Crystallization of the bifunctional proteinase/amylase inhibitor PKI-3 and of its complex with proteinase K

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Received 17 December 1985

One of the three wheat germ inhibitors of proteinase K is bifunctional and inhibits simultaneously proteinase K (or subtilisin but not enzymes of the trypsin family) and insect α-amylase. The molecular mass of this inhibitor called PKI-3 is 21 kDa, and the binding constant for proteinase K is 0.8 nM at pH 8.2, 25°C, in 1:1 molar ratio. PKI-3 was crystallized by microdialysis against 10–12% polyethylene glycol 6000, 50 mM NaH_2PO_4, pH 6.7. The crystals have monoclinic space group $P2_1$ with $a=42.5$, $b=65.3$, $c=31.5$ Å, $\beta=110°$, and diffract beyond 2.0 Å resolution. The complex proteinase K · PKI-3 was crystallized by equilibrium vapor diffusion under the same conditions. The crystals are needle-shaped and still too small for X-ray analysis. Gel electrophoresis established the composition of the crystals.

Bifunctional inhibitor *Amylase* *Enzyme inhibitor* *Proteinase K* *X-ray diffraction* *Crystallization*

1. INTRODUCTION

In all kinds of plant and animal tissues and in microbia, globular proteins are found which act as proteinase inhibitors [1]. Recently, 3 inhibitors for the subtilisin-type enzyme proteinase K have been isolated from wheat germ [2]. According to the sequence of their elution from a CM-Sepharose CL-6B column in one of the purification steps they were named PKI-1, PKI-2 and PKI-3. Each of these inhibitors consists of a single polypeptide chain of 11 kDa for PKI-1, 8 kDa for PKI-2 and 21 kDa for PKI-3. All 3 inhibitors are specific against proteases of the subtilisin family; at high concentrations, PKI-1 and PKI-2 also exhibit modest inhibitory action against trypsin and chymotrypsin [2].

The amino acid composition of inhibitor PKI-3 is similar to that of the subtilisin inhibitor isolated from barley [3]. PKI-3 binds to proteinase K in 1:1 stoichiometry with a dissociation constant of 0.8 nM at pH 8.2 and 25°C. Besides its inhibitory activity towards subtilisin-type proteinases, PKI-3 also binds to and inhibits α-amylase from wheat and several insects but not human or bovine α-amylase. This binding capacity is even retained after PKI-3 is complexed with proteinase K. Comparable bifunctional inhibitors have previously been reported for barley [3] and for the seeds of ragi, the Indian finger millet *Eleusine coracana* Gaertn [4].

Because of these unique properties of PKI-3, an X-ray structure study on the inhibitor and on its complex with proteinase K is of interest. It will provide insight into the molecular mechanism of the inhibitory action of PKI-3, the specific protein-protein recognition in the complex of PKI-3 with proteinase K, and possible conformational differences of proteinase K when in the free [5,6] and complexed state. Similar studies have been performed on the complexes of basic pancreatic trypsin inhibitor BPTI (Kunitz) with bovine typsin [7,8], soybean trypsin inhibitor (STI) with porcine

Dedicated to Professor Georg Manecke on the occasion of his 70th birthday

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

trypsin [9], and *Streptomyces* subtilisin inhibitor (SSI) with subtilisin BPN' [10].

Here, the crystallization of the inhibitor PKI-3 and of its complex with proteinase K are described, and some preliminary X-ray data are presented.

2. MATERIALS AND METHODS

2.1. *Materials*

PKI-3 was purified from wheat germ according to [2] and stored at 4°C as lyophilized material. Proteinase K was purchased from Merck, Darmstadt, and purified further by gel filtration using a Sephadex G-75 column at room temperature. The fractions which exhibited proteinase K activity were pooled, dialysed against a large volume of 1 mM calcium acetate, pH 7.5, and stored as lyophilized material at 4°C.

2.2. *Crystallization of PKI-3*

2.2.1. Microdialysis

15–20 mg lyophilized PKI-3 were dissolved in 1 ml water and centrifuged. 20 μl of the clear supernatant were pipetted into microdialysis tubes [11] and set up for crystallization at room temperature against 5 ml of reservoir solution. This solution contained 10–12% polyethylene glycol 6000 (w/v), or 25–30% saturated ammonium sulfate in 50 mM NaH_2PO_4, pH 6.7.

2.2.2. Vapor diffusion

Equilibrium vapor diffusion of a protein droplet situated on a polyethylene bridge was carried out at room temperature against a large reservoir of 20 ml. The reservoir buffers were the same as mentioned in Section 2.2.1, and the drops were prepared by mixing 10 μl of a 3–4% solution of PKI-3 in water with an equal volume of the reservoir buffer. The bridge and the 20 ml reservoir buffer were placed in a plastic petri dish which was sealed with scotch tape.

