EXAMINATION OF THE EXPRESSION OF NOVEL CHAPERONE PROTEIN AIP2P/DLD2P WITH METHYLOTROPHIC YEAST *PICHIA PASTORIS*

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Abstract

Oligomeric state of the actin interacting protein 2/ D-lactate dehydrogenase protein 2 (Aip2p/Dld2p) isolated from *Saccharomyces cerevisiae* was shown to be able to unfold the conformation of pathogenic highly aggregated polypeptides, and has been supposed to be a novel chaperone protein. However, the mechanism of the unfolding activity is little understood. Since experiments for the structure–function relationships of a protein require an efficient expression system, we attempted to use the methylotrophic yeast *Pichia pastoris* because of its ability of very high expression yield. Changing the vector/protein construct combinations, we examined the secretion expression system of *P. pastoris* with several Aip2p/Dld2p constructs as well as the intracellular expression system. We did not observe significant expression yields in any construct combinations. RT-PCR analyses revealed that mRNA coding Aip2p/Dld2p was successfully synthesized, suggesting that the structure of Aip2p/Dld2p is inherently unstable and susceptible to proteolysis of the cellular quality control system.

Key words: Aip2p/Dld2p, *Pichia pastoris*, protein aggregation, unfolding activity.

1. Introduction

D-lactate dehydrogenase protein 2 (Dld2p) [1, 2] was originally isolated from *Saccharomyces cerevisiae*. Since this protein was initially identified as actin interacting protein 2 (Aip2p) using a two-hybrid screen to search for proteins that interact with actin [3], it is called Aip2p/Dld2p. Monomer Aip2p/Dld2p consists of 530 amino acids and its molecular weight is approximately 60 kDa. It was reported that oligomeric Aip2p/Dld2p assumes a unique grapple-like structure, and bind to actins and modify the protein conformation in the presence of ATP or AMP–PNP [4–7]. It is stressed that Hachiya et al.[4] further identified oligomeric Aip2p/Dld2p was also able to modify the conformation of pathogenic highly aggregated polypeptides such as recombinant prion protein (rPrP) in the beta form, α-synuclein, and Aβ (1–42) in the presence of ATP [6]. The oligomer formation was suggested to be requisite for the unfolding activity, and the deletion of N-terminal residues was reported to inhibit the oligomerization. However, the mechanisms of the oligomer formation and unfolding activity are little understood.

Experiments for the structure–function relationships of a protein require an efficient expression system. The reported yield of the present recombinant Aip2p/Dld2p expression system by *Saccharomyces cerevisiae*, which is several to several tens μg per liter of culture, is not deemed sufficient for a number of biophysical studies. Thus, we attempted to use the methylotrophic yeast *Pichia pastoris* as the expression

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host [8, 9] for its ability to show very high expression yield [8, 10, 11]. In addition, we can control the destination of expressed protein, to be secreted or to stay in cytosol, by selecting the plasmid with or without the signal sequence, α-factor. The advantages of the secretion system are that, as *P. pastoris* secretes very little of its own protein, the purification of the expressed protein will be simplified [12], and that, during the secretion process of the expressed protein, many chaperone proteins, such as protein disulfide isomerase (PDI), will assist correct foldings of the expressed protein.

In the present study we examined several vector/protein construct combinations of *P. pastoris* system (Table 1). We used pPIC Z for expression in cytosol (Table 1, **1**), whereas pPIC11 plasmid for the secreted expression (Table 1, **2**–**4**). Regarding the examination of the secreted expression system, we tried the N-terminal residue-truncated Aip2p/Dld2p construct (ΔN UFG, Table 1, **3**), as well as the full-length construct (fl UFG, Table 1, **2**), to assess the effect of the truncation to the oligomer formation and final expression efficiency. ("UFG" in the vector names stands for oligomeric Aip2p/Dld2p from its unfolding activity.) It is noted that Aip2p/Dld2p is intracellular protein and is supposed to have its cysteinyl residues in the reduced form. Thus, we further examined the no-cysteine mutant of full-length Aip2p/Dld2p (fl UFG noCys, Table 1, **4**) in order to avoid potential formations of non-native disulfide bonds, which might be caused by PDI during the secretion process. For example, for secreted expression of full-length Aip2p/Dld2p, we prepared the pPIC11/fl UFG vector (**2**, Table 1). Then, we investigated the protein expression yields with these plasmids.

Table 1. Vector/protein construct combinations examined.

