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Crystallization and preliminary crystallographic studies of an α-amylase inhibitor from wheat (*Triticum aestivum*)

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Abstract

Crystals of the human salivary α-amylase inhibitor from wheat have been obtained. A native data set was collected to 2.1 Å resolution with 90% completeness at laboratory sources. The crystals belong to the trigonal system, space group $P3_1$ (or enantiomer) with $a = b = 79.31$, $c = 60.56$ Å. Crystal density analysis and self-rotation function studies suggest the presence of four subunits in the asymmetric unit.

α-Amylases are found in many living organisms and are responsible for the degradation of glucosidic bonds of type α(1–4), present in glycogen and starch. Three-dimensional structures have been reported for α-amylases from *Aspergillus oryzae* (Matsuura, Kusonoki, Harada & Kakudo, 1984; Swift *et al.*, 1991), from porcine pancreas (Buisson, Duée, Haser & Payan, 1987; Qian, Haser & Payan, 1993), from *Aspergillus niger* (Boel *et al.*, 1990; Brady, Brzozowski, Derewenda, Dodson & Dodson, 1991) and from barley (Kadziola, Abe, Svensson & Haser, 1994). They all belong to the $(\beta\alpha)_8$-barrel protein family (Farber & Petsko, 1990).

Many substances capable of inhibiting the α-amylase activity have been reported in the literature (Silano, 1987). Among them, substances of proteinaceous or glycoproteinaceous nature are found in cereals, legumes, tubers and other living organisms. It is suspected that these proteins are involved in a natural mechanism of defense of the plants against insect α-amylases, but there still remain many questions to be answered about their function. Besides the existence of great diversity amongst this class of inhibitors, there is a clearly observed difference in the capability of inhibiting α-amylases from different sources. Two crystallographic structures of proteinaceous inhibitors have been reported, one from the yeast *Streptomyces tendae* (Pflugrath, Wiegand & Huber, 1985) and one bifunctional serine protease/α-amylase inhibitor from wheat (Zemke, Müller-Fahrnow, Jany, Pal & Saenger, 1991). Their structures are exclusively composed of β-strands and folded into somewhat unrelated β-barrels. Differently, the inhibitor we have crystallized is highly active against human salivary α-amylase and circular dichroism analysis indicates to be composed mainly of α-helix structure, as was already reported for this family of inhibitors (Sannia, Petrucci, Parlamenti & Silano, 1977). Indeed, the recently determined NMR solution structure of one member of the family, the bifunctional α-amylase/trypsin inhibitor from ragi seeds, shows a new motif composed of α-helices (Strobl *et al.*, 1995).

The research of α-amylase inhibitors has important implications in many areas: diagnosis of pancreatic disorders and other forms of hyperamylasemias, diabetes control, obesity control, nutritional and toxicological aspects of foods, with possible improvement of these. The understanding of the mechanism of inhibition, as well as the different specificities to different α-amylases, essential for planning their technological applications, is strongly dependent on the knowledge of the three-dimensional structures of this class of inhibitors and their complexes with the target enzyme (Whitaker, 1988).

The protein was purified according to the method described by P. E. Granum (Granum & Whitaker, 1977). Initially, *Triticum aestivum* grains (cultivar Iapar 28-Igapó) were triturated and mixed with water for 60 min, in order to extract the protein. This solution was filtered and heated to 343 K to inactivate endogenous α-amylases. The supernatant of the first step of fractionation with 20% $(NH_4)_2SO_4$ was further precipitated with 50% of the same salt, centrifuged and the pellet assayed for inhibitory activity. This fraction was then redissolved in phosphate buffer, concentrated and added to a DEAE-cellulose column. The peak with high inhibitory activity against human salivary α-amylase was rechromotographed on a CM-cellulose column, which led to a highly purified inhibitor solution. Polyacrylamide gel electrophoresis at denaturing conditions revealed a molecular weight of *ca* 13 000 Da for the monomer. This purified inhibitor solution was further concentrated and assayed for crystallization.

