DOCTORAL THESIS

Domain Collaboration of Protein Disulfide Isomerase-P5 Brings Intermolecular Potent Isomerase Activity

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CONTENTS

Abbreviations:

DTT, dithiothreitol

hyLYS, lysozyme multimers with significant intermolecular disulfides and hyper molecular mass

IPTG, isopropylthiogalactoside

2-ME, 2-mercaptoethanol

M. lyso, *Micrococcus lysodeikticus*

mPDI-P5, mature protein disulfide isomerase-P5

NEM, N-ethylmaleimide

NR, non-reducing conditions

PBS, phosphate-buffered saline

Protein disulfide isomerase-P5, PDI-P5 or P5

R, reducing conditions

T-TBS, TBS containing 0.05% Tween 20

ABSTRACT

Protein disulfide isomerase-P5 (P5) is thought to have important functions as an oxidoreductase, however, molecular functions of P5 have not been fully elucidated. P5 has been reported to have insulin reductase activity and inhibit lysozyme refolding by formation of lysozyme multimers with hyper molecular mass inactivated by intermolecular disulfides (hyLYS) in oxidative refolding of reduced denatured lysozyme. To explore the role of each domain of P5, I investigated the effects of domain deletion and Cys-Ala mutants of P5 on insulin reduction and the oxidative refolding of the lysozyme. The mutants of catalytic cysteines, C36/39A, C171/174A, and C36/39/171/174A inhibited the lysozyme refolding almost similarly to P5, and even *b* domain without catalytic cysteines showed moderate inhibitory effect, suggesting that the *b* domain played a key role in the inhibition. Western blotting analysis of the refolding products indicated that the catalytic cysteines in both the *a* and *a'* domains cross-linked lysozyme comparably to form hyLYS resistant to trypsin, in which *b* domain was suggested to capture lysozyme for the significant sulfhydryl oxidation. The mutant of the conserved cysteines in *b* domain, C272/278A, did not form hyLYS, however, showed predominant reductase activity, implying that P5 functioned as a potent sulfhydryl oxidase and a predominant reductase depending on the circumstance around C272/278. These results provide new insight into the molecular basis of P5 function.

Key words: Protein disulfide isomerase-P5; PDIA6; Sulfhydryl oxidase activity; Protein regulation; Intermolecular disulfide cross-linking.

INTRODUCTION

Protein disulfide isomerase (PDI) family, which typically has CXXC active sites, catalyzes the formation, isomerization, and reduction of disulfide bonds of proteins (App. 1 and 2) [1, 2]. PDI plays the centering role for protein folding [3-5], and it is likely that each member plays a distinct role in protein maturation as an oxidoreductase [6]. Recently, Araki *et al.* have reported a hierarchical electron transfer network centering on the PDI-Ero1α complex, including ERp57, ERp46, and PDI-P5 (P5, ERp5 or PDIA6), suggesting a cooperative quality control network in the redox pathway (App. 3) [7].

P5 has also been reported to be involved in asymmetric organogenesis in zebrafish [8], regulation of human platelet function [9], and regulation of Ca^{2+} signaling in mice and *Caenorhabditis elegans* [10, 11], human breast cancer invasion and metastasis [12], human tumor immune evasion triggered by disulfide reduction of the membrane-anchored ligand MICA by P5 [13]. In plants, GmPDIM (a soybean orthologue of human P5) has been reported to be involved in the folding of storage proteins [14]. PDIL2;3 (a rice orthologue of human P5) has been shown to have a function to retain GFP-AB (a protein body I-marker), the N-terminal region of α-globulin fused to GFP, within the endoplasmic reticulum (ER) by accumulating it via intermolecular disulfide cross-linkages, and promote sorting of the

cross-linked α-globulin to protein body I *in vivo* (App. 5) [15, 16]. Boar P5 has been reported to catalyze formation of lysozyme multimers with intermolecular disulfide bonds and hyper molecular mass (hyLYS) in oxidative refolding of reduced denatured lysozyme (App 6) [17]. This property of boar P5 is similar to that of rice P5 to introduce intermolecular disulfides into reduced denatured α-globulins (C79F) to form α-globulin multimers with hyper molecular mass *in vitro* [15].

Considering that P5 is localized not only in the ER but also in mitochondria [18], and membrane surface [13], P5 is thought to have important functions as an oxidoreductase. However, molecular functions of P5 have not been fully understood yet. Kikuchi *et al.* has reported that *a* domain of human P5 is more important for isomerase activity than *a'* domain using Cys-Ser mutants [19]. However, years later, Tian *et al.* have mentioned that Cys-Ser mutants of PDI have reducing activity in scrambled RNase A refolding due to its hydroxyl group [20]. In addition, several protein disulfide isomerases such as ERp44 with an active-site sequence, CRFS [21], and PDILT with those, SKQS and SKKC [22], have been identified. These imply that there is a possibility of different interpretation of the study using Cys-Ser mutants [19].

In the present study, I investigated the effects of domain-deletion and Cys-Ala variants of P5 on insulin reductase activity, and the oxidative refolding of the lysozyme followed by

Western blotting analysis of the refolding products, to elucidate the role of each domain of P5 and its molecular function. This study demonstrated that P5 formed hyLYS resistant to trypsin, in which the redox-active cysteines in both the *a* and *a'* domains participated almost comparably. And also, the conserved C272/278 in the *b* domain were suggested to play a key role to capture the substrate lysozyme in the formation of hyLYS, and implied a possible activity shift of P5 from a potent sulfhydryl oxidase to a predominant reductase vice versa dependent on the circumstance around C272/278.

MATERIALS AND METHODS

1. Plasmid construction for expression of full-length P5 and its variants

DNA fragments encoding the *a*, *aa'*, *a'b*, or *b* domains of boar mature P5 were amplified by PCR using the plasmid pET-mPDI-P5 [17] as a template and subcloned into the *Bam* HI and *Hin*d III sites of the expression vector pET30a(+) (Novagen). The mutations C36/39A, C171/174A, and C36/39/171/174A were introduced by direct mutagenesis using Cys-Ala single mutants as templates, which were produced by direct mutagenesis by PCR with complementary mutagenic primers (Table 1). After PCR, the template DNA was digested with *Dpn* I [23], followed by transformation and plasmid preparation. The double mutation of C272/278A with complementary mutagenic primers (Table 1) was not successfully introduced into pET-P5, thereby, an alternative plasmid, pUC-mPDI-P5 was constructed and used as a template. The DNA fragment encoding C272/278A in pUC118 was subsequently inserted into the *Bam*HI and *Hin*dIII sites of the expression plasmid, pET30a(+). The pUC118 was kindly provided by Professor Miyakawa at Yamanashi University. The nucleotide sequences of the domain and Cys-Ala-substitution variants in pET30a(+) were confirmed by sequencing using the dye terminator method on a 3130x1 genetic analyzer (Applied Biosystems).

2. Expression and purification of P5 and its derivatives

The expression vectors were transformed into competent HMS174 (DE3) *Escherichia coli* (*E. coli.*). Cells harboring the plasmids were grown at 37°C until the OD_{600} reached 0.8 – 1.0. Expression was induced by addition of IPTG to a final concentration of 0.4 mM, and the cells were harvested after 3 hr of incubation at 37°C by centrifugation (12000 × *g*, 10 min, 4°C).