Vector No.	Vector name	Expression destination	Aip2p/Dld2p construct
1	pPIC Z/fl UFG	Intracellular (cytosol)	Full length
2	pPIC11/fl UFG	Extracellular (secretion)	Full length
3	pPIC11/ΔN UFG	Extracellular (secretion)	N-terminal 36 amino acids deleted
4	pPIC11/fl UFG noCys	Extracellular (secretion)	Full length with no cysteine (C121A, C170A, C200A)

2. Materials and Methods

2.1. Matrials

Escherichia coli XL-I Blue was used as the host strain to construct vectors. *Pichia pastoris* GS115 (Invitrogen) was used as the host strain for protein expression [11]. All of the media used for *E. coli* and *P. pastoris* were prepared as described by Kim *et al.* [13]. The extractions of plasmid DNA from *E. coli* cells were performed using a Wizard Plus Miniprep kit (Promega). The DNA sequences of the Aip2p/Dld2p gene on the plasmids were checked using an Applied Biosystems Model 3100 sequencer.

2.2. Construction of an Aip2p/Dld2p expression plasmid with pPIC Z

The vector maps for pPIC Z and pPIC11 are shown in Figure 1. The methods, PCR templates, and primers used for the plasmid constructions were summarized in Table 2.

cDNA encoding full-length Aip2p/Dld2p (**1**) was amplified by PCR with pYES2/UFG as template using KOD plus DNA polymerase (Toyobo), where pYES2/UFG is the expression vector for the *S. serevesiae* expression system. It is noted that a Met residue and a His-tag were present at the N- and C-terminal positions of the recombinant protein, respectively. The amplified DNA fragment was cloned between the *Eco*RI and *Xho*I sites of MCS of pPIC Z. After transformation of XL-1 Blue strain with the plasmid, the cell was cultured and the amplified plasmid was extracted.

After confirmation of the DNA sequence of the Aip2p/Dld2p gene, the obtained plasmid was nicked using the restriction enzyme *Pme*I (New England Biolabs) for transformation of *P. pastoris* strain GS115, which was carried out with electroporation using an *E. coli* pulser (Bio-Rad) at a voltage of 1.7 kV, followed by spreading the cells on YPDS plate medium containing Zeocin to select transformants.

2.3. Construction of Aip2p/Dld2p expression plasmids with pPIC11

First, we constructed the pPIC11 plasmid [14] containing the full-length Aip2p/Dld2p sequence (pPIC11/fl UFG, **2**). cDNA encoding Aip2p/Dld2p was amplified by PCR with pYES2/UFG as template. The amplified DNA fragment was cloned between the *Xho*I and *Eco*RI sites of MCS of pPIC11. The cloned plasmid was selected and amplified with XL-1 Blue strain.

