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GJ: supervised the project, designed the experiments, analyzed the experimental data, and wrote the paper; PJ, RL, and LM: performed the experiments and analyzed the experimental data

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ORIGINAL RESEARCH PAPER

The contribution of individual domains of chloroplast protein AtDeg2 to its chaperone and proteolytic activities

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Abstract

The thylakoid protease AtDeg2 is a non-ATP hydrolyzing chloroplast protease/chaperone peripherally connected with stromal side of thylakoid membrane. Its linear structure consists of protease domain and two PDZ domains. To unveil the significance of individual domains, chaperone and proteolytic activities of AtDeg2, its mutated recombinant versions have been developed and their ability to suppress protein aggregation and resolubilization of protein aggregates as well as the ability to degrade substrate protein was examined in vitro. Our work reveals for the first time that AtDeg2 is able not only to suppress aggregation of denatured proteins, but to resolubilize existing protein aggregates as well. We show that PDZ2 domain contributes significantly to both chaperone and protease activities of AtDeg2, whereas PDZ1 is required for chaperone but superfluous for proteolytic activity. Protease domain – but not S-268 in its catalytic site – contributes to chaperone activities of AtDeg2. These results show an entirely new function of AtDeg2 chaperone/protease (i.e., disaggregation of protein aggregates) and allow to identify structural motifs required for "old" and new functions of AtDeg2.

Keywords

AtDeg2 chloroplast protein; *Arabidopsis thaliana*; PDZ domain; protease domain; chaperone activity; aggregation; disaggregation; protease activity

Introduction

Non-ATP hydrolyzing Deg serine endopeptidases were first discovered and elaborated as a result of studies performed on *E. coli* mutants which had not been able to grow at high temperatures (>37°C) due to inability to degrade periplasmic proteins damaged under such conditions [1,2] and these endopeptidases were later found to occur in cells of all living organisms [3]. According to MEROPS database (release 12.0), Deg proteases belong to DegP peptidase (*E. coli*) subfamily (S1C) of chymotrypsin family (S1) of clan PA.

Sixteen genes coding for proteins orthologous to *E.coli* Deg proteases (DegP, DegQ, and DegS) have been identified in the *A. thaliana* nuclear genome and marked *AtDEG1–16* [4]. It has been confirmed both by GFP tagging and by proteomic studies that products of *AtDEG 1*, *2*, *5*, *8*, and *13* are targeted exclusively to chloroplasts with AtDeg2 being sorted to stromal side and AtDeg1, 5, and 8 to luminal side of thylakoid membrane [5–8]. Contrarily, no data on intrachloroplast location of AtDeg13 has been published. As judged by the results of studies based on GFP tagging approach, remaining *AtDEG*

genes code for proteins which are targeted to mitochondria (AtDeg6, 11, 12, and 14), nucleus (AtDeg9), peroxisome (AtDeg15), or have a dual chloroplast/mitochondrion (AtDeg3 and 10) or nucleus/mitochondrion location (AtDeg7), whereas two of the sixteen genes seem to be pseudogenes (*AtDEG4* and *AtDEG16*) [8]. An ability of a majority of chloroplast AtDegs to degrade in vitro artificial protein substrates has been convincingly proven. Namely, recombinant forms of AtDeg1 and 8 effectively degraded β-casein [6,9], whereas AtDeg2 degraded gelatin [5], fluorescence labeled casein [10], and β-casein [11]. Thus, AtDeg1, 2, and 8 were demonstrated in vitro to be bona fide proteases. More importantly, a few native chloroplast proteins have been shown to be recognized and degraded by chloroplast-targeted AtDegs in response to exogenous stressing factors. Some of this data come from studies on the ability of recombinant chloroplast AtDegs to degrade PsbA (D1) apoprotein in thylakoid samples incubated in vitro under photoinhibitory conditions [5] or in thylakoids isolated from leaves which underwent a photoinhibitory treatment after detachment [9,12]. More direct data were accumulated by assessing degradation of PsbA in high irradiance-exposed leaves of various *deg* mutants [13,14]. By combining the results of both types of studies, it was established that AtDeg1 and AtDeg5/8 (along with AtFtsH heterocomplex) cooperate in catalyzing the degradation of photodamaged PsbA, which in vivo is inherent to PSII repair cycle. A role AtDeg2 in photoinhibition-related PsbA degradation remains less clear [5,15,16]. Furthermore, AtDeg1 has been suggested to be responsible for degradation of PsbD, PsbS, Lhcb4, and cytb6 apoproteins under photoinhibitory conditions [12] and Lhcb6 apoprotein has been found to be a target for short stress-induced degradation catalyzed by AtDeg2, as judged by an inability of *deg2* mutants to cleave Lhcb6 in leaves stressed by elevated irradiance, heat, high salt, and wounding [16]. Using the same approach, PsbF apoprotein was found to be the target for AtDeg5-related degradation in leaves stressed by wounding [17]. Little is known about the physiological significance of chloroplast-targeted AtDegs, and AtDeg2 in particular under nonstressing conditions. AtDeg5 was shown in this respect to be involved in regulation of chronological progression of ontogenetic stages, leaf morphology, and chloroplast ultrastructure [18] and AtDeg2 was demonstrated to be required for morphology and ontogenesis of chloroplasts in juvenile rosette leaves [16].

