Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction studies of a Bowman–Birk inhibitor from *Vigna unguiculata* seeds

K. N. Rao,[a] S. S. Hegde,[a]† R. J. Lewis[b] and C. G. Suresh[a]*

[a]Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India, and [b]Department of Chemistry, University of York, York YO10 5DD, England

† Present address: Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA.

Correspondence e-mail: suresh@ems.ncl.res.in

Received 24 May 1999
Accepted 22 July 1999

A Bowman–Birk type trypsin/chymotrypsin inhibitor isolated from *Vigna unguiculata* seeds has been crystallized. Crystals were grown using the vapour-diffusion method at pH 4.0 using citrate/phosphate as a buffer and 30% saturated ammonium sulfate as precipitant. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 32.4$, $b = 61.8$, $c = 32.9$ Å, $\beta = 114.5°$. The Matthews coefficient calculated assuming two molecules in the asymmetric unit was 1.95 Å^3 Da^{-1}, which corresponds to a 37% solvent content. X-ray data were collected to 2.5 Å resolution from a flash-frozen crystal. The structure was solved using the molecular-replacement method using tracy soybean inhibitor structure (PDB entry 1pi2) as a model.

1. Introduction

Protein–protein recognition is vital to many biological processes, such as those in the immune system, signal transduction, receptor/activator interactions and in the regulation of enzyme activity. Protease–protein inhibitor complexes provide excellent models for the study of molecular mechanisms involving protein–protein recognition (Bode & Huber, 1992). Protease inhibitors play an important role in plant defence mechanisms by inhibiting various digestive proteases present in insects and plant pathogens (Hilder *et al.*, 1987). Therefore, some of these inhibitors are of interest as potential bio-pesticides.

The Bowman–Birk-type protease inhibitors are serine-protease inhibitors (Laskowski & Kato, 1980). The first such inhibitor to be isolated was isolated by Bowman from soybean seeds (Bowman, 1946). Its properties were studied by Birk *et al.* (1963). The Bowman–Birk inhibitors (BBIs) differ from functionally related Kunitz-type inhibitors by their relatively small size (molecular weight 7–10 kDa) and by the presence of seven highly conserved disulfide bridges (Ikenaka & Norioka, 1986). BBIs have two tandem homology domains in the single chain, each having a protease-binding site. BBI molecules are thus capable of simultaneously inhibiting two protease molecules (Harry & Steiner, 1970) and are termed 'double-headed' inhibitors. Usually, the reactive site of the first homology domain binds trypsin and that of the second homology domain binds either trypsin or chymotrypsin. The amino acid at the P1 site in the first domain, which inhibits trypsin, is either arginine or lysine, whereas the analogous residue in the second domain, which inhibits the second protease molecule, is either leucine, phenylalanine or tyrosine (Chen *et al.*, 1992).

BBIs are stable at cooking temperatures as well as under the acidic conditions found in the digestive system of higher order animals (Birk, 1987). Recent studies have shown that a high consumption of plant seeds containing BBIs reduces susceptibility towards breast, colon, prostatic, oral and pharyngeal cancers (Birk, 1993; Kennedy, 1994). Because of their anticarcinogenic properties, plant BBIs could have potential medical applications.

Among the reported three-dimensional structures of BBIs are those of PI-II from tracy soybean (Chen *et al.*, 1992), A-II from peanut (Suzuki *et al.*, 1993) and a pea BBI (de la Sierra *et al.*, 1999) in the apo forms. The structure of BBI from soybean meal has been determined using both NMR (Werner & Wemmer, 1992) and X-ray diffraction (Voss *et al.*, 1996). The crystal structures of BBIs from adzuki beans (Tsunogae *et al.*, 1986) and mung beans (Lin *et al.*, 1993) complexed with porcine trypsin are also available. Only crystallization reports and no structures are available for BBIs from horse gram seeds (Prakash *et al.*, 1994) and barley seeds (Song & Suh, 1998) in the apo forms, for soybean BBI in ternary complex with trypsin and chymotrypsin (Gaier *et al.*, 1981) and for barley BBI in complex with porcine pancreatic trypsin (Kim *et al.*, 1999).

There have been reports on the presence of various isoinhibitors of trypsin and chymotrypsin in the seeds of *Vigna anguiculata* (Gatehouse *et al.*, 1980; Vartak *et al.*, 1980). Of these isoinhibitors, the amino-acid sequence of only one BBI has been reported (Morhy & Ventura, 1987). Here, we report the purification and preliminary crystallographic studies of a BBI from *V. unguiculata*.

© 1999 International Union of Crystallography
Printed in Denmark – all rights reserved

Table 1
Summary of the X-ray data collected from a single crystal of *V. unguiculata* BBI in the resolution range 20.0–2.50 Å.

Values in parentheses refer to data in the highest resolution shell, 2.59–2.50 Å.