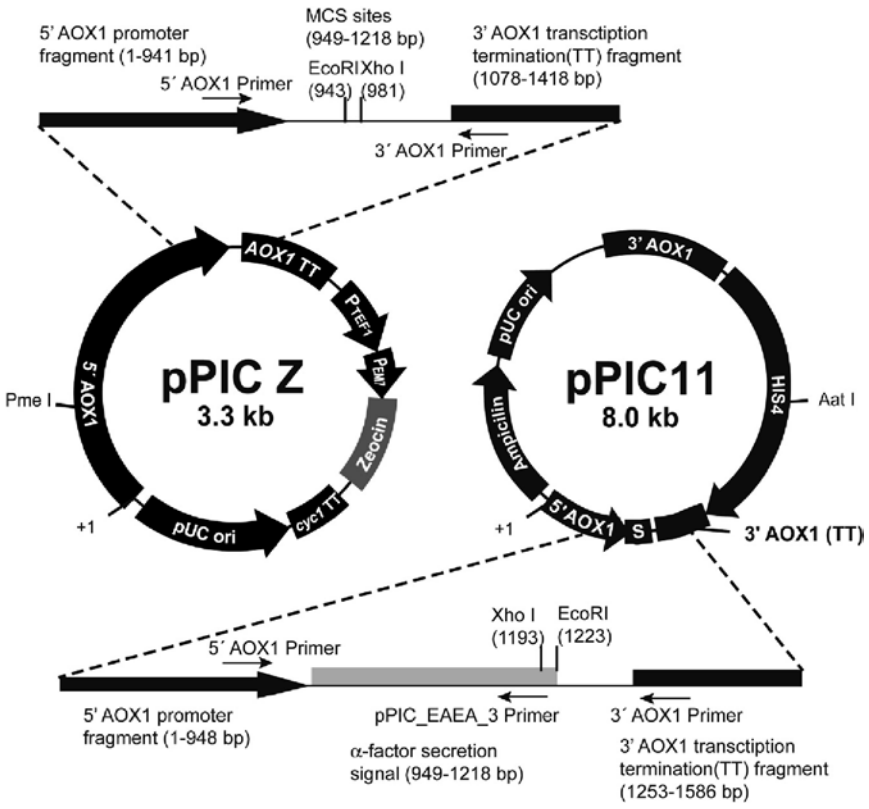

Figure 1. The vector maps of pPIC Z and pPIC 11. The restriction enzyme sites and positions of primers related to the present study were shown.

Table 2. Methods and primers used for construction of the expression vectors.

Vector No.	Method	PCR Template	Primer Name	Primer Sequence
1	Subcloning	pYES2 /UFG	Y2Z_5_fl UFG2	GCTTGAATTCATGCTAAGAAACATTTTGGTG
			CYC1TT_3	ACATAACTAATTACATGATGCGGCCC
2	Subcloning	pYES2 /UFG	YES2PIC_5_flUFG	GTATCTCTCGAGAAAAGAGAGGCTGAAGCTATGCTAAGAAAC ATTTTGG
			YES2PIC_3_UFG	GCCTCTGGAATTCTCAATGGTGATGGTGATGATG
3	KOD plus mutagenesis	pPIC11 /fl UFG	pPIC_EAEA_3	AGCTTCAGCCTCTCTTTTCTCGAG
			dNUFG_Nterm_5	TCGACCAAGATACAAACCAGACTGAC
4	KOD plus mutagenesis	pPIC11 /fl UFG	UFG_C121A_5	GTTTCTTTAATCTTAAATTATGCTAATGATGAAAAAATTGCCG
			UFG_C121A_KOD(-)	TTTTTCCACTGACTTAGGTCTCAATACTAACTTG
			UFG_C170A_5	GGTATCTTGAAGGCTGATGCTGGTG
			UFG_C170A_KOD(-)	TGATACAGGGTCAAAATCTCTTATTTTGTTTAAATTTGC
			UFG_C200A_5	CTAAAGGTTCCGCCCATGTTGGTG
			UFG_C200A_KOD(-)	CTCCCAGATCCAACGGAAACATATAATTTTGTTC

For construction of pPIC11/ΔN UFG (**3**), the cDNA portion coding the N-terminal 36 amino acid residues were deleted by the KOD plus mutagenesis protocol (Toyobo) with pPIC11/fl UFG (**2**) as a template. For construction of pPIC11/fl UFG noCys (**4**), the corresponding codons were substituted by the KOD plus mutagenesis protocol with pPIC11/fl UFG (**2**) as a template. As Aip2p/Dld2p contains cysteine at residue positions 121, 170, and 200, the substitutions were repeated three times.

The obtained plasmid was nicked using the restriction enzyme *Aat* I (Toyobo) for transformation of *P. pastoris* strain GS115. After electroporation, the cells were spread on RD plate medium containing no His to select transformants.

2.4. Direct PCR of *Pichia* clones

Several colonies of the transformants on the selection plate were picked and resuspended in 50 μl of TE buffer containing 200 Unit/mL lyticase. The solutions were incubated at 37℃ for 70 minutes. The solution was further incubated 95℃ for 10 minutes. Then PCR amplifications with the cell lysate as template were performed using UFG_750_5 and 3' AOX1 primers, where sequence of UFG_750_5 was 5'-TTATGATCTGAAACAGCTGTCATTGGC-3' encoding a part of the Aip2p/Dld2p sequence. The PCR extensions were analyzed by agarose gel electrophoresis (Fig. 2).

