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Short communication

Crystallization and preliminary crystallographic studies of a new crystal form of papain from *Carica papaya**

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A new crystal form of papain from the latex of *Carica papaya*, complexed with an inhibitor (Z-Arg-Leu-Val-Gly-CHN_2) was obtained by the vapor-diffusion method using a methanol/ethanol mixture as a precipitant. The slat-like crystals are monoclinic, space group $P2_1$, with unit cell parameters a = 42.6 Å, b = 49.8 Å, c = 50.5 Å, β = 111.9°, and contain one molecule in the asymmetric unit. The crystals are stable in the X-ray beam and diffract beyond 1.8 Å. A molecular model has been placed in the unit cell by molecular replacement.

Papain from the latex of *Carica papaya* is the best known representative of a superfamily of cysteine proteases, also known as the papain superfamily. Cysteine proteases are a major component of lysosomal proteolytic systems and play a significant role in intracellular proteolysis. Abnormalities in the activity of cysteine proteases lead to a variety of serious diseases, such as muscular dystrophy [1], osteoporosis [2], pulmonary emphysema [3] and tumors [4]. Papain is homologous to other plant cysteine proteases whose three-dimensional structures are known: actinidin [5], caricain (papaya protease omega) [6], glycyl endopeptidase (papaya protease IV) [7], and to mammalian cathepsin B [8]. The single polypeptide chain of the papain molecule is 212 amino acids long and is cross-linked by three disulfide bonds. The active site contains a highly reactive thiol group, residing on Cys-25. Papain is a good model for the development of new inhibitors that can modify the proteolytic activity of cysteine proteases and is, therefore, useful in drug design. The crystal structures of papain complexed with many different inhibitors (leupeptin [9], chloromethyl ketone analogue [10], E64-c and E64 [11–13], succinyl-Gly-

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Abbreviations: BMCD, Biological Macromolecule Crystallization Database; DAM, diazomethane; MPD, 2-methyl-2,4-pentanedial; PDB, Protein Data Bank.

Val-Val-Ala-Ala-*p*-nitroanilide [14], 2-mercaptoethanol [15]) have been described in the literature. We have crystallized a complex between papain and a new irreversible synthetic inhibitor: Z-Arg-Leu-Val-Gly-diazomethane (DAM). We have prepared the inhibitor according to a procedure described earlier [16]. The inhibitor molecule (Fig. 1) contains a peptidic fragment, Arg-Leu-Val-Gly, modelled after the N-terminal binding sequence of human cystatin C (which is a natural protein inhibitor of cysteine proteases) and a DAM group which reacts chemically with the active thiol group of the enzyme.

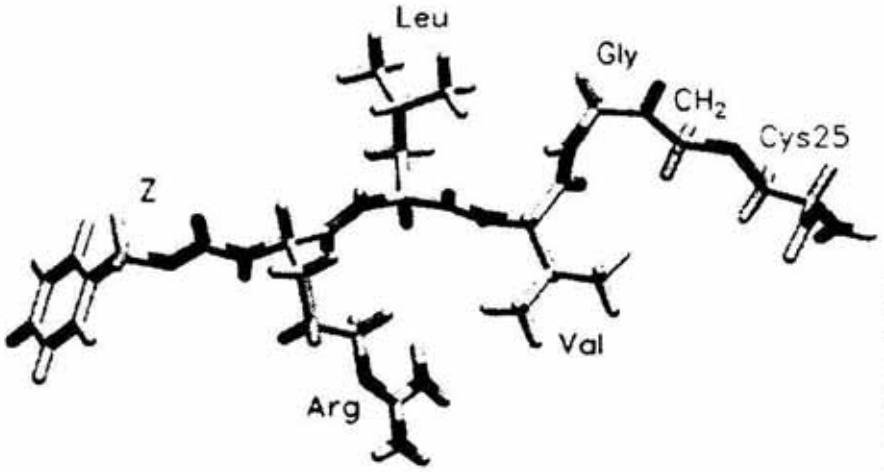

Figure 1. Chemical structure of the inhibitor (Z-Arg-Leu-Val-Gly-CH_2-) in a covalent complex with the enzyme.

The chemical bond is formed between Cys-25 Sγ and the reacted diazomethane (DAM) group.

PREPARATION OF THE PAPAIN-INHIBITOR COMPLEX

Papain (purchased from Sigma) was purified by column chromatography using CNBr-activated Sepharose 4B to which the peptide Gly-Gly-Tyr(Bzl)-Arg was attached [17]. Papain was dissolved in an activating solution (0.5 mM sodium acetate, 30 mM 2-mercaptoethanol, 25 mM EDTA, pH 8.0) and then run through the column, which was pre-equilibrated with buffer 1 (20 mM EDTA, 10 mM 2-mercaptoethanol, pH 4.3). Activated papain was washed from the column by the following procedure: buffer 1 (removal of inactive enzyme), buffer 2 (5 mM EDTA, pH 6.0), and water. Papain activity was assayed by acidimetric titration of the products of enzymatic hydrolysis of benzoylarginine ethyl ester [17]. The activity of purified papain was estimated as 30 U/mg of the enzyme. Activated papain was treated with a solution of the inhibitor (Z-Arg-Leu-Val-Gly-CHN_2) until no enzymatic activity could be detected.

