41. Effect of Dimethylsulfoxide on the Crystallization of *Aspergillus niger* Proteinase A

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Abstract: Proteinase A from *Aspergillus niger* var. *macrosporus* is a non-pepsin-type acid proteinase, whose catalytic site and enzymatic mechanism remain to be clarified. As the first step toward elucidating its three-dimensional structure by X-ray crystallography, proteinase A was crystallized in ammonium sulfate solutions by the hanging-drop vapor diffusion method. In the absence of an organic solvent, only the aggregates of long, thin needles were formed. On the other hand, thicker rodlike single crystals were grown at around pH 1.75 when dimethylsulfoxide (DMSO) was added to a concentration of around 5%. The single crystals of proteinase A thus obtained were suitable for X-ray diffraction measurements with a precession camera.

Key words: Crystallization; *A. niger* proteinase A; DMSO.

Introduction. Proteinase A is one of the two acid proteinases secreted by the fungus *Aspergillus niger* var. *macrosporus*,¹⁾ and belongs to a group of non-pepsin-type acid proteinases as judged from its clear differences in various properties from the ordinary aspartic proteinase family, including pepsin, *A. niger* acid proteinase B, renin, and human immunodeficiency virus proteinase.^{2)–6)} Proteinase A is rather insensitive to common, specific inhibitors for the pepsin-type aspartic proteinases.²⁾ It has a relative molecular mass of 22,300,^{4),7)} about half the size of ordinary pepsin-type aspartic proteinases (M_r ~35,000), and shows fairly different substrate specificity.^{3),8)} The amino acid sequence determination^{4),6)} revealed that no proteins in the GenBank database are homologous with proteinase A except for *Scytalidium lignicolum* proteinase B⁹⁾ that shows about 50% identity. Proteinase A has no consensus sequences, i.e., Asp-Thr/Ser-Gly, including the active-site aspartic acid residues, present in the family of pepsin-type aspartic proteinases. It, thus, remains to be elucidated which residues participate in the catalysis and how the mechanism operates. In order to approach this question, it is of utmost importance to elucidate the three-dimensional structure of the enzyme.

In the present study, we examined various conditions to grow the crystals of proteinase A for X-ray crystallography with special attention to the effect of dimethylsulfoxide (DMSO).

Materials and methods. Proteinase A was purified from the crude enzyme mixture obtained from the culture medium of *Aspergillus niger* var. *macrosporus* by repeated chromatography on DEAE-Toyopearl 650S columns. The hanging-drop vapor diffusion method was employed at 4°C and 25°C to survey crystallization conditions, with ammonium sulfate, over the range of pH values from 1.0 to 3.0 at every 0.25 pH unit. The

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enzyme solution contained 50 mg/ml protein in 5 mM sodium acetate buffer, pH 4.5. The reservoir solution contained 1.3–1.6 M ammonium sulfate, 50 mM buffer, and DMSO. The concentrations of DMSO examined were 0, 1, 3, 5, 7, and 10% (v/v). The enzyme solution (8 μ l) was mixed with 8 μ l of a reservoir solution, placed on the underside of a glass cover slip over 1 ml of the same reservoir solution in a plastic well, and then sealed.

Preliminary X-ray diffraction experiments were performed using a Huber precession camera with Ni-filtered CuK α radiation generated by a Rigaku RU-200 X-ray generator (40 kV, 70 mA, focus size 0.2 $\times$ 2 mm).

Results. Fig. 1(a) shows the crystals of proteinase A grown in the absence of DMSO at pH 1.75. Crystals grew to about 1 mm in length in a few days at 25°C, or in a week at 4°C. Under these conditions, only the aggregates of long, thin needles were obtained. The diameters of these needles were less than 20 μ m, so X-ray diffraction data for crystallography could not be collected using such thin crystals.

Fig. 1. Crystals of *A. niger* proteinase A grown from 1.4 M ammonium sulfate solutions at pH 1.75 (a) in the absence and (b) in the presence of 5% DMSO.

Fig. 2. X-ray precession photograph of the 0 kl zone for a crystal of *A. niger* proteinase A grown at pH 1.75. The crystal-to-film distance, precession angle, and exposure time were 100 mm, 12.0°, and 8 h, respectively.

When 1% DMSO was present, rodlike single crystals were grown in addition to the aggregates of needles. As the concentration of DMSO was increased up to 5%, the single crystals formed grew thicker and more crystals appeared. In solutions containing 5% DMSO, the size of the single crystals became maximum ($0.15 \times 0.15 \times 1$ mm). Single crystals were also grown in solutions containing 3% or 7% DMSO, which were almost comparable in diameters to those obtained in a solution containing 5% DMSO. No single crystals were obtained when the concentration of DMSO was 10% at 25°C.

The change in pH was less effective than that in the concentration of DMSO. At around pH 1.75, thick single crystals appeared in the presence of 1 to 10% DMSO except for 10% DMSO at 25°C. Single crystals obtained at pH between 1.0 and 2.5 had similar thicknesses although fewer single crystals appeared as the pH of the solution was increased or decreased from 1.75. The range of pH in which single crystals were obtained was wider in 5% DMSO solutions than in 3% or 7% DMSO solutions.

The single crystals obtained at 4°C and 25°C were indistinguishable although the rate of crystal growth was slower and fewer single crystals appeared at 4°C than at 25°C. Crystals grown at 25°C were used for the measurements of X-ray diffraction. The typical size of single crystals used was $0.1 \times 0.1 \times 1$ mm. An X-ray precession photograph of the $0kl$ zone is shown in Fig. 2.

Discussion. The presence of around 5% DMSO favored the growth of single crystals of proteinase A which could be subjected to the collection of X-ray diffraction data. In contrast, only the aggregates of thin needles were formed in the absence of DMSO. This may be due to the non-specific hydrophobic interaction between proteinase A molecules, and DMSO may weaken such non-specific interaction.^{10),11)}

In addition to DMSO, 1,4-dioxane and poly(ethyleneglycol) 600 were also examined for their effects on the formation of the single crystals of proteinase A. 1,4-Dioxane was less effective than DMSO and poly(ethyleneglycol) 600 was not effective on the formation of thick single crystals. Such differences may be due to the difference in the polarity of these organic compounds, or due to the fact that DMSO is a good precipitation agent for proteins without denaturation.¹⁰⁾

As will be detailed elsewhere on the crystallographic data,¹²⁾ precession photographs revealed that the proteinase A crystals belong to orthorhombic space group $P2_12_12_1$ with unit cell dimensions of $a=54.7$ Å, $b=70.4$ Å, and $c=38.0$ Å.

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