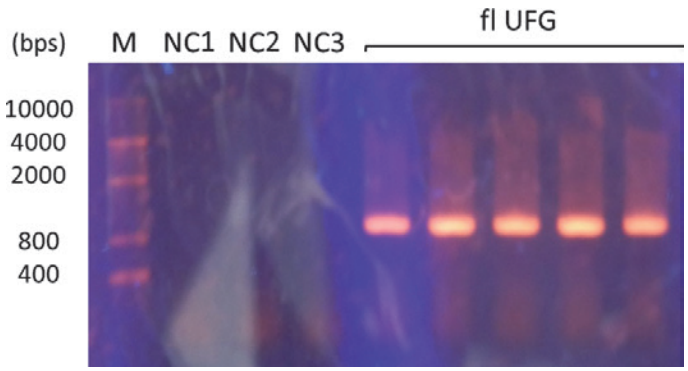

Figure 2. Confirmation of integration of Aip2p/Dld2p gene into the *P. pastoris* genome by direct PCR. The representative data performed for pPIC11/fl UFG (**2**) system were shown. All of the five selected transformants showed the bands at 1300 bps, suggesting the successful integration of the Aip2p/Dld2p gene. As negative controls, the samples from untransformed GS115 (NC1), transformed GS115 with pPIC11 containing β-lactoglobulin C121A gene (NC2), and transformed GS115 with pPIC Zα containing LacZ gene with His-tag sequence (NC3) were included, all of which did not show any bands.

2.5. Protein expression check

The obtained transformants were cultured on a small scale to check their protein expressions. Several colonies of the transformants were inoculated to 2 mL BMGY and vigorously shaken at 30℃ for two days. After the cell was centrifuged and the supernatant was discarded, the cell was resuspended in 1 mL BMMY and shaken for one day, where BMMY involves methanol for induction of target protein expression. Regarding the intracellular expression, the harvested cell was broken with 0.3 mm glass beads in breaking buffer (50 mM phosphate buffer (pH 7.4), 1 mM PMSF, 1 mM EDTA) on ice, and the obtained lysate was subjected to dot blot analysis. In the dot blot analysis, the obtained lysate was spotted onto the PVDF membrane (Atto, Japan) and treated with monoclonal anti-polyHistidine–alkaline phosphatase antibody produced in mouse (Sigma). After the antibody solution was rinsed, antibody bindings were detected with BCIP-NBT Solution Kit (Nacalai tesque). Regarding the secretion system, the supernatant of the obtained culture was investigated by SDS-PAGE.

2.6. RT-PCR

Total RNA was isolated from GS115 strain and its transformant with pPIC11/fl UFG (**2**) cultured with/without methanol induction by phenol extraction. After DNase treatment, the isolated RNA was reverse-transcribed with ReverTra Ace (Toyobo) and random primer. Adding the obtained DNA extensions template, PCR amplifications with 5' AOX1 and pPIC_EAEA_3 primers were performed, where sequence of pPIC_EAEA_3 was 5'-AGGTTCAGCCTCTCTTTTCTCGAG-3' encoding a complementary sequence of the C-terminal end of α-factor (Fig. 1).

3. Results and Discussion

3.1. Results of intracellular expression of Aip2p/Dld2p with *P. pastoris*

We first examined the intracellular expression of Aip2p/Dld2p with the plasmids pPIC Z/fl UFG (**1**). Before the expression check, we confirmed the gene integrations of our target proteins (section 2.4.). Then, the transformants were cultured with BMGY media and the protein expression was induced with BMMY media. Then, the harvested cells were broken and the lysate were subjected to the dot blot analysis with anti-His-tag antibody as described in subsection 2.5. However, we did not observe any expressions other than that of a positive control (Fig. 3).

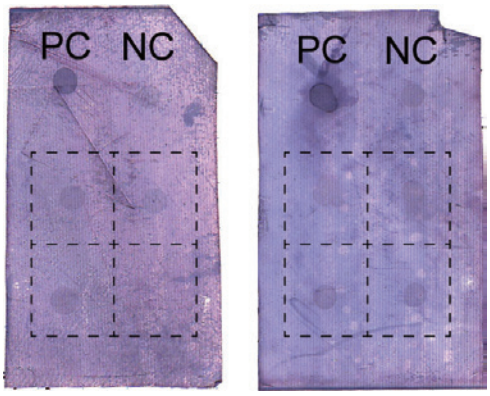

Figure 3. The results of dot blot analysis for small-scale expression check of the intracellular expression systems with pPIC Z/fl UFG (**1**). There are 8 spots of lysates of the harvested cells derived from different transformants enclosed with the broken lines. PC and NC are those of GS115 transformant with pPIC Zα containing LacZ gene with His-tag sequence and untranformed GS115 strain, respectively.