I combined ammonium sulfate precipitation and three types of chromatography to highly purify P5 and its variants, as described in Table 2. Briefly, the cells from 50 mL of culture were sonicated on ice, followed by centrifugation (12000 × *g*, 10 min, 4°C). Supernatant was subjected to ammonium sulfate precipitation (80% saturation; Table 2) for 1 hr on ice. The precipitate was resolubilized and applied to a phenyl-Sepharose CL-4B column (GE Healthcare Bio-Sciences). Proteins including P5 were eluted with Tris-AcOH basal buffer containing 1% Triton-X 100 (pH 6.5), then loaded onto a column of Chelating Sepharose Fast Flow (GE Healthcare Bio-Sciences). His-tagged P5 was eluted with phosphate basal buffer containing 500 mM imidazole (pH 7.8). After buffer-exchange with 20 mM Tris-HCl (pH 7.0) by ultrafiltration (4000 × *g*, 10 min, at 4°C) (Amicon-Ultra-4 centrifugal filter unit, Millipore), the N-terminal His-tag was digested with recombinant enterokinase (Novagen) for 16 hr at

20°C. For further purification, (Ala-Met-Ala-Asp-Ile-Gly)-P5 was applied to a column of DEAE-Sephacel (Sigma). P5 was eluted with Tris-HCl buffer containing 300 mM NaCl (pH 7.0), and buffer-exchanged with PBS (pH 7.4) by ultrafiltration as described above. Purification of Cys-Ala mutants except for C272/278A was performed in the same manner as described above. The *a*, *aa'*, *a'b*, and *b* domains and C272/278A were also purified in a similar manner as full-length P5 with a slight modification (Table 2).

3. Insulin turbidity assay

Insulin turbidity assay was carried out essentially according to Holmgren's method [24] with a slight modification [25]. Insulin solution in the presence or absence of P5 or Cys-Ala mutants or *a'b* was preincubated in a sample cuvette for 10 min at 25°C. The reactions catalyzed by P5 or the variants were initiated by addition of DTT, giving a final concentration of 1.0 mM insulin/100 mM sodium phosphate/1.0 mM EDTA·2Na/1.0 mM DTT (pH 7.0) in the presence of P5 or the variants. The absorbance at 540 nm at 25°C was monitored for 2200 s. Measurements were performed in duplicate with a dual wavelength spectrophotometer (U-2001, Hitachi).

4. Oxidative refolding assay of reduced denatured lysozyme

Lysozyme refolding assay was performed essentially according to the method of Puig and Gilbert [26, 27] as described previously [17] (Fig. 1). Lysozyme was reduced and denatured by incubation in denaturation buffer (8 M urea/130 mM 2-mercaptoethanol/25 mM Tris-HCl, pH 8.6) for 90 min at 37°C. Denaturation was quenched by 10-fold dilution with 0.1 M acetic acid, pH 4.0. The denatured lysozyme was refolded in refolding buffer for 60 min at 37°C, giving its final composition of 1.4 μM lysozyme/100 mM HEPES/20 mM NaCl/2 mM EDTA/5 mM $MgCl_2$/5 mM GSH/0.5 mM GSSG, pH 7.0. All refolding experiments were performed in a volume of 200 μL in siliconized polypropylene tubes to minimize protein adsorption [17, 26]. Aliquots of 30 μL of the lysozyme refolding solution were sampled every 20 min and added to the *Micrococcus lysodeikticus* cell wall suspension (0.5 mg/mL of 50 mM sodium phosphate, pH 6.2) to initiate bacteriolysis, accompanying the decrease in absorbance at 540 nm. Measurements were performed in triplicate in the presence or absence of P5 or its variants using a dual wavelength spectrophotometer (U-2001, Hitachi) for 304 sec at 25°C. Lysozyme activity was derived from the slope of the decrease in absorbance against that of native lysozyme.

5. Western blotting analysis

Lysozyme refolding products, after incubation for 0 – 60 min in the presence or absence of P5 or its variants, were boiled in 2% SDS-PAGE sample buffer containing 3 mM 2-ME or 5 mM N-ethylmaleimide (NEM) and separated in a Tris-Tricine buffer system on 7.5% polyacrylamide gels [28]. Proteins in the gel were transferred onto PVDF membranes (Millipore) and analyzed by Western blotting with antibodies against both lysozyme and P5. The following primary antibodies were used: anti-lysozyme rabbit polyclonal antibody (α-LYS) (ab34799; Abcam) (diluted 1:10000 with 3% ECL blocking agent/T-TBS); anti-human PDI-P5 rabbit polyclonal antibody (α-P5), raised against amino acids 192-440 of human P5 (ERp5 (H-249): sc-292227; Santa Cruz Biotechnology) (diluted 1:500 with 3% ECL blocking agent/T-TBS). After washing three times with T-TBS, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG) antibody (Sigma) solution (diluted 1:5000 with 3% ECL blocking agent/T-TBS), followed by ECL Western blotting detection system (GE Healthcare Biosciences).

6. Trypsin sensitivity of refolding lysozyme in the presence or absence of P5 and C36/39/171/174A

For the negative control, 1.4 μM of lysozyme was prepared by dissolving with 50 mM sodium phosphate, pH 6.2, to be the sample of native lysozyme. Recued denatured lysozyme was prepared and quenched in the same manner as described in MATERIALS AND METHODS 4. The quenched lysozyme was used as a sample of reduced denatured lysozyme, while the lysozyme refolded for 60min in the presence or absence of P5 and C36/39/171/174A was used as a sample of refolding lysozyme. The native lysozyme and reduced denatured lysozyme were treated with trypsin (V511A; Promega, Madison, WI) at enzyme/substrate weight ratio of 1:25 for 60 min at 37°C. The refolding lysozyme in the presence or absence of P5 or C36/39/171/174A was treated with trypsin for 30min under the same condition. The digested products were boiled in an equal volume of 2% SDS-PAGE sample buffer containing 5 mM NEM and subjected to Western blotting analysis as described in MATERIALS AND METHODS 5.

RESULTS AND DISCUSSION

1. Purification of P5 and its mutants

To investigate the mechanism by which P5 inhibits oxidative refolding of lysozyme, we constructed plasmids carrying the DNA fragments of the *a*, *aa'*, *a'b*, and *b* domains and Cys-Ala mutants (Fig. 2). The pET *E. coli* expression system was utilized to generate P5 variants. The expression of the resultant N-terminal His-tagged and (Ala-Met-Ala-Asp-Ile-Gly)-added proteins was induced with IPTG. I highly purified P5 and its variants by a combination of ammonium sulfate precipitation and three types of chromatography as shown in Fig. 3.

2. Reducing activities of P5 variants

P5 has been shown to have thiol-dependent reductase activity, which catalyzes the reduction of insulin disulfides by DTT [17]. I investigated the thiol-dependent reductase activities of P5 variants. The reductase activity of C171/174A was 12% relative to P5, showing the greatest decrease among C36/39A, C171/174A, and *a'b* (Fig. 4). These results

suggested that the *a'* domain had greater reductase activity than the *a* domain. C36/39/171/174A was catalytically impaired, indicating that the *b* domain had not insulin reductase activity (Fig. 4).

3. Effects of domain deletion and Cys-Ala mutants of P5 on oxidative refolding of reduced denatured lysozyme

It has been previously reported that ERp57 (PDIA3), the closest homolog of PDI, promotes oxidative refolding of reduced denatured lysozyme, while P5 inhibits oxidative refolding of the lysozyme in a dose-dependent manner at an equimolar ratio to lysozyme (Fig. 5) by forming lysozyme multimers with hyper molecular mass inactivated by significant intermolecular disulfides (hyLYS) under the same conditions as ERp57 (App. 6) [17]. Then, I investigated the effects of domain deletion or Cys-Ala mutants on the oxidative refolding of the lysozyme to explore the essentials of the refolding inhibition. I used Cys-Ala mutants as Cys-substituted variants because serine has been reported to have residual reducing activity [20].

In the absence of P5, the lysozyme activity was 38% after 60 min incubation in the refolding conditions used, while the activity in the presence of P5 was 2 %. The activities

after 60 min incubation in the presence of domain deletion and Cys-Ala mutants at 0.7 μM were summarized in Fig. 6. In the presence of C36/39A and C171/174A, the activities were 8% and 12%, respectively (Figs. 6 and 7), suggesting that not only cysteines in the *a* domain but also those in the *a'* domain participated comparably in the refolding inhibition. The inhibition in the presence of the double mutants showed dose-dependence (Figs. 8 and 9). C36/39/171/174A and *b* domain fragment, having none of catalytic cysteines, unexpectedly inhibited the refolding (Figs. 6 and 10-12), implying the importance of the *b* domain in the refolding inhibition. In contrast, *aa'* domain fragment enhanced lysozyme refolding (Fig. 13). However, under the coincubation with *aa'* domain and *b* domain, only the inhibitory effect of *b* domain was observed (Fig. 14). Similarly, *a'b* fragments inhibited the refolding (Fig. 15) even when *a'b* was coincubated with *a* domain (Fig. 16), which accelerated the refolding (data not shown). These observations suggested that *b* domain had a large impact on the refolding inhibition.