R_{sym}†	0.083 (0.202)
$I/\sigma(I)$	13.9 (5.8)
Number of measurements	12514
Number of unique reflections	4072
Completeness (%)	99.0 (94.7)

† $R_{sym} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i (hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of the hkl reflection and $\langle I(hkl) \rangle$ is its mean.

Table 2
Details of the molecular-replacement calculations.

(α,β,γ) are Eulerian angles, (T_x, T_y, T_z) are fractional translations and Cc is the correlation coefficient. In the translation-function solution, the Cc and R (R factor) shown for S2-T are for the two solutions together.

Rotation function, resolution 10–3.0 Å.

Solution	α (°)	β (°)	γ (°)	Cc (%)
S1	220.93	97.54	57.34	15.7
S2	160.94	100.85	32.96	15.3

Translation function, resolution 10–2.5 Å.

Solution	T_x	T_y	T_z	Cc (%)	R
S1-T	0.4074	0.0000	0.2589	34.4	55.0
S2-T	0.9669	0.3996	0.5185	46.0	49.9

2. Methods and materials

V. unguiculata seeds were obtained from a local market. These seeds (1 kg) were first crushed in a grinder with 1 l of 50 m*M* sodium acetate buffer pH 5.7, 100 g KCl in 2 l distilled water, 1 l of 0.6 *M* HCl and 180 ml 2.0 *M* potassium bicarbonate. The solution was then filtered and centrifuged. The inhibitor was precipitated by ammonium sulfate fractionation between 30 and 90% saturation. It was further purified by ion-exchange chromatography using DEAE-Sephadex and by gel filtration using Sephadex-G50. The fraction with inhibitor activity was finally applied to an FPLC MonoQ anion-exchange column, where it separated into six isoinhibitors, of which there were two major ones. The purity of each fraction was analyzed by SDS–PAGE. The crystals analyzed here were from one of the two major fractions. The yield of this fraction was 16.0 mg of protein. The molecular weight of the inhibitor was 15.5 kDa, as determined by SDS–PAGE. When considering the previous studies on BBIs from *V. unguiculata* (Gatehouse *et al.*, 1980) and comparing with the known molecular weights of other BBIs, the weight determined here could be associated with a dimer. This was found to be the case by inference from the crystal structure solution. The association between the monomers in the dimer seems to be very strong and in all probability is resistant to denaturation by boiling in the presence of SDS and β-mercaptoethanol.

The sample used in crystallization trials using the hanging-drop vapour-diffusion method had a protein concentration of 50 mg ml^{-1}. The well solution was prepared by mixing 0.1 *M* citrate/phosphate buffer at pH 4.0 with ammonium sulfate to obtain a saturation of 30%. Hanging drops consisted of 2 µl well solution and 2 µl protein solution. The drops were set up at room temperature on siliconized glass cover slips suspended over 1 ml well solution. It took around three to four weeks for the crystals to appear and grow to their final maximum size of 0.2 mm in the longest dimension. Typically, each drop contained several tiny crystals. The crystals for X-ray data collection were transferred directly into a cryoprotectant comprising the well solution supplemented with 30% glycerol. After some 10 s in the solution, a single crystal was mounted in a thin rayon loop and flash-frozen in a stream of boiling liquid nitrogen at a temperature of 120 K. X-ray data were collected from this frozen sample at the University of York, England, using a MAR Research imaging-plate detector mounted on a Rigaku copper rotating-anode X-ray generator equipped with double focusing mirrors.

3. Results and discussion

The frozen crystals diffracted X-rays to beyond 2.5 Å resolution using incident Cu $K\alpha$ radiation. Data was processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The diffraction data were concordant with monoclinic space group $P2_1$ and unit-cell parameters a = 32.4, b = 61.8, c = 32.9 Å, β = 114.5°. The X-ray data statistics are summarized in Table 1. The Matthews coefficient calculated in the $P2_1$ unit cell with two molecules in the asymmetric unit was V_m = 1.95 Å^3 Da^{-1}, corresponding to a crystal solvent content of 37%, which is within the range normally observed in protein crystals (Matthews, 1968).

The molecular-replacement method was used to solve the structure using the monomeric tracy-bean inhibitor structure as a search model. Calculations were carried out using the program *AMoRe* (Navaza, 1994), as implemented in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Details of the solution are presented in Table 2. The reported sequence of *V. unguiculata* BBI (Morhy & Ventura, 1987) was assumed for the present inhibitor. However, the unmatched residues in the model were replaced by alanines. Two molecules were present in the asymmetric unit, corresponding to the two independent solutions obtained by molecular replacement (Table 2). Ten cycles of rigid-body refinement using 17–2.5 Å resolution data improved the values of the correlation coefficient and R factor to 53.5 and 48.4%, respectively. Non-crystallographic averaging was used in the initial stages of the refinement. Repeated cycles of refinement were carried out using the maximum-likelihood method as implemented in the program *REFMAC* (Murshudov *et al.*, 1997), followed by model fitting in the graphics program *QUANTA*. The present values of R and R_{free} are 20.0 and 30.0%, respectively. The two molecules in the asymmetric unit were found close to each other and formed a stable dimer in the crystal. The packing diagram of the molecules in the unit cell of *V. unguiculata* BBI is shown in Fig. 1. Further refinement and the detailed analysis of the structure are in progress.

