{hkl}(j)|/\sum_{hkl} I_{hkl}$, where I_{hkl} is the mean intensity of the reflections *hkl*.

The concentrations of the porcine pancreatic elastase, the hybrid inhibitor HEI-TOE I, citrate buffer pH 6.0 and ammonium acetate were varied and different component ratios were tested. In all variations an excess of the inhibitor was added to the protease. The optimization process shows that crystals were obtained at a PPE:HEI-TOE I ratio of between 1:2.5 and 1:3.3 (the concentration of the stock solutions were 1.4 m*M* for PPE and 2.3 m*M* for HEI-TOE I). In the hanging drop, the citrate buffer pH 6.0 concentration was varied between 2 and 5.3 m*M* and ammonium acetate concentration between 100 and 70 m*M*. In all cases, the reservoir contained only 20 m*M* citrate buffer pH 6.0. The pH value of one crystallization condition was determined to be 6.2 and is similar to the pH of the citrate buffer used. Interestingly, in some conditions resulting in crystals of the complex the concentration of PPE (0.3 m*M*) is lower than the concentration needed for crystallization of free PPE (0.4 m*M*).

The resulting crystals, which grew within 2 d, look similar to those stemming from the native PPE (Fig. 1). Both types of crystals are shaped similarly, but on average the crystals of the complex are significantly smaller in volume. Crystals used for X-ray measurements were soaked with paraffin oil and flash-frozen.

Diffraction data (Table 1) were collected at 100 K to 1.8 Å resolution at the synchrotron beamline. Unit-cell parameters and space group are given in Table 1. The space group of the crystals is the same as that of crystals of free elastase grown in sodium sulfate (Wuertele *et al.*, 2000). The unit-cell parameters of the complex (a = 56.33, b = 56.44, c = 72.76 Å) are also similar to those of the free elastase (a = 49.91, b = 57.82, c = 74.27 Å) (Wuertele *et al.*, 2000). The volume of the unit cell of the complex is 7.2% larger than that of the elastase.

There is one elastase–inhibitor complex in the crystal asymmetric unit with a $V_{\rm M}$ value of 1.98 Å^3 Da^{-1}, corresponding to a solvent content of 37.8%. A molecular-replacement solution with free porcine pancreatic elas-



(*a*)

(*b*)

Figure 1
Pictures of crystals of (*a*) the complex between HEI-TOE I and porcine pancreatic elastase and (*b*) elastase only. Crystals of the elastase were established by mixing 2 µl 1.4 m*M* PPE in pure water with 2 µl 20 m*M* citrate buffer. The approximate dimensions of the crystals of the complex are 0.1 × 0.05 × 0.3 mm and of the free elastase are 0.2 × 0.1 × 0.3 mm.

tase (PDB code 1qnj) as a search model was obtained using the program *AMoRe* (Navaza, 1994). The solution confirmed that there is one elastase–inhibitor complex in the crystal asymmetric unit. After orienting and positioning of the elastase molecule by rigid-body refinement and calculating an $(F_o - F_c)$ electron-density map, well defined electron density of the inhibitor peptide was visible. The model building and refinement of this structure is in progress.

References

Favel, A., Mattras, H., Coletti-Previero, M. A., Zwilling, R., Robinson, E. A. & Castro, B. (1989) *Int. J. Pept. Protein Res.* **33**, 202–208.

Helm, K. von der, Korant, B. D. & Cheronis, J. C. (2000). Editors. *Handbook of Experimental Pharmacology*, Vol. 140, *Proteases as Targets for Therapy*. Berlin/Heidelberg/New York: Springer-Verlag.

Hilpert, K., Hansen, G., Wessner, H., Schneider-Mergener, J. & Hoehne, W. (2000). *J. Biochem. (Tokyo)*, **128**, 1051–1057.

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

Otlewski, J. & Krowarsch, D. (1996). *Acta. Biochim. Pol.* **43**, 431–444.

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

Saxena, I. & Tayyab, S. (1997). *Cell. Mol. Life Sci.* **53**, 13–23.

Schechter, I. & Berger, A. (1967). *Biochem. Biophys. Res. Commun.* **27**, 157–162.

Volkmer-Engert, R., Landgraf, C. & Schneider-Mergener, J. (1998). *J. Pept. Res.* **51**, 365–369.

Wuertele, M., Hahn, M., Hilpert, K. & Hoehne, W. (2000). *Acta Cryst.* D**56**, 520–523.