4. Western blotting analysis of refolding products

In the previous study with Western blotting analysis of refolding products, P5 forms lysozyme multimers with significant intermolecular disulfides to be hyper molecular mass (hyLYS), which are not detected in the presence of ERp57 (App. 6-B) [17]. The hyLYS is too large to enter not only the gel for separating proteins but also the gel for concentrating them, and reduced to be monomeric form under the reducing conditions. However, Cys-Ala mutants did not show clear differences in the inhibition of lysozyme refolding, although C36/39/171/174A did not have catalytic cysteines. To interpret the results of the inhibition by the mutants with catalytic cysteines (C36/39A, C171/174A and *a'b*) and the mutants without catalytic cysteines (C36/39/171/174A and *b*), the refolding products in the presence of the variants were analyzed by Western blotting using anti-lysozyme antibody and anti-P5 antibody, which did not cross react to each other (Fig. 17).

In the absence of P5 and its variants, a portion of the denatured lysozyme formed intermediates with intermolecular disulfides (Fig. 18), which explained 38% of lysozyme activity after 60 min refolding. Lysozyme products after 60min refolding in the absence or presence of P5 and its variants were compared as shown in Fig.19. In the presence of P5, C36/39A and C171/174A, the lysozyme intermediates, detected when lysozyme alone, were

faded, while hyLYS were detected in the hyper molecular region (Fig. 19-A, lanes 1 vs 2-4). The difference between P5 and the double mutants became clearer with twice as much as the refolding products, showing the intermediates remained in the presence of the double mutants, while those did not in the presence of P5 (Fig. 20-A). In the presence of *a'b*, the amount of hyLYS were relatively less and the molecular mass of the lysozyme intermediates were smaller than that detected in the presence of C36/39A (Fig. 19-A, lanes 3 and 6 and Fig. 20-B). It was reasonable that both of the catalytic cysteines and the proper conformation of *a* domain were important for efficient formation of hyLYS, being in agreement with the result that C36/39A inhibited lysozyme refolding more greatly than *a'b* did (Fig. 6). The hyLYS were reduced to be a monomeric form under the reducing condition (Fig. 19-B). These observations evidenced that P5 catalyzed the formation of the hyLYS to inactivate lysozyme via significant intermolecular disulfide cross-linking, and demonstrated that the catalytic cysteines in both the *a* and *a'* domains participated in the sulfhydryl oxidation almost comparably.

C36/39/171/174A or *b* domain without catalytic cysteines unexpectedly inhibited the lysozyme refolding (Fig. 6). In the Western blotting, lysozyme intermediates indicated by the square bracket, which were similar to those in the presence of lysozyme alone (Fig. 18), were detected in the presence of C36/39/171/174A (Fig. 19-A, lane 5). These intermediates were detected in the presence of *b* domain as well (Fig. 19-A, lane 7). In addition, in the presence

of C36/39/171/174A, the amount of lysozyme multimers at the hyper molecular region was greater than those in the presence of P5 (Fig. 19-A, lanes 2 and 5). Because C36/39/171/174A did not have catalytic cysteines, the multimers in the presence of C36/39/171/174A were not the product due to the significant intermolecular cross-linking by catalytic cysteines. As shown by the results detected by anti-P5 antibody, the amount of C36/39/171/174A at the hyper molecular region was also greater than that of P5 (Fig. 19-A, lanes 9 and 12). These lysozyme multimers and C36/39/171/174A at the hyper molecular region detected by anti-lysozyme antibody and anti-P5 antibody, respectively, in the presence of C36/39/171/174A were independent of refolding buffer (Fig. 21) and SDS concentration (Fig. 22, lanes 1-4), but reduced to be a monomeric form under the reducing condition (Fig. 22, lane 5). These data suggested a possible arrest of formation of hyLYS due to loss of catalytic cysteines to stay at the hyper molecular region as a mixed multimers via covalent bond(s). As shown in Fig. 23, most of *a'b* were in monomeric form (Fig. 23, lanes 1-4) throughout 60 min refolding, while the bands including *b* domain gradually migrated to the hyper molecular region (Fig.23, lanes 5-8), supporting the possible interaction of *b* domain with lysozyme to capture lysozyme in the formation of hyLYS. Considering that *a'* domain showed a higher reductase activity toward insulin reduction than *a* domain (Fig. 4), a reason for less amount of *a'b* than *b* staying at the hyper molecular region was that *a'* domain of *a'b* fragment might

reduce the mixed covalent bond(s) between lysozyme and *a'b*.

5. Trypsin sensitivity of lysozyme multimers with hyper molecular mass formed by P5 and C36/39/171/174A

P5 and C36/39/171/174A formed the lysozyme multimers at the hyper molecular region after 60 min refolding (Fig. 19-A, lanes 2 and 5). The lysozyme multimers in the presence of P5 progressed with time (Fig. 24, lanes 2-5), while those in the presence of C36/39/171/174A did not (Fig. 24, lanes 6-9), suggesting that these multimers were different in quality. To investigate whether P5 formed lysozyme multimers with the significant disulfide cross-linkages, while C36/39/171/174A formed a huge complex with lysozyme via *b* domain, I analyzed the sensitivities of the multimers formed in the presence of P5 and C36/39/171/174A toward trypsin.

Activity of native lysozyme was retained throughout trypsin treatment for 60 min (Fig. 25) and correspondingly, the results of Western blotting showed that native lysozyme was not affected by trypsin (Fig. 25, lanes 1-5). When the reduced denatured lysozyme was treated with trypsin right after the addition of refolding buffer, lysozyme activity did not recover (1%) (Fig. 25), and the reduced denatured lysozyme was found to be digested by trypsin at

least for 30 min treatment (Fig. 25, lanes 6-10), indicating that native lysozyme with disulfide cross-linkages was relatively resistant to trypsin.

Activity of the lysozyme after 60 min refolding, followed by 30 min incubation with trypsin, slightly decreased from 38% to 29% (data not shown), and the lysozyme treated with trypsin for 30 min did not show notable change in Western blotting (Fig. 26, lanes 1 and 2 vs 7 and 8), indicating that the regeneration of disulfides by refolding made the denatured lysozyme more resistant to trypsin (Fig. 26, lane 8 vs Fig. 25, lane 9). In the presence of P5 and C36/39/171/174A, the lysozyme multimers with hyper molecular mass in the absence of trypsin increased slightly during additional refolding for 30min (Fig. 26, lanes 3-6), showing that formation of the multimers were progressed. With trypsin treatment for 30min after incubation for 60 min, the lysozyme multimers in the presence of P5 slightly decreased (Fig. 26, lanes 3 and 4 vs 9 and 10), while the multimers in the presence of C36/39/171/174A notably decreased (Fig. 26, lanes 5 and 6 vs 11 and 12). These results indicated that P5 formed hyLYS to be registrant to trypsin by the significant intermolecular disulfides, but C36/39/171/174A did not.

PDIL2;3 (a rice orthologue of human P5) introduces intermolecular cross-linkages into reduced denatured α-globulin (C79F) *in vitro* [15], and retains GFP-AB within the ER by accumulating it via intermolecular disulfide cross-linkages, and promote sorting of the

cross-linked α-globulin to protein body I *in vivo* (App. 5) [16]. Considering these findings together with the results of this study, P5 may function as a regulator to pack proteins to be ready for transport by intermolecular disulfide cross-linking.