CRYSTALLIZATION AND DATA COLLECTION

Single crystals of the covalent papain-inhibitor complex were grown by the vapor diffusion method at 19°C in 4–10 μl hanging drops, using a modification of the procedure described for the complex papain-E64-c [11, 12]. The ingredients of the solution were as in [11, 12] but the concentrations were changed as follows. The complex was dissolved in 10 mM 2-aminoethanol/HCl buffer, pH 9.2, and the protein solution mixed with an equal volume of reservoir solution containing 50–68% methanol/ethanol (2:1, v/v) and 38 mM NaCl in 50 mM 2-aminoethanol/HCl buffer (pH 9.2). The final protein concentration was 1.5%. Crystals appeared after 3–4 days and measured up to 0.1 mm × 0.2 mm × 0.4 mm (Fig. 2). They had slat-like habit and reached maximum dimensions of about 0.2 mm × 0.3 mm × 1.5 mm within a month. The crystals were protected against drying by short (10 min) soaking in 30% MPD (2-methyl-2,4-pentanediol) solution (mother liquor). The crystal for X-ray diffraction experiments (a fragment measuring 0.2 mm × 0.2 mm × 0.4 mm) was sealed in a thin-walled quartz capillary tube with a small volume of mother liquor. X-ray

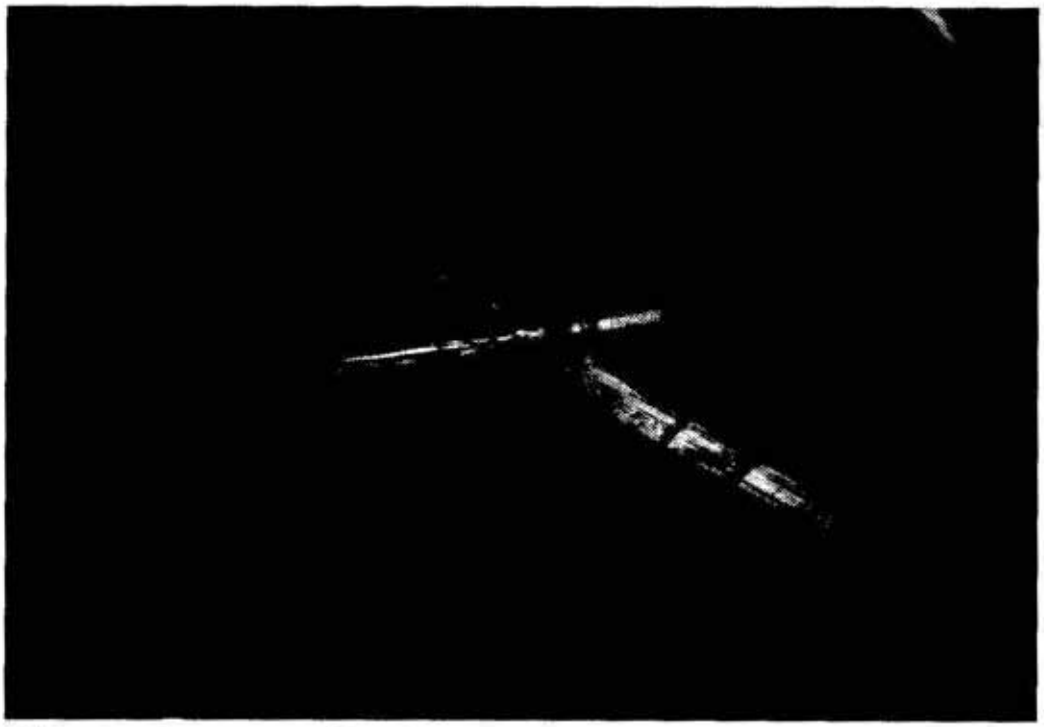

Figure 2. Single crystals of the papain-inhibitor complex.

The slat-like crystals in the center of this photograph are about 0.5 mm long.

diffraction data were collected at room temperature in 1.0° oscillations using a 300-mm imaging plate (MarResearch) and graphite-monochromated CuKα radiation generated from a Siemens SRA2 rotating anode operated at 45 kV and 112 mA.