A crystal structure of AtDeg2 has been solved recently based on X-ray diffraction analysis of a recombinant version of the protease [11]. A primary structure of pre-AtDeg2 molecule consists of a catalytic triad (H-159 D-190 S-268) – containing protease domain (positions 110–313) as well as two PDZ domains (post synaptic density protein, *Drosophila* disc large tumor suppressor, zonula occludens-1 protein) marked PDZ1 (positions 314–422) and PDZ2 (positions 423–577), with protease region being preceded (positions 70–109) and PDZ2 domain being ensued (positions 578–607) by stretches which do not contain any conserved motifs. It is regarded that in vivo AtDeg2 forms a hexamer (supposedly representing proteolytically inactive state) of a "sealed cage" type, comprising two trimer rings which are stacked upon each other and form a catalytic chamber, the access to which is limited to six small pores located within PDZ1 domains. It was suggested that trimeric units may be rearranged into higher oligomeric states (12-mers and 24-mers, thought to be proteolytically competent) by disruption of protease domain/PDZ2 interaction [11]. Besides proteolytic function, AtDeg2 may have chaperone activity as well, consisting in an ability to prevent aggregation of DTT-denatured lysozyme in vitro [11]. This would imply that the regulatory functions of AtDeg2 in vivo may be fulfilled due to the interplay between its protease and chaperone activity. We hereby demonstrate that AtDeg2 may act not only as a protein aggregation preventing agent, but has a potency to resolubilize protein aggregates as well. In order to establish what is the contribution of structural motifs to proteolytic and two chaperone activities of AtDeg2, its mutants in which individual domains have been changed or deleted were prepared and tested. Our results indicate that both PDZ domains and the protease domain excluding catalytically active S-268 are required for AtDeg2 to exhibit chaperone activities and that PDZ1 is unnecessary to maintain proteolytic activity.