6. Effects of the mutations of absolutely conserved C272/278 on formation of the lysozyme multimers with intermolecular disulfides and hyper molecular mass and on insulin reduction

The *b* domain was suggested to interact with refolding lysozyme via covalent bond(s) as described above (Figs. 19, 22 and 23). The cysteines (C272 and C278) in the *b* domain are absolutely conserved among animals and plants (Fig. 27), thereby, I speculated that the cysteines could be the key for the formation of hyLYS. To further investigate a role of *b* domain, I examined effects of C272/278A on lysozyme refolding and insulin reduction. The refolding activity in the presence of C272/278A was 16±3% (Fig. 28-A). In Western blotting analysis, neither of hyLYS nor C272/278A at the hyper molecular region were detected by anti-lysozyme antibody and anti-P5 antibody, respectively in the presence of C272/278A having the catalytic cysteines (Fig. 28-B, lanes 4-7), while in the presence of P5 and C36/39/171/17A, having C272/278, hyLYS, and P5 and C36/39/171/17A in the hyper

molecular regions were detected by anti-lysozyme antibody and anti-P5 antibody, respectively (Fig. 28-B, lanes 2 and 3). These results indicated the C272 and/or C278 played a key role to capture lysozyme in forming hyLYS. Prediction of secondary structures using bioinformatics tool, SOPMA, showed that the mutations of C272/278 to C272/278A caused conformational changes of extended strands and beta turns around C272/278 to alpha helixes. C272 and/or C278 was/were thought to be necessary to maintain the conformation to provide P5 a platform to form hyLYS, although possible covalent interactions with lysozyme via C272/278 for formation of hyLYS was also considered as described above (Figs. 19, 22 and 23).

Furthermore, C272/278A showed predominant reductase activity toward insulin (Fig. 28-C), suggesting a possibility that P5 functions as a potent sulfhydryl oxidase and a predominant reductase, depending on the circumstance around C272/278.

7. Significance of *b* domain with the conserved C272/278 in P5 activity

Some protein disulfide oxidoreductases (PDOs) with only *a* and *a'* domains have been reported to catalyze the refolding of scrambled RNase A [29-31]. In the present study, *aa'* domain of P5 promoted the refolding of lysozyme (Fig. 13). However, P5 with *a, a',* and *b*

domains inhibited the refolding by formation of hyLYS, in which catalytic cysteines in the *a* and *a'* domains of P5 participated comparably, and the conserved C272/278 in *b* domain played a key role to capture the substrate lysozyme.

Jessop *et al.* have studied on substrates of PDI family, finding that P5 non-covalently interacts with immunoglobulin binding protein (BiP) [6]. The binding is redox-dependent, and P5 present with BiP, functions as a reductase in hyperoxidative conditions, suggesting that BiP may regulate P5 activity [6]. The hydrophobic cluster analysis using the drawhca program by Luc CANARD showed that P5 had hydrophobic-rich regions around C272/278 in *b* domain. Therefore, it is likely that the hydrophobic-rich regions of P5 could be associated with BiP to provide proper conformation to function as a reductase. The functional shift caused by C272/278A mutations (Fig. 28) suggests a possible allosteric regulation of P5 activity, in which the conserved C272/278 are structurally necessary to decide whether P5 functions as a significant sulfhydryl oxidase and a predominant reductase.

CONCLUSION

Using domain and Cys-Ala variants, I demonstrated that P5 had potent sulfhydryl oxidase activity to catalyze formation of the trypsin-resistant lysozyme multimers with hyper molecular mass inactivated via intermolecular disulfide cross-linkages, in which the catalytic cysteines in both the *a* and *a'* domains of P5 participated comparably, and the conserved C272/278 in *b* domain played a key role to capture the substrate lysozyme. Furthermore, the C272/278A variant lost the potent sulfhydryl oxidase activity, however, showed predominant insulin reductase activity, implying the activity shift of P5 dependent on the circumstance around C272/278. These results provide new insight into the molecular basis of P5 function.

TABLES

Table 1. Primers for P5 variants.

	Forward	Reverse
a	5’-CGGGATCCCTCTATTCATCTAGTGACGATGTC-3’	5’-CCCAAGCTTCTAATCCTTCACGAGCTGGCGCA-3’
aa’	5’-CGGGATCCCTCTATTCATCTAGTGACGATGTC-3’	5-CCCAAGCTTTCAAAGAGCCCGGGTAAC-3’
a’b	5’-CGGGATCCGGTTCGGGTAAGAAGGACGTG-3’	5’-CCCAAGCTTTCACAACTCATCCTTCTCCAG-3’
b	5’-CGGGATCCACCCGGGCTCTTGATCTG-3’	5’-CCCAAGCTTTCACAACTCATCCTTCTCCAG-3’
C36A	5’-CTATGCTCCTTGGGCTGGCCACTGAACCCG-3’	5’-CTTTGGCAGTGGCCAGCCCAAGGAGCATAG-3’
C39A	5’-CTTGGTGTGGCCACGCCCAAAGGTTGACAC-3’	5’-GTGTCAACCTTTGGGCGTGGCCACACCAAG-3’
C171A	5’-GCTCCTTGGGCTGGACACTGCAAAAATC-3’	5’-GATTTTTGCAGTGTCCAGCCCAAGGAGC-3’
C174A	5’-CTTGGTGTGGACACGCCAAAAATCTAGAGC-3’	5’-GCTCTAGATTTTTGGCGTGTCCACACCAAG-3’
C36/39A	5’-CTTGGGCTGGCCACGCCCAAAGGTTGACAC-3’	5’-GTGTCAACCTTTGGGCGTGGCCAGCCCAAG-3’
C171/174A	5’-GGGCTGGACACGCCAAAAATCTAGAGC-3’	5’-GCTCTAGATTTTTGGCGTGTCCAGCCC-3’
C272/278A	5’-CACCAGCTCGCCGTAGTGGCCGT-3’	5’-ACGGCCACTACGGCGAGCTGGTG-3’

Primers used for domain-deleted or Cys-Ala mutants are listed.

Table 2. Conditions for purification of domain-deletion and Cys-Ala mutants.

Alternative conditions for ammonium sulfate precipitation, hydrophobic, affinity (IMAC) and anion exchange chromatography are listed.

Ammonium sulfate precipitation (%) (g/ 15 mL suspension)					
	Full-length	***a***	***aa'***	***a'b***	***b***
1st	80 (8.41)	42 (3.82)	37 (3.32)	37 (3.32)	
2nd	—	60 (2.03)	70 (3.76)	60 (2.46)	
Hydrophobic chromatography: Basal buffer; 100 mM Tris-AcOH (pH 6.5)					
	Full-length*[1]	***a***	***aa'***	***a'b***	***b***
Loading buffer including	2M NaCl	2M NaCl	1M $(NH_4)_2SO_4$		
Washing buffer including	100 mM NaCl*[2]	—		—	
Elution buffer including	100 mM NaCl/ 1% Triton-X100	100 mM NaCl		100 mM NaCl	

Affinity chromatography (IMAC): Basal buffer; 50 mM sodium phosphate/ 150 mM NaCl (pH 7.8)					
	Full-length*[1]	***a***	***aa'***	***a'b***	***b***
Loading buffer	100 mM sodium phosphate/ 300 mM NaCl*[3]				
Washing buffer including	50 mM imidazole			—	
Elution buffer including	500 mM imidazole			50 mM imidazole	
Anion exchange chromatography: Basal buffer; 100mM Tris-HCl (pH7.0)					
	Full-length	***a***	***aa'***	***a'b***	***b***
Loading buffer	100 mM Tris-HCl		20 mM Tris-HCl		
Washing buffer	100 mM Tris-HCl	—	20 mM Tris-HCl	—	
Elution buffer including	300 mM NaCl	150 mM NaCl		150 mM NaCl	

Shaded boxes: conditions for full-length P5, unshaded boxes: the same conditions as full length, dashes: non-operated. *[1]: Except for C272/278A. *[2]: C272/278A was eluted with washing buffer. *[3]: After loading of eluate including C272/278 A from hydrophobic chromatography, column was pre-washed with the loading buffer.