3.2. Results of secreted expression of Aip2p/Dld2p with *P. pastoris*

Next, we examined the secreted expression of Aip2p/Dld2p with the plasmids pPIC11/fl UFG (**2**), pPIC11/ΔN UFG (**3**) or pPIC11/fl UFG noCys (**4**). Before the expression check, we also confirmed the gene integrations of our target proteins (Fig. 1 for plasmid pPIC11/fl UFG **2**, and see section 2.4.). The transformants were cultured with BMGY media and the protein expression was induced with BMMY media. Then, the supernatant of the obtained cultures were subjected to SDS-PAGE. However, for all constructs, we did not observe any expressions, too (data not shown).

We supposed the negative results for the secreted expression systems were due to proteolysis of expressed protein. Therefore, we examined expressions under proteolysis-suppressed conditions for the transformants with the plasmids pPIC11/fl UFG (**2**) and pPIC11/ΔN UFG (**3**). We tested two expression conditions. One is cultivating in the presence of 0.4% casamino acids in the BMMY medium and adding 0.5 mM PMSF to the supernatant of the culture. Another is cultivating at lower temperature and shaking speed (*i.e.*, 25℃ and 110 rpm, respectively). However, under the former (data not shown) and latter (Fig. 4) conditions, the band patterns from these transformants did not show significant differences from that of the negative control, indicating the difficulty of Aip2p/Dld2p expression by the *P. pastoris* system.

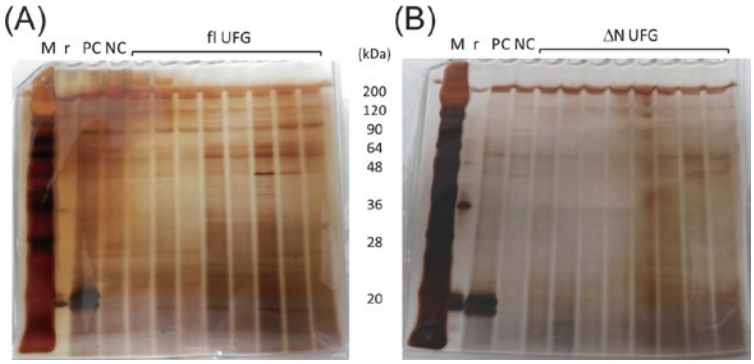

Figure 4. The sliver-stained SDS-PAGE gel images for small-scale expression check of the secreted expression systems with (A) the plasmids pPIC11/fl UFG (**2**) and (B) pPIC11/ΔN UFG (**3**). The expression conditions were at 25℃ and at a shaking speed of 110 rpm. The positive (PC) and negative (NC) controls were the samples from transformed GS115 with pPIC11 containing β-lactoglobulin C121A gene and untransformed GS115, respectively. The reference, indicated by "r", was 5 μL of 0.3 mg/mL β-lactoglobulin C121A, whose molecular weight is 18.4 kDa. For all conditions, different 8 transformants were examined.

3.4. Identification of the step at which the secretion process stopped

Then, we attempted to identify the step at which the secretion process possibly stopped. First, we examined the transcription step. We harvested the transformed GS115 with pPIC11/fl UFG (**2**) after methanol induction. As negative controls, we also prepared the same cell sample without induction and the cell sample of untransformed GS115 with methanol induction. From these cells, we extracted total mRNA, and carried out RT-PCR with the obtained mRNA as templates. We observed a band with a size of

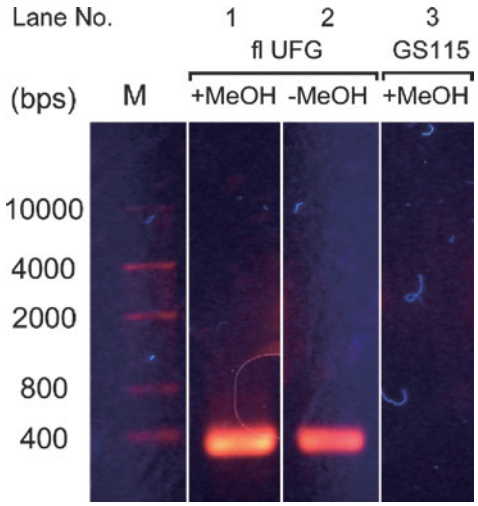

Figure 5. RT-PCR analysis for Aip2p/Dld2p-encoding mRNA in the transformed GS115 with pPIC11/fl UFG after methanol induction (lane 1). As negative controls, the same cell sample without methanol induction (lane 2) and the cell sample of untransformed GS115 with methanol induction (lane 3) were subjected to electrophoresis.

