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Improved Crystals of the Toxic Protein MAP by Protein Engineering Towards the Host Specificity

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Abstract

Mirabilis anti-viral protein (MAP) is a ribosome-inactivating protein from *Mirabilis jalapa* L. Since MAP is effective over a broad spectrum of species, the protein is difficult to express in heterologous hosts such as *Escherichia coli*. Recently, we obtained a MAP mutant, Y72F which exhibits a lower (1/100) activity against *E. coli* ribosomes while retaining almost full activity against mammalian cells [Habuka, Miyano, Kataoka, Tsuge & Noma (1992). *J. Biol. Chem.* **267**, 7758–7760]. For the crystallographic studies, the Y72F MAP expression vector with an *OmpA* leading sequence was constructed and expressed in *E. coli*. The Y72F MAP mutant was then isolated and purified from the cell culture medium. Crystals were grown using the crystallization conditions for the native MAP crystals [Miyano *et al.* (1992). *J. Mol. Biol.* **226**, 281–283]: 50% ammonium sulfate containing 50 m*M* ammonium citrate and 2 m*M* adenine sulfate, pH 5.4. The crystals belong to space group $P3_121$ (or $P3_221$) with $a = b = 104.1$ and $c = 134.3$ Å. The crystals are isomorphous with the wild-type crystals but diffract to higher resolution. Imaging-plate photographs of the Y72F mutant showed sharp intense spots without the streaking observed in the native crystals.

Introduction

Mirabilis anti-viral protein (MAP) isolated from the root of *Mirabilis jalapa* L. inhibits not only the mechanical transmission of plant viruses, such as tobacco mosaic virus (Ikeda *et al.*, 1987), but also the *in vitro* protein synthesis of eucaryotic systems (Habuka *et al.*, 1990). The specific *N*-glycosidase activity of MAP proteins inactivates the ribosomes (Habuka, Miyano, Kataoka & Noma, 1991). This class of ribosome-inactivating proteins (RIPs) are found in a variety of plants (Barbieri & Stirpe, 1982). Some RIPs such as trichosanthin and pokeweed antiviral protein have been reported to inhibit the replication of human immunodeficiency virus (McGrath *et al.*, 1989; Zarling *et al.*, 1990).

MAP exhibits *N*-glycosidase activity toward ribosomes from various sources including the *Mirabilis* plant (Kataoka, Habuka, Miyano, Masuta & Koiwai, 1992). MAP has been shown to inhibit prokaryotic protein synthesis as well (Habuka *et al.*, 1990). This broad range of activity has made recombinant MAP difficult to express in *E. coli*. For example, only small amounts of recombinant MAP could be expressed in *E. coli* (Habuka, Murakami, Noma, Kudo & Horikoshi, 1989) while the recombinant ricin *A*-chain, another well known RIP, which has no activity against *E. coli* ribosomes, could be expressed in large quantities (Piatak & Habuka, 1992). In systematic mutation studies on MAP several mutants, whose activity modulated the *N*-glycosidase function, were identified (Habuka, Miyano, Kataoka, Tsuge & Noma, 1992). Of these a Y72F mutant that retained almost full activity in mammalian cells was found to have a much lower (1/100) activity against *E. coli* ribosomes (Habuka *et al.*, 1992). This mutant, which can be overexpressed in *E. coli*, presents an excellent opportunity for the structural study and an understanding of the specificity of MAP *N*-glycosidase activity.

A few RIP structures such as trichosanthin (Pan *et al.*, 1987) and ricin (Montfort *et al.*, 1987) have been determined by X-ray crystallography. A comparative structural study on the diverse RIP family would

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Table 1. *Yield of recombinant MAP (wild/Y72F) under various expression conditions by E. coli*

MAP gene	Expression vector/ host strain	Induction	Secretion to medium	Yield ($\mu g\ l^{-1}$)	References
Wild	pMH2/JM109	IPTG	No	3.6	(1)
Wild	pSH6/MM294	315 K	No	20	(2)
Wild(*OmpA*)	pSH7/MM294	351 K	Yes	140	(3)
Y72F(*OmpA*)	pSH7/MM294	351 K	Yes	400	(4)
Y72F(*OmpA*)	pSHompA/MM294	IPTG	Yes	2570	(5)

References: (1) pMH2 with *tac* promoter and the JM109 carries *lacI*^{q} (Habuka *et al.*, 1989). (2) pSH6 with *P*L promoter and *cI*857 repressor (Habuka *et al.*, 1990). (3) pSH7 inserted with *OmpA* signal sequence of pSH6 (Habuka *et al.*, 1990). (4) Habuka *et al.* (1992). (5) pSHompA with *tac* promoter and *lacI*^{q} repressor (this paper).

also be interesting in terms of molecular evolution and it is noteworthy that the MAP genomic gene has been reported to possess an intron (Kataoka, Miyano, Habuka, Masuta & Koiwai, 1993).