FIGURES

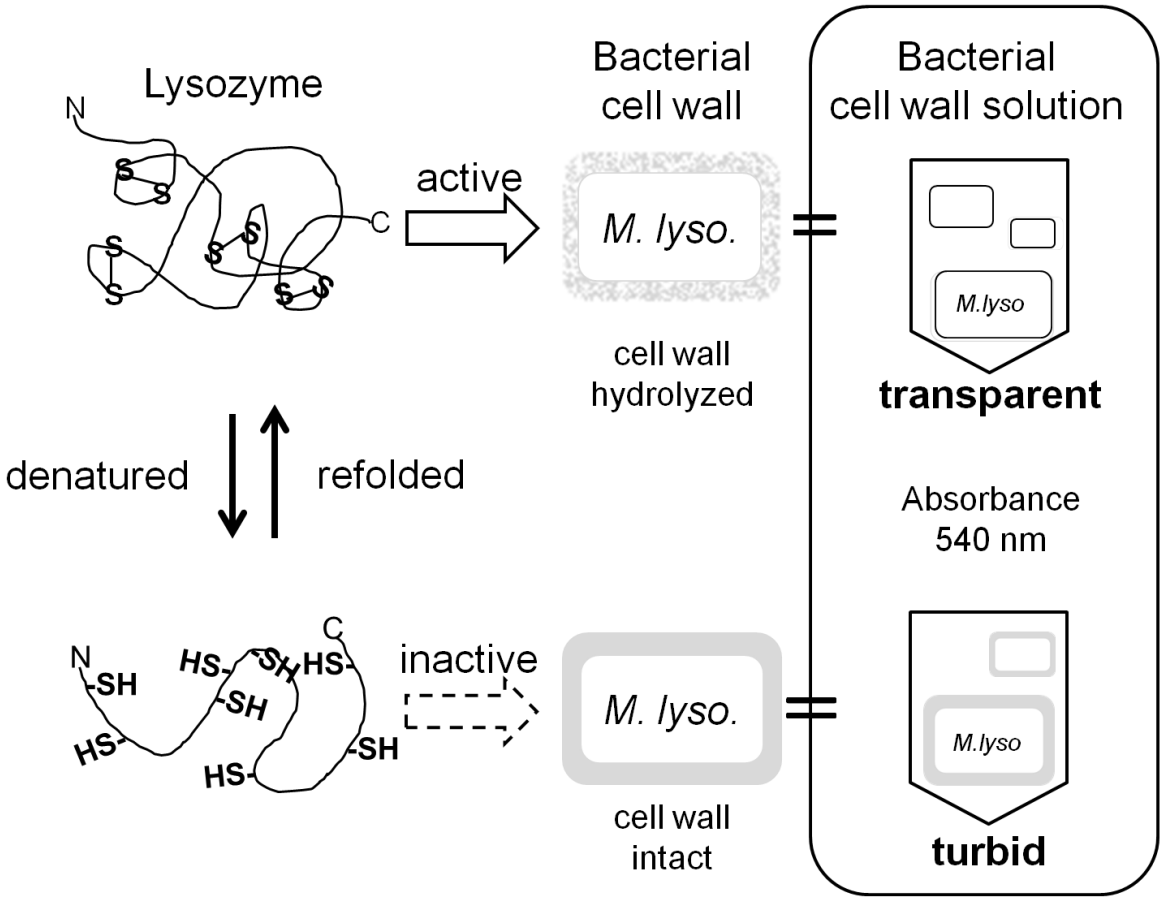


Fig. 1. Scheme of lysozyme refolding assay.

To investigate isomerase activity of P5, the refolding of denatured lysozyme was monitored. Refolding was measured as the bacteriolytic effect against *Micrococcus lysodeikticus* cell wall suspension, with the accompanying decrease in absorbance at 540 nm. The denatured lysozyme was refolded for 60 min at 37℃ in refolding buffer containing GSSG/GSH in the presence or absence of P5 or its variants.

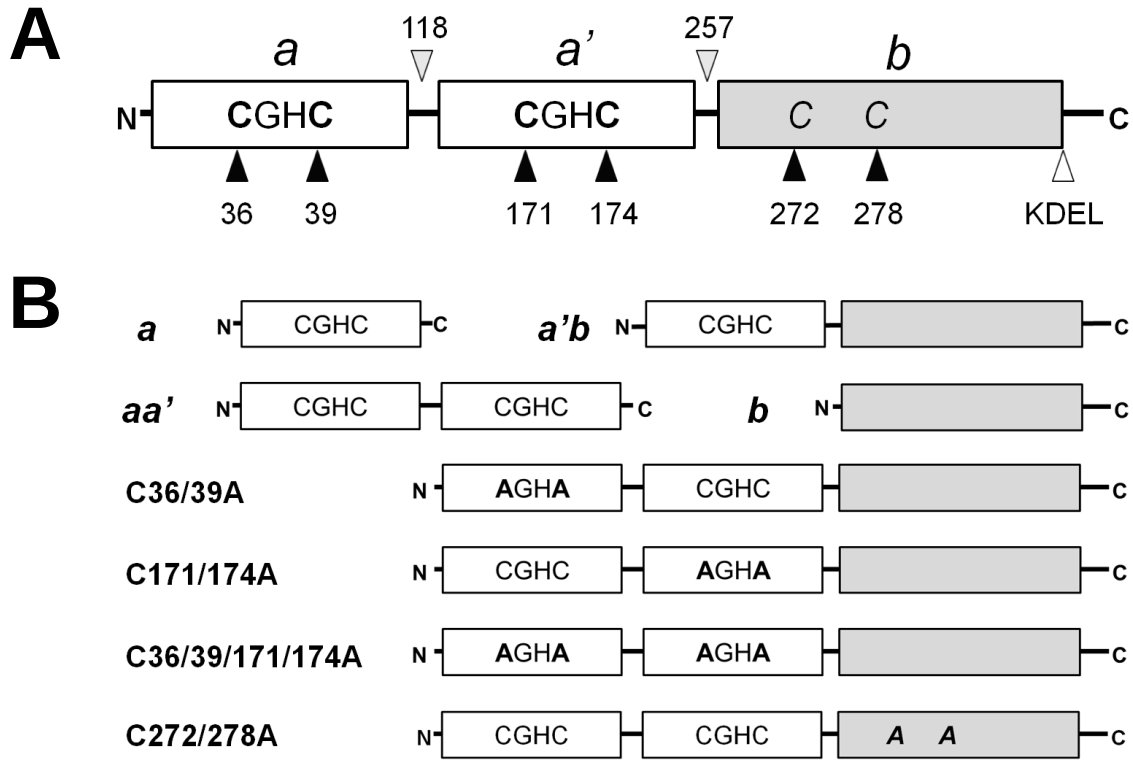


Fig. 2. Domain structures of PDI-P5 and its variants.

(A) The domain structure of P5. The catalytic *a* and *a'* domains (open boxes) followed by the non-catalytic *b* domain (shaded box). The catalytic and non-catalytic cysteines are shown in bold and italic, respectively. Filled arrowheads indicate Cys positions with numbers. Shaded arrowheads show amino acid positions at domain boundaries. (B) Domain-deletion and Cys-Ala variants used are shown. Mutated catalytic and non-catalytic cysteines are represented in bold and italic bold, respectively.