RESULTS AND DISCUSSION

The crystals are very fragile and prone to drying. Nevertheless, they turned out to be very stable in the X-ray beam allowing collection of a complete data set from a single

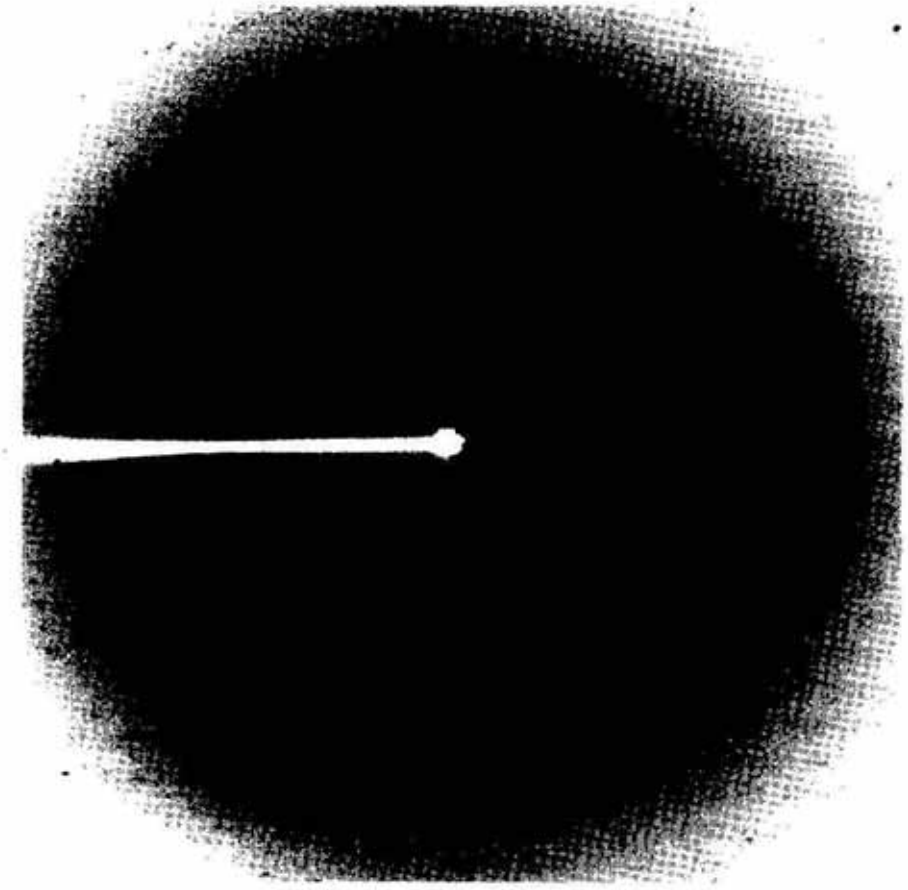

Figure 3. A diffraction pattern of the papain-inhibitor complex recorded at a 1° oscillation.

Table 1. Statistics of X-ray data collection

Resolution limits [Å]	R factor	⟨I⟩/⟨σ⟩	Number of unique reflections	Completeness [%]
20.00–3.83	0.097	12.3	1861	95.5
3.83–3.04	0.110	10.4	1879	97.9
3.04–2.66	0.126	9.3	1829	97.0
2.66–2.41	0.130	8.7	1827	96.3
2.41–2.24	0.140	8.5	1770	93.3
2.24–2.11	0.156	7.3	1741	92.3
2.11–2.00	0.186	5.6	1698	89.8
2.00–1.92	0.225	3.9	1622	85.8
1.92–1.84	0.253	2.9	1484	79.1
1.84–1.78	0.306	2.6	897	48.0
all hkl	0.114	9.7	16608	87.6

specimen. A noticable deterioration of the diffracting power was observed only after 100 h exposure. The data extend to 1.78 Å d-spacing. The images (Fig. 3) were indexed and integrated using the HKL program package [18]. 161875 reflections were merged to give 16608 unique data R_{int} = 0.114. The experimental data represent 87.6% of theoretically possible reflections (48.0% in the last resolution shell, 1.84–1.78 Å). The statistics of the data collection are given in Table 1.

So far, six different crystal forms have been reported for papain and its complexes with low molecular mass inhibitors (BMCD [19] codes: A [20], B,C,D [21], E [22] and S [23]). The present papain-(Z-Arg-Leu-Val-Gly-CH_2-) complex is a new, seventh, polymorphic modification. It belongs to the monoclinic system, space group $P2_1$, and has the following lattice parameters a = 42.6 Å, b = 49.8 Å, c = 50.5 Å and β = 111.9°. Using the 1.6-Å resolution model of papain [24] available in the Protein Data Bank (PDB access code 1PPN) as a molecular replacement probe, a rotation function has been calculated using the AMoRe package [25]. It has only one outstanding solution (correlation coefficient 0.275, next peak 0.078, 15–3.5 Å data) and indicates that there is only one copy of the complex in the asymmetric unit, corresponding to a Matthews volume of 2.2 $Å^3$/Da [26].

Some of the calculations were performed in the Poznań Supercomputing and Networking Center.

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