Crystals of the wild-type MAP were obtained from *Mirabilis* plants (Miyano *et al.*, 1992) and native data sets were collected up to 2.5 Å resolution. However, these crystals showed lattice defects at 3 Å and higher resolution as evidenced by streaks in the diffraction pattern. In addition, very often the crystals were twinned, making it difficult to search for heavy-atom derivatives. Crystallization trials of the Y72F MAP mutant were begun in the hope of obtaining better diffraction-quality crystals.

Materials and methods

Restriction endonucleases and DNA-modification enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Bethesda Research Laboratory (Tokyo, Japan). CM-Sepharose, Blue-Sepharose, Mono-Q and Mono-S columns were purchased from Pharmacia LKB (Tokyo, Japan). Rabbit reticulocyte lysate system was purchased from Amersham. All other chemicals were of reagent grade.

The construction and the DNA sequence of the *Y72F* gene with the *OmpA* signal sequence for secretion has been described previously (Habuka *et al.*, 1992). The expression vector pSHompA was derived from pSH7 by substitution of the *tac* promoter and *lacI*^{q} repressor for P_L and *cI*857, respectively (Habuka *et al.*, 1990).

Expression and purification of the Y72F mutant were perfomed according to Habuka *et al.* (1990). The *E. coli* strains HB101, JM103, JM109, JM115, MM294, N99Cl$^+$, TG1, YA21 and W3110 were transformed using the expression vector. The transformant was cultured at 303 K in LBroth, 0.1 m*M* isopropyl-β-D-thiogalactoside (IPTG) was added when the $OD_{550\ nm}$ of the culture medium reached 0.5, and the culture was incubated further for 5 h. Cells were removed by centrifugation and the Y72F mutant in the supernatant was collected by centrifugation in 90% saturated ammonium sulfate. The precipitant was purified using three-step column chromatography on CM-Sepharose, Mono-Q and Blue-Sepharose followed by a final pass through a Mono-S column.

Protein analysis and activity measurements were performed according to Habuka *et al.* (1990) and analyzed by SDS–PAGE. The NH_2-terminal sequence was determined using a 447A protein sequencer (Applied Biosystems Incorporated) using automatic Edman degradation. The inhibitory activity of the protein synthesis was measured using *E. coli* and rabbit reticulocyte systems as described by Habuka *et al.* (1991). Quantification of total Y72F was determined by enzyme-linked immunosorbent assay using the antisera against wild-type MAP.

The hanging-drop method (McPherson, 1985) using Crystal Screen™ (Hampton Research, USA) was used in the initial crystallization trials. Although, small crystals were obtained from the Crystal Screen™ trials, diffraction-quality crystals were only obtained using conditions similar to those used to grow the wild-type crystals (Miyano *et al.*, 1992).

The crystals were mounted in thin-walled glass capillaries containing a small amount of mother liquor to prevent dehydration of the crystals. Diffraction data were collected on a MacScience MXC18 diffractometer equipped with a San Diego multiwire area-detector system (Hamlin, 1985) using Cu $K\alpha$ (λ = 1.5418 Å) X-rays generated on a rotating anode. The data were collected and reduced using a modified version of the *UCSD* software (Howard, Nielsen & Xuong, 1985). In addition, a native data set was collected at beamline $BL6A_2$ (KEK Photon Factory, Tsukuba, Japan) using monochromitized synchrotron radiation (λ = 1.00 Å) with a Weissenberg camera imaging-plate system (Sakabe, 1991). The exposed imaging plates were scanned by a BA100 scanner (Fuji Film Co.). The data reduction was performed with the program *WEIS* (Higashi, 1989) at the KEK Photon Factory computer center.

Results and discussion

The *Y72F* gene was successfully inserted into the pSHompA expression vector. Of the nine strains used, the transformant of *E. coli* strain MM294

showed the best production at 303 K, where the Y72F mutant shows a low toxicity to the host (data not shown, Habuka *et al.*, 1992). After induction of IPTG, the Y72F production reached a plateau in 5 h at 2.57 mg l^{-1} with half of the mature Y72F mutant secreted into the culture medium. However, wild-type MAP reached a plateau in 1 h from induction at 0.14 mg l^{-1} by the pSH7 system (Habuka *et al.*, 1990). Table 1 shows the improvement in the production of recombinant MAP in our laboratory.