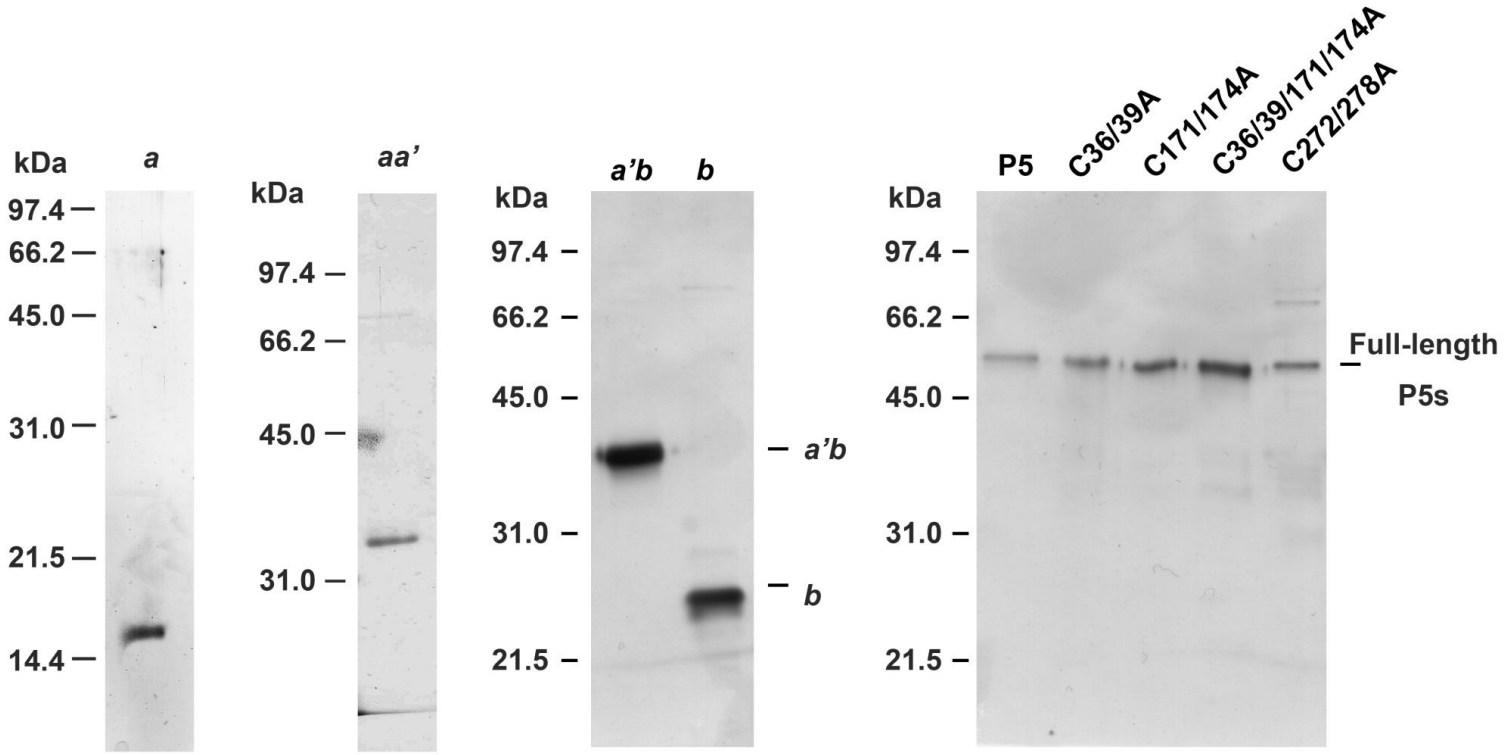


Fig. 3. SDS-PAGE of the purified recombinant boar P5 and its mutants.

Proteins were detected by silver staining. The motilities of molecular mass markers (rabbit muscle phosphorylase B, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase, soy bean trypsin inhibitor, and hen egg white lysozyme) are indicated in kDa on the left side of the gel (*a* domain: 12.5%, the other variants: 10% gel).

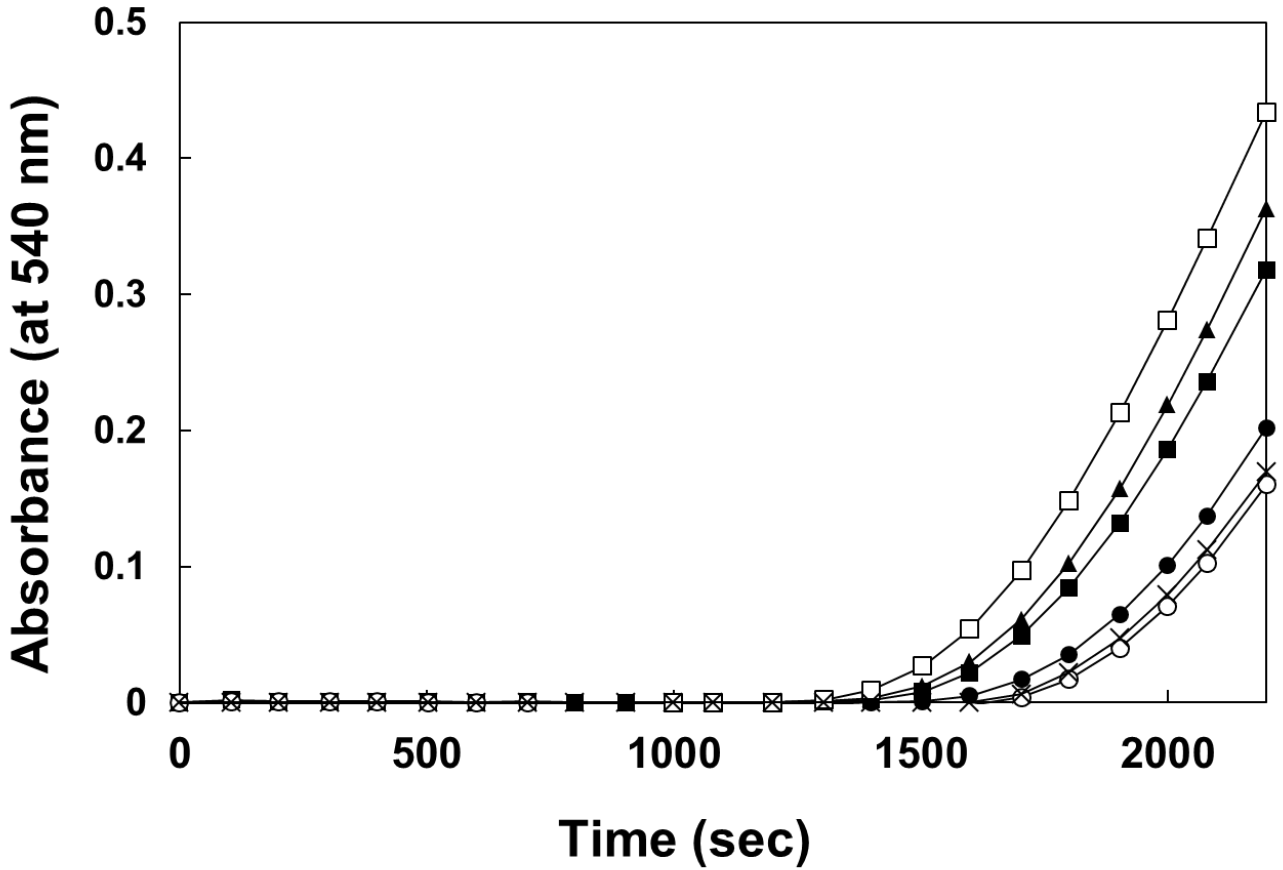


Fig. 4. Reductase activities of P5 and its variants.

Reductive activities toward insulin in the presence of 1.0 mM DTT were examined by monitoring absorbance at 540 nm. The assays were performed in duplicate in the presence or absence of P5 or its variants (0.19 μM). Representative data are shown in the graph. □, P5; ▲, *a'b*; ■, C36/39A; ●, C171/174A; ×, C36/39/171/174A; ○, DTT only.