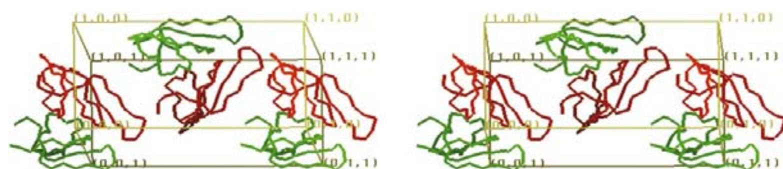

Figure 1
Stereo diagram showing the packing of the inhibitor molecules in the unit cell. The two molecules in the asymmetric unit forming the dimer are shown in different colours. The outline of the unit cell is also shown.

KNR is a senior research fellow of the Council of Scientific and Industrial Research, India. RJL is supported by the Wellcome Trust and the British Council. The use of the computation facility provided by the Department of Biotechnology, New Delhi is acknowledged. The authors thank

Drs K. N. Ganesh and P. K. Ranjekar for their support and encouragement.

References

Birk, Y. (1987). *Hydrolytic Enzymes*, edited by A. Neuroberger & K. Brocklehurst, pp. 257–300. Amsterdam: Elsevier.

Birk, Y. (1993). *Protease Inhibitors as Cancer Chemopreventive Agents*, edited by W. Troll & A. R. Kennedy, pp. 97–106. New York: Plenum.

Birk, Y., Gertler, A. & Khalef, S. (1963). *Biochem. J.* **87**, 281–284.

Bode, W. & Huber, R. (1992). *Eur. J. Biochem.* **204**, 433–451.

Bowman, D. E. (1946). *Proc. Soc. Exp. Biol. Med.* **63**, 547–550.

Chen, P., Rose, J., Love, R., Wei, C. H. & Wang, B.-C. (1992). *J. Biol. Chem.* **267**, 1990–1994.

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

Gaier, J. R., Tulinsky, A. & Liener, I. E. (1981). *J. Biol. Chem.* **256**, 11417–11419.

Gatehouse, A. M. R., Gatehouse, J. A. & Boulter, D. (1980). *Phytochemistry*, **19**, 751–756.

Harry, J. B. & Steiner, R. F. (1970). *Eur. J. Biochem.* **16**, 174–179.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. & Boulter, D. (1987). *Nature (London)*, **330**, 160–163.

Ikenaka, T. & Norioka, S. (1986). *Proteinase Inhibitors*, edited by A. J. Barrett. & G. Salvesen, pp. 361–374. Amsterdam: Elsevier.

Kennedy, A. R. (1994). *Cancer Res.* **54**, 1999S–2005S.

Kim, Y. S., Song, H. K. & Suh, S. W. (1999). *Acta Cryst.* D**55**, 1244–1246.

Laskowski, M. & Kato, I. (1980). *Annu. Rev. Biochem.* **49**, 593–626.

Lin, G., Bode, W., Huber, R., Chi, C. & Engh, R. A. (1993). *Eur. J. Biochem.* **212**, 549–555.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Morhy, L. & Ventura, M. M. (1987). *Ann. Acad. Brasil. Cienc.* **59**, 71–81.

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.

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

Prakash, B., Murthy, M. R. N., Sreerama, Y. N., Sharma, P. R. & Rao, D. R. (1994). *J. Mol. Biol.* **235**, 364–366.

Sierra, I. L. de la, Quillien, L., Flecker, P., Gueguen, J. & Brunie, S. (1999). *J. Mol. Biol.* **285**, 1195–1207.

Song, H. K. & Suh, S. W. (1998). *Acta Cryst.* D**54**, 441–443.

Suzuki, A., Yamane, T., Ashida, T., Norioka, S., Hara, S. & Ikenazka, T. (1993). *J. Mol. Biol.* **234**, 722–734.

Tsunogae, Y., Tanaka, I., Yamane, T., Kikkawa, J., Ashida, T., Ishikawa, C., Watanabe, K., Nakamura, S. & Takahashi, K. (1986). *J. Biochem. (Tokyo)*, **100**, 1637–1646.

Vartak, H. G., Bodhe, A. M., Paranjpe, S. V. & Jagannathan, V. (1980). *Arch. Biochem Biophys.* **204**, 129–133.

Voss, R. H., Ermler, U., Essen, L. O., Wenzl, G., Kim, Y. M. & Flecker, P. (1996). *Eur. J. Biochem.* **242**, 122–131.

Werner, M. H. & Wemmer, D. E. (1992). *Biochemistry*, **31**, 999–1010.