Material and methods

Protein expression and purification

AtDEG2 was obtained by RT-PCR from *A. thaliana* cDNA using D2MNtagF (CACCGATGAAAGTTCCAATCCTCCTC) and D2NtagR (TTATGCCCACACCAGTCCATCAAAGC) primers, whereas deletion *AtDEG2* mutants were synthesized by GeneArt Gene Synthesis service (Thermo Fisher Scientific, USA). *AtDEG2* and its deletion mutants were cloned into pENTR/SD/D-TOPO (Thermo Fisher Scientific, USA) to generate entry clones. In order to obtain *AtDEG2 S268G* entry clone, site-directed mutagenesis of *AtDEG2* pENTR/SD/D-TOPO was performed using D2S268GF (CCAGGGAATGGTGGTGGCCCT) and D2S268GR (AGGGCCACCACCATTCCCTGG) primers. All expression clones were generated by an LR recombination reaction between entry clones and destination vector Champion pET300/NT-DEST (Thermo Fisher Scientific, USA) using Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific, USA). Each AtDeg2 recombinant version possessed N-terminal His-tag and was expressed in *E. coli* BL21 (CodonPlus) at 37°C. Briefly, 100 mL of LB medium containing 100 μg/mL rifampicin, 36 μg/mL chloramphenicol and 12.5 μg/mL tetracycline was inoculated with *E. coli* cells transformed with recombinant expression clones and shaken at 37°C until the medium reached OD_{600} of 0.4–0.5. Expression of each AtDeg2 recombinant version was induced in the presence of 1 mM isopropyl-β-D-1-thiogalactopyranoside for 3 h and the cells were harvested by centrifugation at 10,000 *g* for 10 min at 4°C. The cell pellet was suspended in an ice-cold buffer A (8 M urea, 100 mM Na_3PO_4, and 10 mM Tris HCl pH 8.0) and lysed by performing three freeze-thaw cycles using a liquid nitrogen and 42°C water bath. Afterwards, the cell lysate was sonicated on ice using three 1-minute pulses and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant then was added to 3 mL of Ni-NTA agarose resin (Roche Diagnostics, Germany), the mixture was gently agitated for 30 min, washed two times with buffer A, two times with buffer B (containing 8 M urea, 100 mM Na_3PO_4, and 10 mM Tris HCl pH 6.3), and once with buffer B supplemented with 10 mM imidazole. Subsequently, the recombinant proteins were eluted with buffer B containing 100 mM imidazole and purified by a preparative SDS-PAGE – the appropriate bands were excised and desirable polypeptides electroeluted (Model 422 Electro-Eluter; BIO-RAD, Italy). The eluates were equilibrated with a buffer containing 20 mM NaH_2PO_4 and 50 mM Tris HCl pH 7.6 by performing several buffer exchange steps on Amicon Ultra Centrifugal Filter Units (Merck Millipore, Ireland). Purified proteins were flash-frozen in liquid nitrogen and stored at −80°C.

Chaperone activity assay

The chaperone activity of AtDeg2 consisting in preventing a formation of aggregates of DTT-denatured lysozyme was studied by incubating 200 μL mixtures of 40 μg lysozyme and 0, 3, or 6 μg of AtDeg2 or BSA (bovine serum albumin) at 28°C in a buffer containing 50 mM PBS (phosphate-buffered saline), 3 mM NaH_2PO_4, 7.5 mM Tris HCl pH 7.6, and 20 mM DTT (dithiotreitol).

The chaperone activity of AtDeg2 consisting in disaggregation of protein aggregates was studied by monitoring disaggregation of aggregates of DTT-denatured lysozyme. A 200-μL solution containing 40 μg lysozyme was incubated at 28°C for 220 min, so that the aggregation was at saturation point, and then 0, 3, or 6 μg of AtDeg2 or BSA was added and the incubation continued for next 220 min.

The aggregation of lysozyme and disaggregation of lysozyme aggregates was recorded as changes in light absorption at 360 nm due to light scattering [11].

Proteolytic activity assay

The proteolytic activity of AtDeg2 was measured by monitoring degradation of β-casein as a substrate protein. A 10-μL mixture of 0.5 μg of AtDeg2 or BSA and 1 μg β-casein in a buffer containing 20 mM NaH_2PO_4 and 50 mM Tris HCl pH 7.6 was incubated at

37°C. The samples of the incubation mixtures were withdrawn at 0, 6, and 12 h, subjected to SDS-PAGE, and the gels were stained with silver method.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to [19] using 12% acrylamide as a resolving gel. After the electrophoresis, the polypeptides were either silver-stained essentially according to [20] or electrotransferred onto PVDF membranes and reacted with anti-Deg2 (used at 1:1,000) and anti-His-tag (used at 1:2,500) antibodies and immunodecorated applying goat antirabbit HRP conjugated IgG (1:50,000) (Agrisera, Sweden) or antimouse HRP conjugated IgG (1:50,000) (GE Healthcare Life Sciences, UK), respectively.

Antibodies

Anti-Deg2 specific affinity-purified polyclonal antibody (rabbit) was custom produced (GenScript, USA) against a KLH-conjugated synthetic peptide corresponding to C-terminal sequence TQALDQGIGDSPVS (positions 582–595 in pre-AtDeg2). The anti-His6 specific polyclonal antibody (mouse) was purchased from GE Healthcare Life Sciences (Little Chalfont, UK).