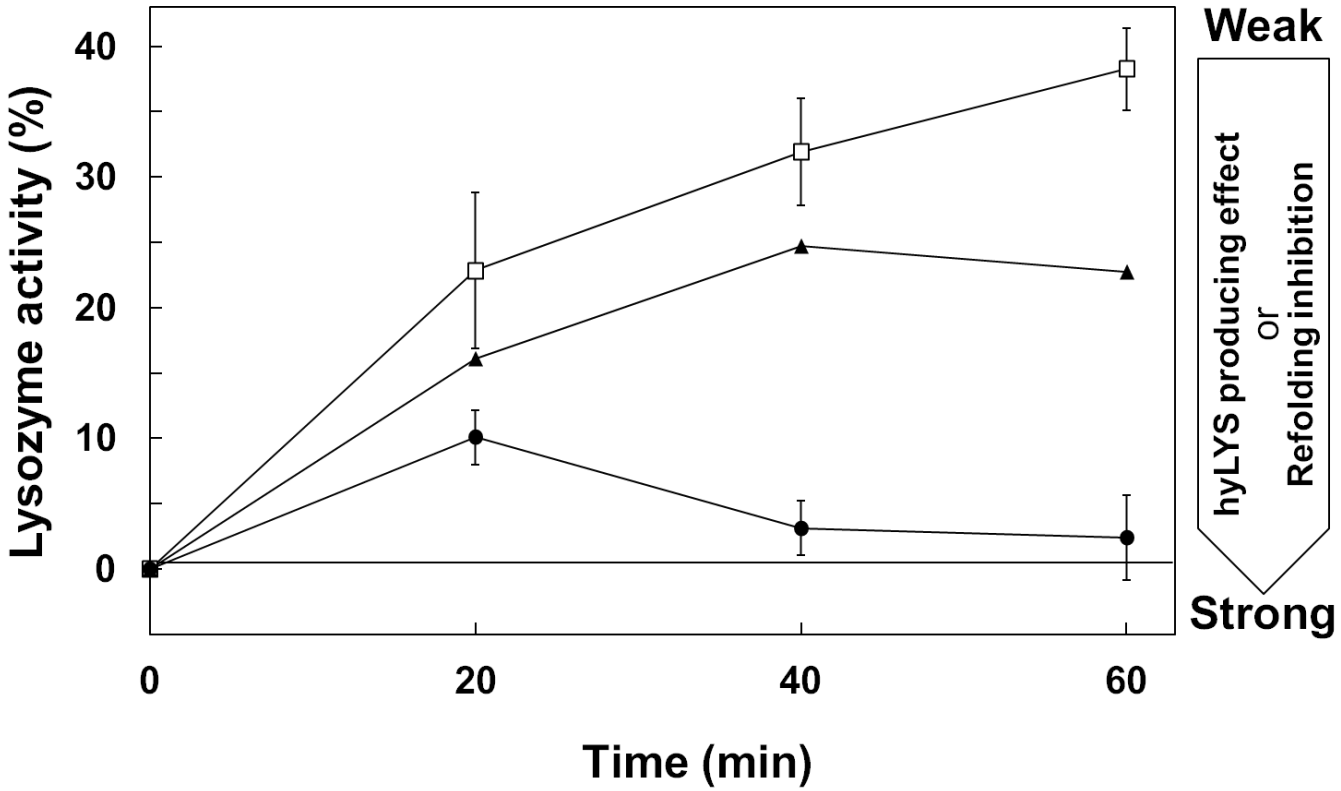
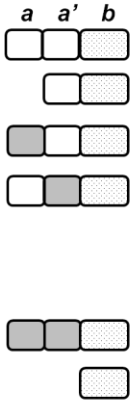


Fig. 5. Dose-dependent effects of P5.

Lysozyme activities in the presence of P5 at 0.7 and 0.15 μM are shown.

□, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM. hyLYS, lysozyme multimers with intermolecular disulfides and hyper molecular mass.

A



P5s	Lysozyme activity (%)
LYS alone	38±3
nP5	2±3
a'b	22±2
C36/39A	8±1
C171/174A	12±0
C36/39/171/17A	4±2
b	16±1

B

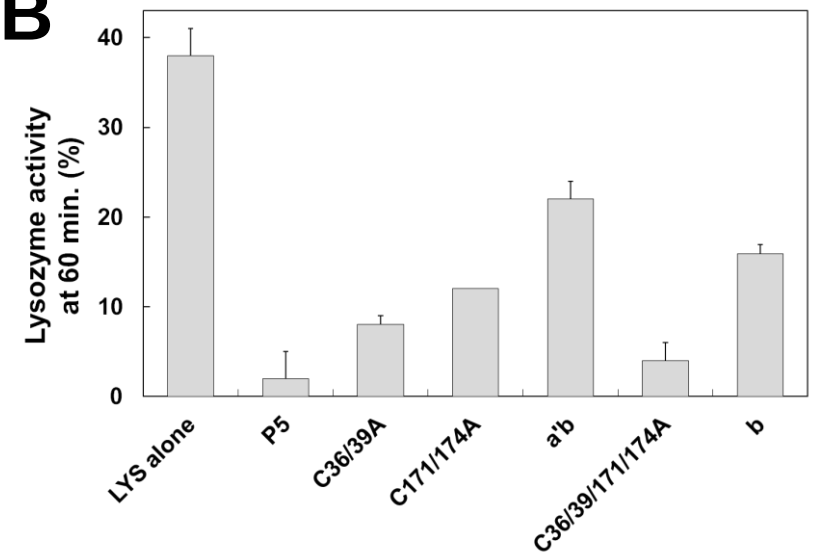


Fig. 6. Summary of lysozyme activities after 60min refolding in the presence or absence of P5 and its variants

Lysozyme activities are shown in a table (A) and in a column chart (B). The refolding was monitored by the bacteriolytic effect of refolding lysozyme on *Micrococcus lysodeikticus* cell wall suspension. Reduced denatured lysozyme (1.4 μM final concentration) was added to refolding buffer containing glutathione redox agents (5 mM GSH/0.5 mM GSSG) and 0.7 μM recombinant P5 or its variants at 37℃, pH 7.0 (100 mM HEPES/5 mM $MgCl_2$/20 mM NaCl/2 mM EDTA). The assays were performed in triplicate in the presence or absence of P5 and its variants (0.7 μM) at 25℃. LYS, lysozyme. P5s, P5 or its mutants. Open boxes, catalytic domains without mutations. Shaded boxes, catalytic domains with Cys mutations. Dotted boxes, *b* domains.