2.3. *Crystallization of the complex between PKI-3 and proteinase K*

The complex between proteinase K and the inhibitor PKI-3 was prepared by mixing the two protein solutions in equimolar ratio so that the final protein concentration was 1%. Crystallization experiments were set up in microdialysis and vapor diffusion techniques as described above for the inhibitor.

2.4. *X-ray examination of crystals*

For X-ray crystallographic studies the crystals were mounted in quartz capillaries with a drop of mother liquor. Diffraction photographs were taken with a Nonius precession camera using Ni-filtered CuKα radiation generated by an Elliott-Marconi GX6 rotating anode source run at 40 kV, 40 mA, focal size $2 \times 0.2\ mm^2$.

3. RESULTS AND DISCUSSION

Stout, plate-like crystals of the inhibitor PKI-3 appeared within 1 day in the microdialysis technique with 10–12% polyethylene glycol 6000 dissolved in 50 mM NaH_2PO_4, pH 6.7. They grew in 3–5 days to the full size of $2 \times 1 \times 0.6\ mm^3$ (fig.1). Using vapor diffusion and microdialysis against ammonium sulfate, smaller crystals grew as clusters and not in single form.

A large single crystal of PKI-3 diffracts X-rays to at least 2.0 Å resolution on still photographs. Systematic extinctions ($0k0$, $k = 2n + 1$) on precession photographs (fig.2) suggest the monoclinic space group $P2_1$. The unit cell dimensions are $a = 42.5$ Å, $b = 65.3$ Å, $c = 31.5$ Å, $\beta = 110°$. Assuming one molecule of 21 kDa per asymmetric

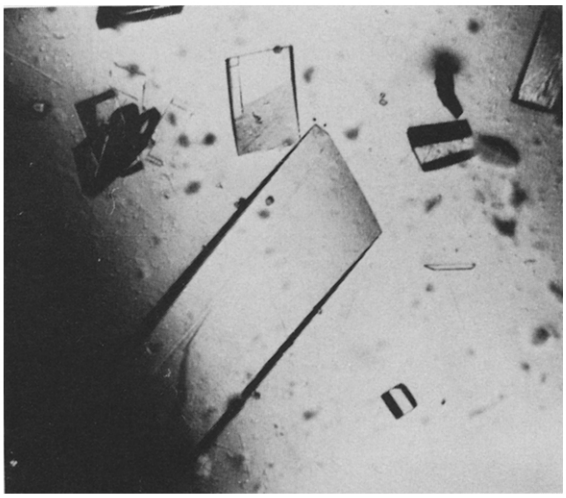

Fig.1. Crystals of proteinase K inhibitor PKI-3 grown from polyethylene glycol 6000. Maximum dimension is 2 mm.

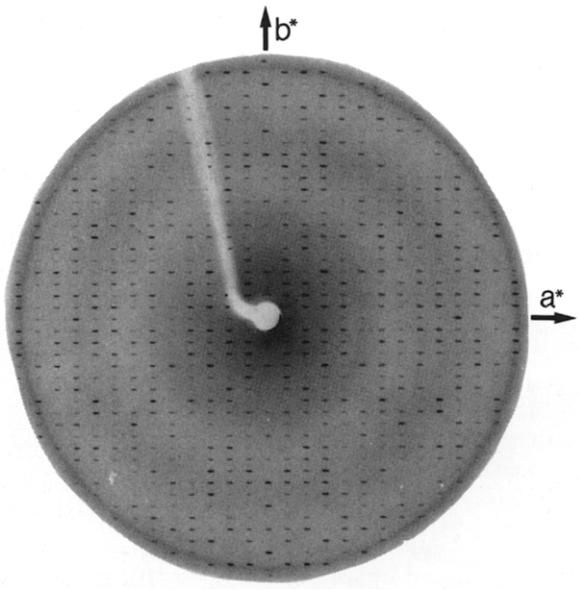

Fig.2. Precession X-ray diffraction photograph of the *hk*0 zone of the inhibitor PKI-3. Taken with CuKα radiation from rotating anode generator, 100 mm crystal to film distance, precession angle $\mu = 15°$.

unit of 41 000 Å^3, the ratio of volume to unit protein mass is $V_M = 1.95$ Å^3/Da. This is in good agreement with V_M values known from other protein crystals [12]. The fraction of the volume occupied by solvent, V_{solv}, is calculated to be 41%, based on the partial specific volume of PKI-3 calculated as 0.723 cm^3/g from its amino acid composition (K.-D. Jany and G. Lederer, unpublished).

Crystals of the complex between proteinase K and the inhibitor PKI-3 grew as bundles of fine needles within 3–4 days in the vapor diffusion experiments. Although the crystals are up to 2 mm in length, their diameters are minute (fig.3) so that the diffraction in the X-ray beam is still too poor to derive unit cell constants and space group.