Results

The contribution of individual domains of AtDeg2 to its chaperone activity

To get the idea of where chaperone activity of AtDeg2 is located, several AtDeg2 mutated versions were developed in which individual structural motifs were changed or deleted. It was demonstrated previously that chaperone (foldase) activity of AtDeg1 was significantly reduced by substitution of proteolytically active serine by alanine (S282A) and this result suggested that chaperone activity of AtDeg1 had been located within a protease domain [21]. Therefore, we generated both AtDeg2 S268G mutant and the one lacking whole protease domain (ΔP). Besides, the mutants were obtained in which PDZ domains were deleted either individually (ΔPDZ1 and ΔPDZ2) or simultaneously [Δ(PDZ1+PDZ2)]. The mutant proteins were recombinantly expressed in *Escherichia coli* and purified to homogeneity, as judged by the results of SDS-PAGE and immunoblotting (Fig. S1).

The ability of AtDeg2 to prevent a formation of protein aggregates (= holdase chaperone activity) was assayed in vitro using lysozyme getting unfolded due to the reduction of disulfide bridges. In preliminary experiments, it was demonstrated that the incubation in the presence of DTT stimulated a formation of lysozyme aggregates with a halftime of about 80 min and that there was no unspecific suppression of formation of lysozyme aggregates in the presence of any protein (as exemplified by BSA); moreover, BSA did stimulate the extent of the aggregation (Fig. 1A). In accordance with the results of a previous report [11], wild type AtDeg2 displayed an ability to prevent a formation of lysozyme aggregates from the start of incubation, which resulted in 3.5/4-fold decrease (with regard to lysozyme alone assay) in light absorption at 360 nm, at a saturation point (Fig. 1A). In contrast to what was found for recombinant AtDeg1 [21], the substitution of proteolytically active S-268 by G did not lead to any loss of AtDeg2 ability to suppress the aggregation of lysozyme. The deletion of the whole protease domain reduced the efficiency of the suppression but AtDeg2 ΔP still significantly suppressed lysozyme aggregation with regard to lysozyme alone assay (Fig. 1B). In turn, the deletion of any PDZ domain or both resulted in a complete loss of the aggregation-suppressing activity (Fig. 1B). Together, the results displayed in Fig. 1 point to the absolute importance of PDZ domains, but not of S-268 for AtDeg2 ability to prevent a formation of protein aggregates. The remaining parts of protease domain were shown to contribute to the preventing activity.