Trigonal prismatic crystals were obtained in a 100 m*M* Tris–HCl buffer at pH 7.3, 100 m*M* $MgCl_2$ and 14 to 20% (*w*/*v*) of PEG 20000 as the precipitant agent with the hanging-drop vapour-diffusion technique. The protein concentration on the initial drop was 5 mg ml^{-1}. Crystallization was complete after 2 weeks with typical crystal size of 0.2 × 0.2 × 0.4 mm.

The crystals belong to the trigonal system, with unit-cell dimensions $a = b = 79.31$, $c = 60.56$ Å. The Laue group was uniquely determined by the analysis of equivalent reflections from diffraction data sets collected at 277 K from three crystals, on an R-AXIS IIC image-plate area detector. Processing the data from the three crystals, adopting the minimal Laue symmetry for the crystal system ($\bar{3}$), resulted in an overall $R_{merge} = 8.9\%$, when averaging the 57 019 measured reflections into 23 688 independent reflections. The other possible Laue groups $\bar{3}1m$ and $\bar{3}m1$ resulted in an R_{merge} of about 46% in both cases. The final data set shows 90% completeness at 2.1 Å resolution and data-collection statistics are presented in Table 1. The observed systematic absences are compatible with space groups $P3_1$ or $P3_2$.

Crystal density calculations indicate that the asymmetric unit could contain two, three or four molecules, yielding

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Table 1. *Statistics of data collection for the α-amylase inhibitor*

Resolution shell lower limit (Å)	R_{sym}*	R_{cum}	Reflections measured	Independent reflections	Percentage of completeness in shell
6.51	0.050	0.050	2296	827	99.1
4.61	0.061	0.057	4264	1532	100.0
3.76	0.067	0.061	5336	1950	98.8
3.26	0.079	0.066	6210	2302	98.5
2.91	0.103	0.071	6831	2571	97.0
2.66	0.125	0.075	6946	2773	94.7
2.46	0.143	0.079	7024	2925	91.8
2.30	0.174	0.083	7002	3069	89.7
2.17	0.196	0.087	6353	3007	82.5
2.06	0.216	0.089	4757	2732	70.9

* $R_{sym} = (\sum_h \sum_i |I_{hi} - \langle I_h \rangle|)/(\sum_h \langle I_h \rangle)$ where I_{hi} is the scaled intensity of the ith measurement of the reflection h or its equivalent and $\langle I_h \rangle$ is the average intensity of reflection h.

calculated V_m values and percentual solvent contents of 4.11, 2.74, 2.05 Å^3 Da^{-1} and 70.1, 55.1, 40.1%, respectively. To further investigate the presence of non-crystallographic symmetry elements in this structure, a self-rotation function analysis was performed using the program *AMoRe* (Navaza, 1994). The data included were within the resolution range 15–2.5 Å, and expansion coefficients with l lower than 6 were filtered out. Three significant peaks were found, one with final correlation coefficient of 79% and the other two with 34%, all corresponding to twofold rotations ($\kappa = 180°$, in polar angles). All remaining peaks on the self-rotation map had correlation coefficients below 18%. In Fig. 1, the stereographic projection of the self-rotation function map showing the significant peaks is plotted. The strongest one, indicated as *P* in the figure, is on the *ab* plane, approximately 2.5° away from the *a* axis. The next two, which show equal heights, are peak *R*, parallel to the *c* axis, and peak *Q*, which lies at right angles to peaks *P* and *R*. These peaks are consistent with a local 222 symmetry, and could be compatible with the presence of four subunits per asymmetric unit with a solvent content of 40.1%. The observed high mechanical resistance of the crystals and diffraction limit to at least 1.7 Å resolution also support the lower solvent content for the unit cell.