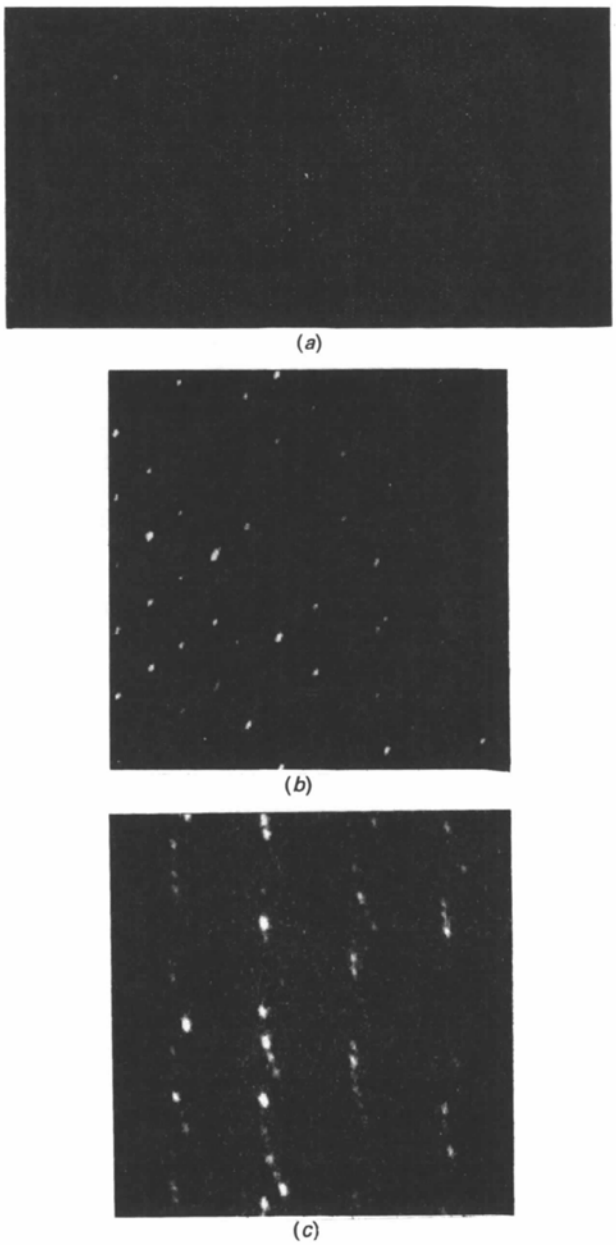

Fig. 1. Imaging-plate Weissenberg photos of MAP and Y72F (d = 429.7 mm). (*a*) A full frame of Y72F crystal diffraction (0.1 mm per pixel, original size 200 × 400 mm). (*b*) The close-up image around 3.0 Å in Bragg spacing of Y72F MAP crystal and (*c*) wild-type MAP crystal.

After the cells had been removed by centrifugation, the cell medium was fractionated with 90% saturated ammonium sulfate. After purification on CM-Sepharose, Mono-Q and Blue-Sepharose columns, the protein gave a single band on SDS–PAGE. The sample was further purified on a Mono-S column in order to obtain a higher concentration of the sample. The final yield for the Y72F mutant was 7.5 mg from a six litre culture. The NH_2-terminal sequence of the Y72F mutant (APTLETIASLDLNNP) was found to be identical to the wild-type sequence (Habuka *et al.*, 1992).

The rod-like crystals of Y72F mutant, grown according to Miyano *et al.* (1992), belong to space group $P3_121$ (or $P3_221$) with $a = b = 104.1$ and $c = 134.3$ Å. The crystals are isomorphous with the wild-type crystals, but diffract to higher resolution and exhibit much less twinning. A full native data set to 2.5 Å resolution was collected using the imaging-plate Weissenberg camera at beamline $BL6A_2$, KEK Photon Factory, Japan. The imaging-plate photographs of the Y72F-mutant crystals showed no obvious defects at high resolution (Figs. 1*a* and 1*b*), while the wild-type crystals showed some extra diffraction between layers along the c axis (Fig. 1*c*). In addition, the diffraction pattern of the Y72F mutant crystals showed sharper more intense spots without the streaking that is observed in the diffraction patterns of the wild-type crystals. The structure determination is underway using both molecular-replacement and multiple isomorphous-replacement techniques.

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