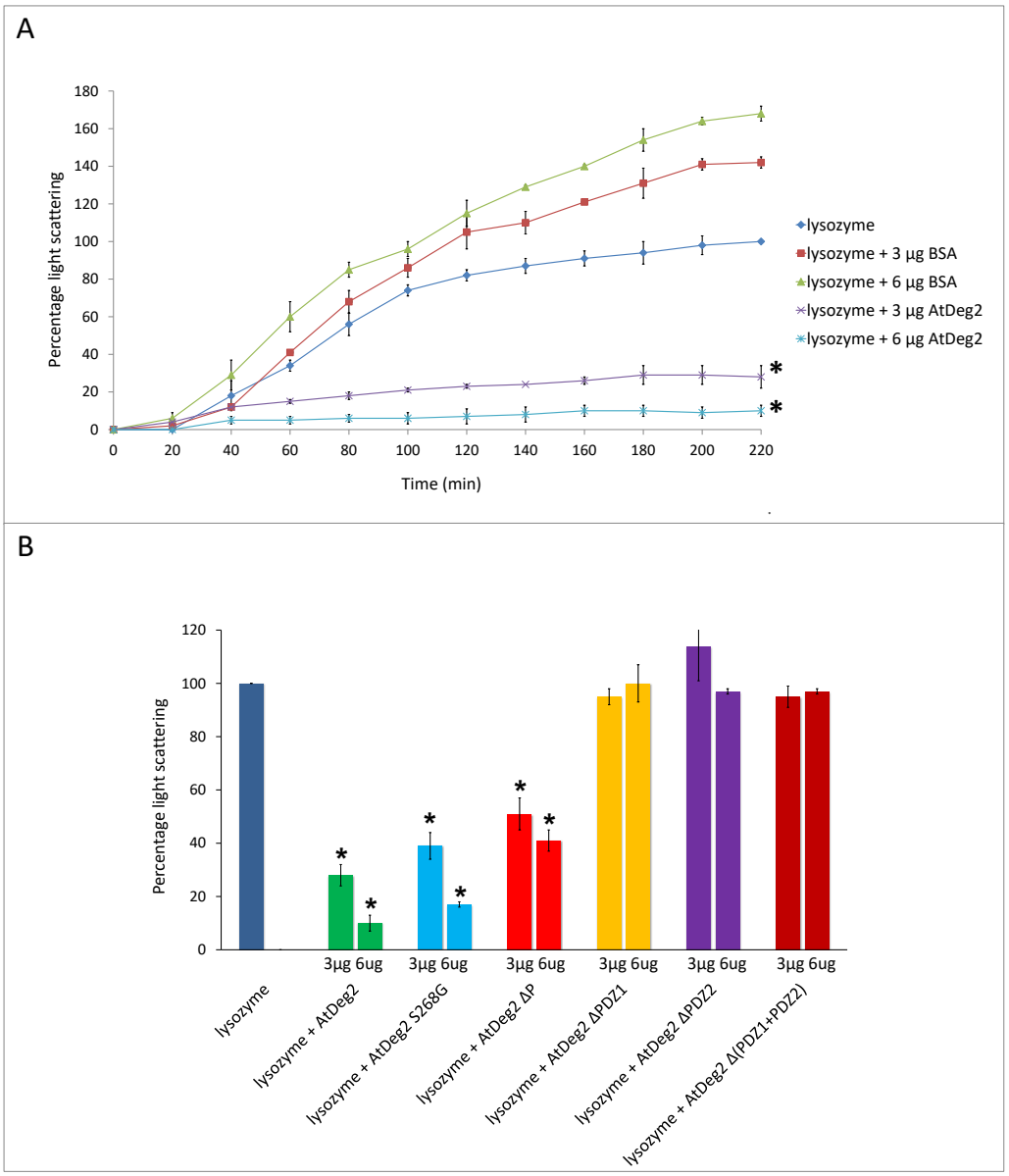

Fig. 1 The role of individual domains of AtDeg2 in preventing formation of aggregates of DTT-denatured lysozyme. Time course of aggregation (0–220 min) of 40 µg of DTT-denatured lysozyme was followed in the presence of 0, 3, and 6 µg of BSA or AtDeg2 (**A**), and the aggregation 40 µg of DTT-denatured lysozyme in the presence of 0, 3, and 6 µg of AtDeg2, AtDeg2 S268G, AtDeg2 ΔP, AtDeg2 ΔPDZ1, AtDeg2 ΔPDZ2, and AtDeg2 Δ(PDZ1+PDZ2) was measured at a saturation time point in the assay containing lysozyme alone, i.e., at 220 min (**B**). The aggregation was recorded as changes in light absorption at 360 nm due to light scattering by the aggregates and the values indicate percentages of absorption signal at 360 nm identified in the assay containing lysozyme alone at a saturation time point (220 min, 100%). The values represent means ±*SD* of three assays. The asterisks indicate the data for which recombinant AtDeg2 versions vs. lysozyme alone differences were significant ($p < 0.01$).

Next, we tested whether AtDeg2 exhibit a potency to resolubilize already existing protein aggregates. To do so, lysozyme was allowed to aggregate in the presence of DTT up to the moment when the absorption signal was saturated (220 min); then, various AtDeg2 versions or BSA were added and the incubation was continued for subsequent 220 min. It was found that BSA did not have any disaggregase activity, conversely, the 360-nm absorption signal rose modestly during 220 min up to 130–150% of the value recorded at the moment of BSA addition (Fig. 2A). In contrast to this, both wild type AtDeg2 and its S268G mutant allowed aggregated lysozyme to resolubilize, so that 360-nm absorption signal decreased 1.5 to 2.5-fold within 220 min of incubation of