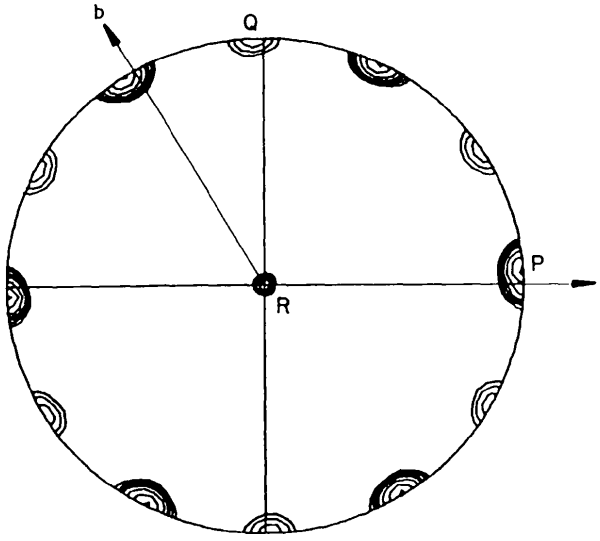

Fig. 1. Stereographic projection down the *c* axis of the $\kappa = 180°$ polar section of the self-rotation function, calculated with data in the range 2.5–15 Å. Contours are made at the following values of correlation coefficient: 20, 25, 30, 35, 45, 60, 75%.

The strong non-crystallographic twofold axis indicated as *P*, close to a cell axis, led us to carefully check if the diffraction pattern also reflected that symmetry. Indeed, when further averaging the unique $\bar{3}$ data set enforcing a twofold axis along crystal axis *a*, and allowing rejection of reflections when the pairs differed by more than 3σ, a subset of 7652 reflections could be merged into 3826 reflections with an R_{merge} of 12.2%, evidence that the non-crystallographic symmetry is also being reflected in reciprocal space.

As all attempts to solve the structure by molecular replacement with the available coordinates of the other α-amylase inhibitors described in the literature have so far failed, preparation of isomorphous derivatives is under way in order to solve the structure by the multiple isomorphous replacement technique.

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References

Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Thim, L. & Woldlike, H. F. (1990). *Biochemistry*, **29**, 6244–6249.

Brady, R. L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E. J. & Dodson, G. G. (1991). *Acta Cryst.* B**47**, 527–535.

Buisson, G., Duée, E., Haser, R. & Payan, F. (1987). *EMBO J.* **6**, 3909–3916.

Farber, G. K. & Petsko, G. A. (1990). *Trends Biochem. Sci.* **15**, 228–234.

Granum, P. E. & Whitaker, J. R. (1977). *J. Food Biochem.* **1**, 385–401.

Kadziola, A., Abe, J., Svensson, B. & Haser, R. (1994). *J. Mol. Biol.* **239**, 104–121.

Matsuura, Y., Kusunoki, M., Harada, W. & Kakudo, M. (1984). *J. Biochem.* **95**, 697–702.

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

Pflugrath, J. W., Wiegand, G. & Huber, R. (1986). *J. Mol. Biol.* **189**, 383–386.

Qian, M., Haser, R. & Payan, F. (1993). *J. Mol. Biol.* **231**, 785–799.

Sannia, G., Petrucci, T., Parlamenti, R. & Silano, V. (1978). *Biochem. J.* **173**, 229–235.

Silano, V. (1987). *Enzymes and Their Role in Cereal Technology*, edited by J. E. Kruger, D. Lineback & C. E. Stauffer, ch. 6. Saint Paul: Am. Assoc. Cer. Chem.

Strobl, S., Mühlhahn, P., Berstein, R., Wiltscheck, R., Maskos, K., Wunderlich, M., Huber, R., Glockshuber, R. & Holak, T. A. (1995). *Biochemistry*, **34**, 8281–8293.

Swift, H. J., Brady, L., Derewenda, Z. S., Dodson, E. J., Dodson, G. G., Turkenberg, J. P. & Wilkinson, A. J. (1991). *Acta Cryst.* B**47**, 535–544.

Whitaker, J. R. (1988). α-Amylase Inhibitors of Higher Plants and Microorganisms, 79th. Annual Meeting of the American Oil Chemists Society, Phoenix, Arizona.

Zemke, K. J., Müller-Fahrow, A., Jany, K., Pal, G. P. & Saenger, W. (1991). *FEBS Lett.* **279**(2), 240–242.