In order to clarify the nature of these needle-shaped crystals, they were washed repeatedly with crystallization buffer and dissolved in SDS solution. SDS-polyacrylamide gel electrophoresis was run in the presence of β-mercaptoethanol. It showed two distinct bands corresponding to proteinase K and inhibitor PKI-3 (fig.4) and proved

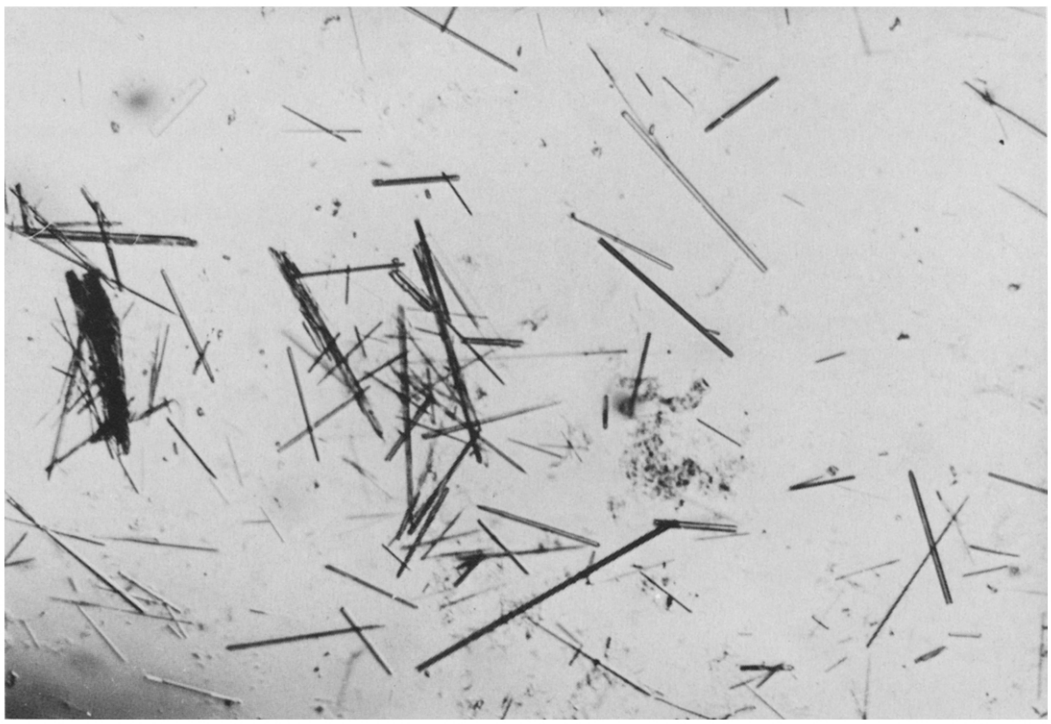

Fig.3. Crystals of the complex between proteinase K and the inhibitor PKI-3. The diameter of the crystals is less than 0.05 mm.

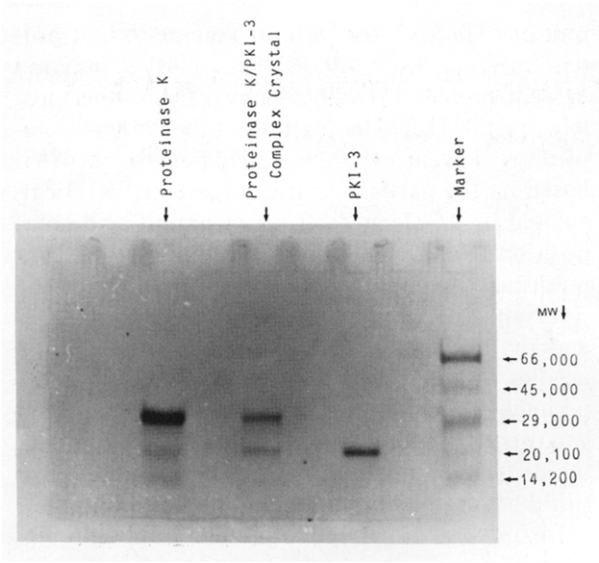

Fig.4. SDS-polyacrylamide gel electrophoresis pattern of the crystals shown in fig.3. The two bands correspond to proteinase K and inhibitor PKI-3. Minor bands of low M_r in the proteinase K lane are probably due to autodigestion even in presence of SDS.

that the complex was indeed present in the needle-shaped crystals.

For X-ray diffraction analysis, the crystal growth of the enzyme-inhibitor complex has to be optimized. As to the crystals of the inhibitor PKI-3 alone, data collection and the search for heavy atom derivatives have begun.

ACKNOWLEDGEMENTS

This work was supported financially by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 9, Bundesministerium für Forschung und Technologie (FKZ: B72C050) and Fonds der Chemischen Industrie. The authors thank Dr Kashyap for help with the gel electrophoresis.

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