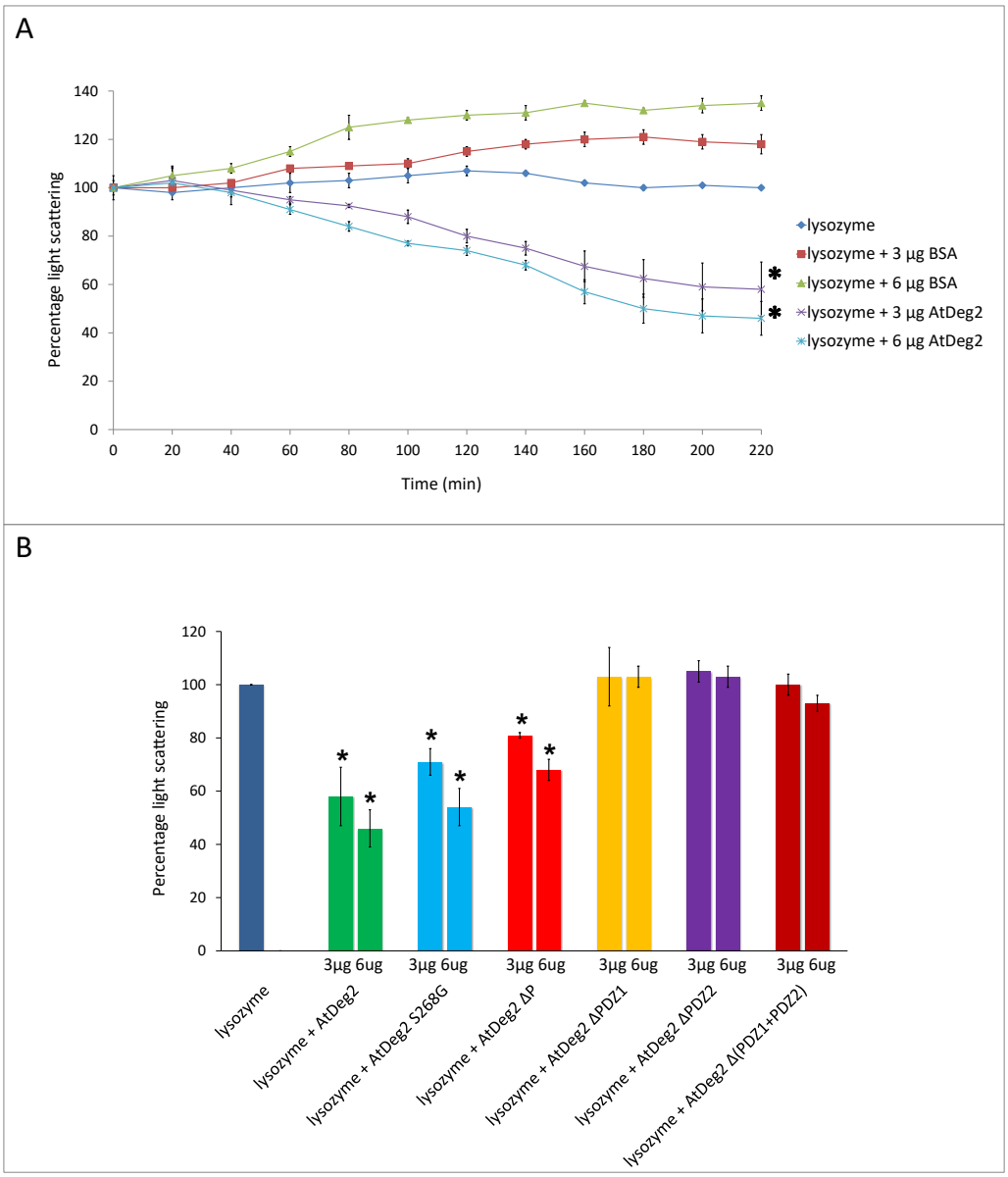

Fig. 2 The role of individual domains of AtDeg2 in disaggregation of aggregates of DTT-denatured lysozyme. Time course of disaggregation (220–440 min) of aggregates of 40 µg of DTT-denatured lysozyme was studied by the addition of 0, 3, and 6 µg of BSA and AtDeg2 at a saturation time point in the aggregation assay containing lysozyme alone (220 min) (**A**), and the disaggregation of aggregates 40 µg of DTT-denatured lysozyme in the presence of 0, 3, and 6 µg of AtDeg2, AtDeg2 S268G, AtDeg2 ΔP, AtDeg2 ΔPDZ1, AtDeg2 ΔPDZ2, and AtDeg2 Δ(PDZ1+PDZ2) was measured at 440 min time point (**B**). The disaggregation was recorded as changes in light absorption at 360 nm due to light scattering by the remaining aggregates. The moment of addition of BSA or AtDeg2 is indicated as 0 min and the values represent the percentages of the absorption signal at 360 nm which was recorded at 0 min (100%). The values represent means ±*SD* of three assays. The asterisks indicate the data for which recombinant AtDeg2 versions vs. lysozyme alone differences were significant ($p < 0.01$).

aggregated lysozyme (Fig. 2A,B). The disaggregation efficiency of AtDeg2 ΔP was reduced in comparison with wild type AtDeg2 and AtDeg2 S268G, still the decrease was significant with regard to lysozyme alone assay (Fig. 2B). The deletion of any PDZ domain or both abolished the disaggregase activity of AtDeg2 