～400 bps, consistent with expected size of 360 bps, whereas the untransformed GS115 did not show the band (Fig. 5, lanes 1 and 3). It is somewhat unexpected that, even without methanol induction, the mRNA synthesis was occurring to some extent (Fig. 5, lane 2). Anyway, we confirmed that the mRNA for Aip2p/Dld2p was successfully synthesized in the transformant.

Then, the possibility was raised that the protein is expressed but not secreted albeit the presence of the secretion signal, and remained within the cell. In order to assess this possibility, we performed dot blot analyses for cell lysate of the harvested cells. However, no Aip2p/Dld2p was detected (Fig. 6).

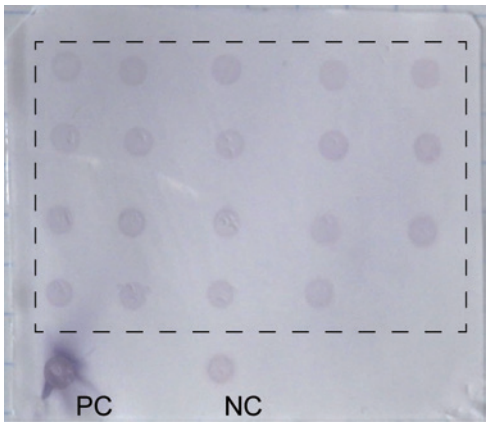

Figure 6. The results of dot blot analysis for cell lysates of the transformants with pPIC11/fl UFG (**2**) after methanol induction. There are 19 spots of lysates of the harvested cells derived from different transformants, indicated by the broken line box. PC and NC are those of GS115 transformant with pPIC Zα containing LacZ gene with His-tag sequence and untranformed GS115 strain, respectively.

3.5. Discussion for the expression of Aip2p/Dld2p with *P. pastoris*

It was shown in this study that, although mRNA encoding Aip2p/Dld2p was synthesized upon induction by methanol, Aip2p/Dld2p was present neither in extracellular media nor in the intracellular compartment. Two possibilities were raised from these observations. One is that the translation was prohibited for some reasons. Another is that, even though translation from the mRNA to polypeptide occurred, the synthesized polypeptide was immediately hydrolysed. As the present information is limited, we are not able to conclude which the case is. However, our recent examinations of Aip2p/Dld2p expression with *E. coli* system showed that the expressed Aip2p/Dld2p was obtained as inclusion body and, if Aip2p/Dld2p was co-expressed with chaperone proteins, such as GroEL and DnaK/J/GrpE, its yield was rather decreased (unpublished data), indicating that the structure of Aip2p/Dld2p is inherently unstable and susceptible to proteolysis of the cellular quality control system. Thus, we suppose that the latter possibility is more plausible. If it is the case, it will be necessary to isolate the expressed polypeptide immediately and to refold them under *in vitro* conditions in the absence of proteases. Although the examination of expression of Aip2p/Dld2p with the *P. pastoris* system was not successful, the results might provide useful information for future works for expression, physicochemical experiment, and structural analysis of this protein.

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和 文 抄 録

メタノール資化酵母を用いた蛋白質異常凝集解離新規シャペロンの発現系の検討

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出芽酵母 *Saccharomyces cerevisiae* から単離されたオリゴメリック蛋白質 Aip2p/Dld2p は病原性の蛋白質異常凝集体を解きほぐす活性を持ち、新規のシャペロン蛋白質であることが示唆されている。しかしながら、その解きほぐし活性の物理化学的メカニズムについてはほとんど調べられていない。蛋白質の物理化学的解析や構造解析には数十ミリグラム程度の試料が必要であり、そのためには高効率の蛋白質発現系を要する。そこで今回、我々は他の蛋白質で高収量が報告されているメタノール資化酵母 *Pichia pastoris* による大量発現を試みた。種々の Aip2p/Dld2p コンストラクト（全長体、N 末端欠損体、システイン→アラニン置換体）とベクター（細胞内発現系、分泌発現系）の組み合わせを試したところ、Aip2p/Dld2p をコードする mRNA の合成は見られたが、蛋白質の発現は細胞内発現系でも分泌発現系でも見られず、さらに分泌発現系の場合の細胞内にも見られなかった。Aip2p/Dld2p の天然構造は非常に不安定で、ヘテロロガスな発現の場合、翻訳直後に細胞内の品質管理機構によって加水分解を受けているのではないかと考えられる。

キーワード：Aip2p/Dld2p、メタノール資化酵母 *Pichia pastoris*、異常凝集体、解きほぐし活性。

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