entirely (Fig. 2B). Thus, AtDeg2 not only prevented formation of lysozyme aggregates in the presence of DTT, but had an efficient disaggregase activity, the exhibition of which requires both PDZ domains but not S-268 at the protease catalytic site. Remaining part of protease domain seemed to contribute to the disaggregating activity.

The contribution of individual domains of AtDeg2 to its proteolytic activity

To examine the proteolytic activity of recombinant versions of AtDeg2, they were incubated in the presence of β-casein as a model substrate and the results are presented in Fig. 3 (a very faint β-casein degradation product is visible already at 0 min of incubation, it makes as little as 4% of the β-casein band). In preliminary experiments, it was demonstrated that β-casein is devoid of any autoproteolytic activity and that it is not unspecifically hydrolyzed by arbitrarily selected protein, exemplified by BSA. The majority of β-casein was degraded within first 6 h and 12 h of incubation in the presence of wild type AtDeg2, whereas S268G mutation as well as deletion of protease domain abolished efficiently the degradation. The deletion of PDZ1 domain had almost no impact on the efficiency of degradation of β-casein, whereas ΔPDZ2 and Δ(PDZ1 and PDZ2) mutants exhibited no degradation activity. This demonstrates that PDZ2, but not PDZ1, is required for the exhibition of AtDeg2's proteolytic activity.

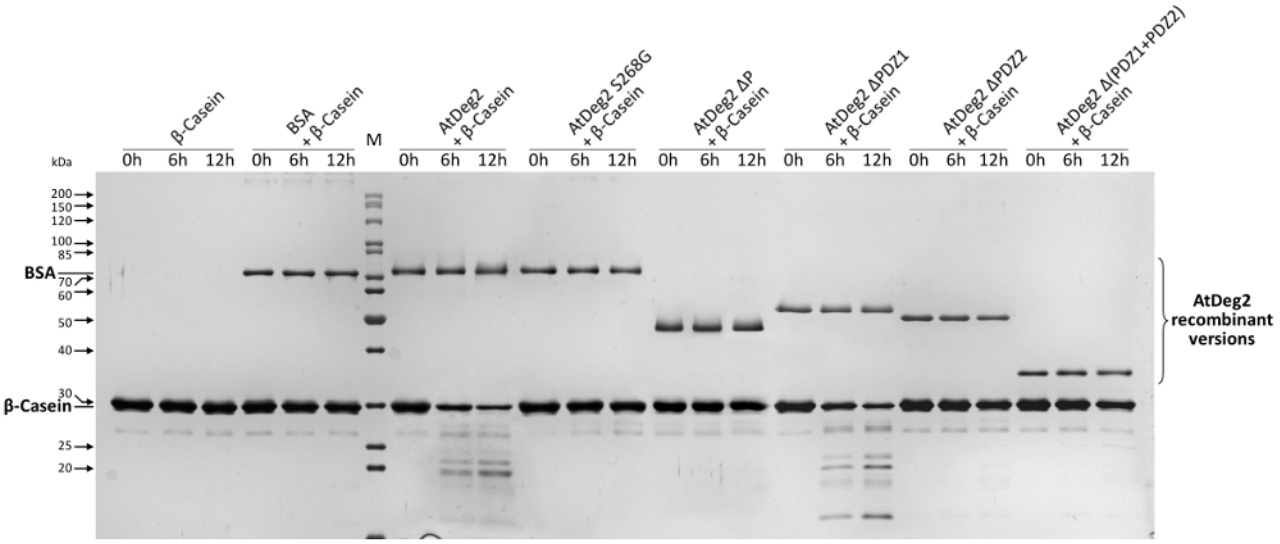

Fig. 3 The role of individual domains of AtDeg2 in its proteolytic activity. The proteolytic activity was assayed by incubation for 0, 6, and 12 h of mixtures containing 1 μg β-casein alone or with the addition of 0.5 μg of individual recombinant AtDeg2 versions or 0.5 μg of BSA. The image shows the results of one typical proteolytic assay out of three assays which were performed for recombinant AtDeg versions and BSA.

Discussion

In this study, we have corroborated that recombinant AtDeg2 is a chaperone, able to suppress DTT-induced protein aggregation (lysozyme) in vitro (Fig. 1). Even more importantly, our study revealed for the first time that AtDeg2 exhibits a potency to resolubilize already existing lysozyme aggregates as well (Fig. 2). This finding provides significant insight into AtDeg2 function since only one non-ATP hydrolyzing chaperone able to induce protein disaggregation has been identified previously, namely the 43-kDa subunit of a chloroplast cpSRP43/cpSRP54 heterodimer, which was found to be responsible for targeting of precursors of LHCPII to thylakoid membrane [22,23]. It was demonstrated that cpSRP43 may bind a L18 motif of LHCPII and resolubilize efficiently LHCPII aggregates [24,25]. Here, we demonstrate that recombinant, non-ATP hydrolyzing AtDeg2, is able to resolubilize lysozyme aggregates. The results of the studies on the contribution of individual structural motifs to AtDeg2's chaperone activities show that the motifs are not located in the catalytic center, in contrast to the finding that the substitution of proteolytically active S-282 by A in AtDeg1 strongly decreased MalS refolding activity [21]. The remaining parts of protease domain seem to contribute to the chaperone activities of AtDeg2 and the deletion of one or both PDZ domains abolished aggregation-suppressing as well as disaggregase activities of AtDeg2 (Fig. 1 and Fig. 2). This leads to the conclusion that PDZ domains and protease domain

(but not S-268 at the protease catalytic site) are required for both types of chaperone activity. It may stem from the fact that AtDeg2 12- and 24-mers, which are proposed to be active states in terms of chaperone activity, arise by the interaction of trimeric subunits through interfaces involving PDZ1 and PDZ2 and loop LA of the protease domain, but not S-268 site [11]. Another point of interest was to determine the contribution of AtDeg2's structural domains to its protease activity. It was rather unexpected that the deletion of PDZ1 had no impact on protease activity, whereas this activity was abolished completely by the removal of PDZ2 (or both PDZ domains) (Fig. 3) since both PDZ domains are thought to be engaged in formation of interfaces necessary for assembly of AtDeg2 proteolytically active oligomeric states. Thus, ΔPDZ1 as well as ΔPDZ2 forms should be locked in a trimeric state. The interfaces mediating monomer trimerization are proposed to consist of protease domains only, thus trimerization of ΔPDZ1 and ΔPDZ2 forms seems to be fully possible [11]. It may be assumed that in the absence of peptide, substrates may still be bound to AtDeg2 inside a groove located in PDZ2 between helix αI and strand β22 – this would elucidate why trimers of AtDeg2 ΔPDZ1 form remain proteolytically active, in a way similar to ΔPDZ2 mutant of DegP protein [26,27]. On the other hand, in AtDeg2 ΔPDZ2 and Δ(PDZ1+PDZ2) mutants no grooves located in PDZ domains may be available for the substrates and this would explain why these forms make proteolytically inactive trimers.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3570/0:

Fig. S1 SDS-PAGE and immunoblot analyses of individual recombinant AtDeg2s confirm the identity and homogeneity of AtDeg2, AtDeg2 S268G, AtDeg2 ΔP, AtDeg2 ΔPDZ1, AtDeg2 ΔPDZ2, and AtDeg2 Δ(PDZ1+PDZ2).

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