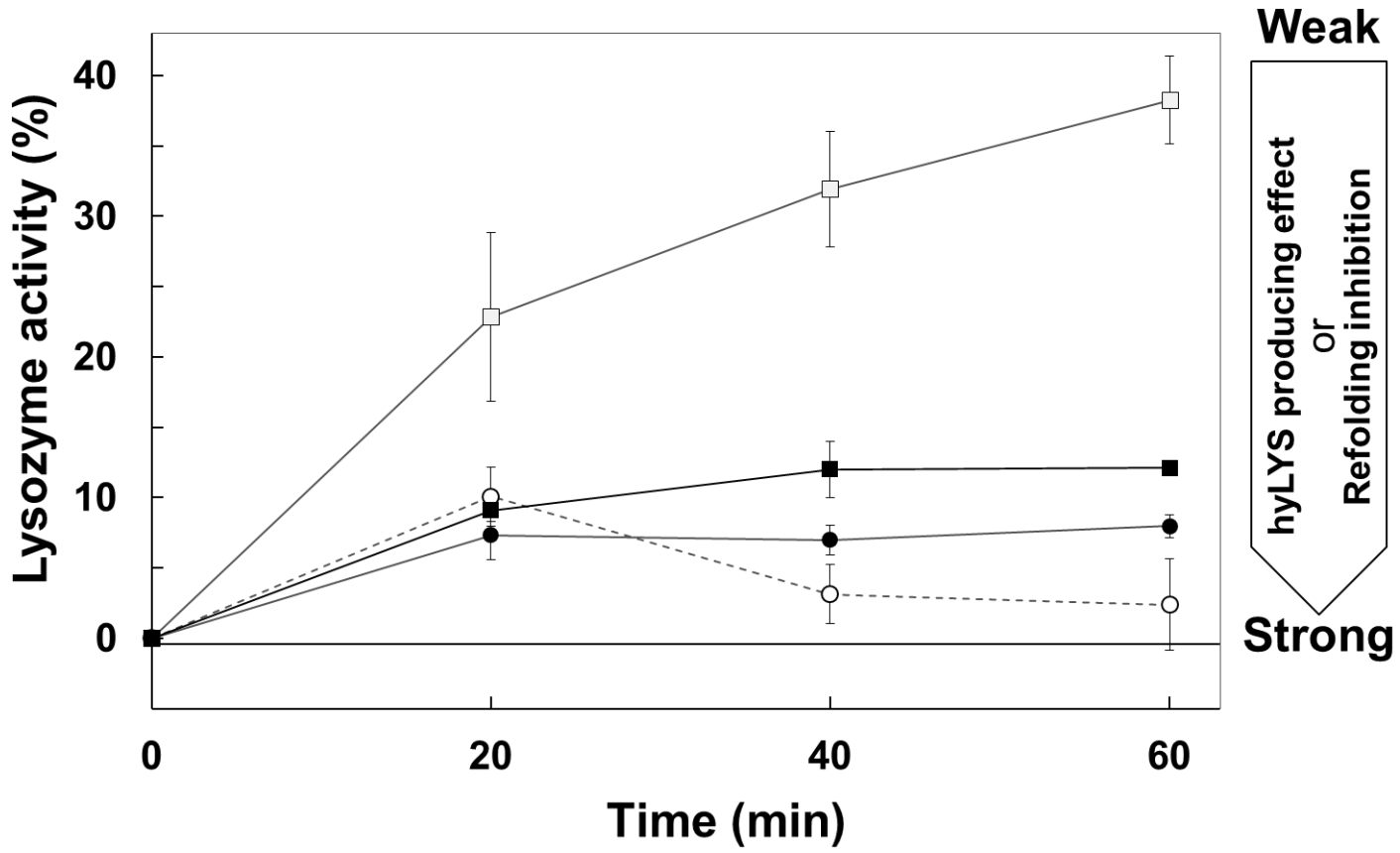


Fig. 7. Effects of C36/39A and C171/174A on lysozyme refolding.

Comparable effects of the double mutants at 0.7 μM □, lysozyme only; ●, C36/39A; ■, C171/174A; ○, P5. hyLYS; lysozyme multimers with intermolecular disulfides and hyper molecular mass.

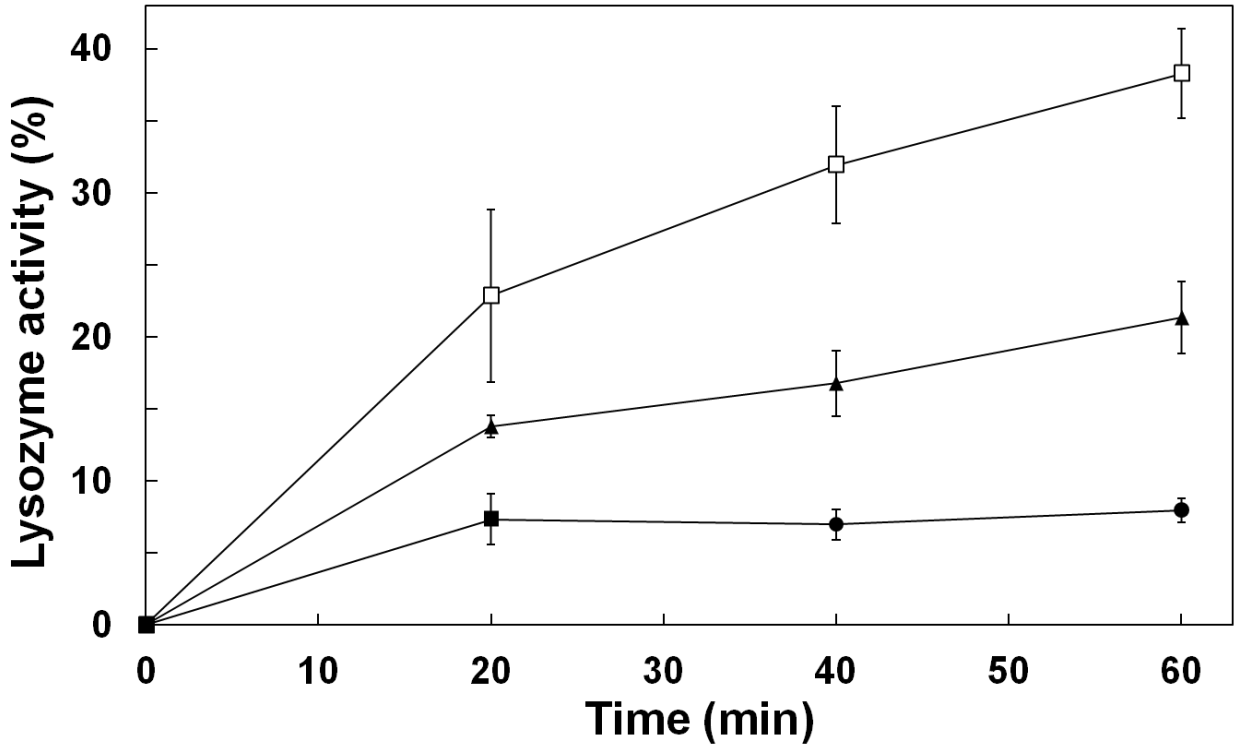


Fig. 8. Dose-dependent effects of C36/39A.

Lysozyme activities in the presence of C36/39A at 0.7 and 0.15 μM are shown.

□, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM

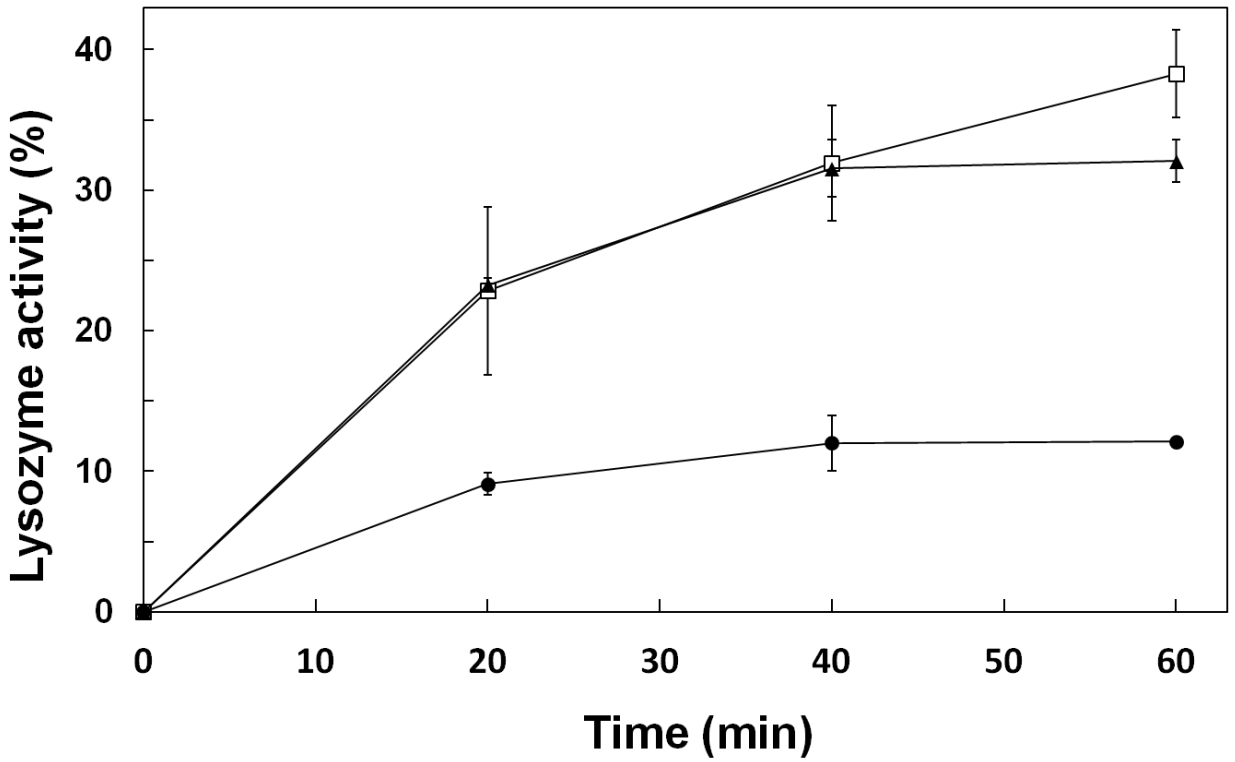


Fig. 9. Dose-dependent effects of C171/174A.

Lysozyme activities in the presence of C171/174A at 0.7 and 0.15 μM are shown. □, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM

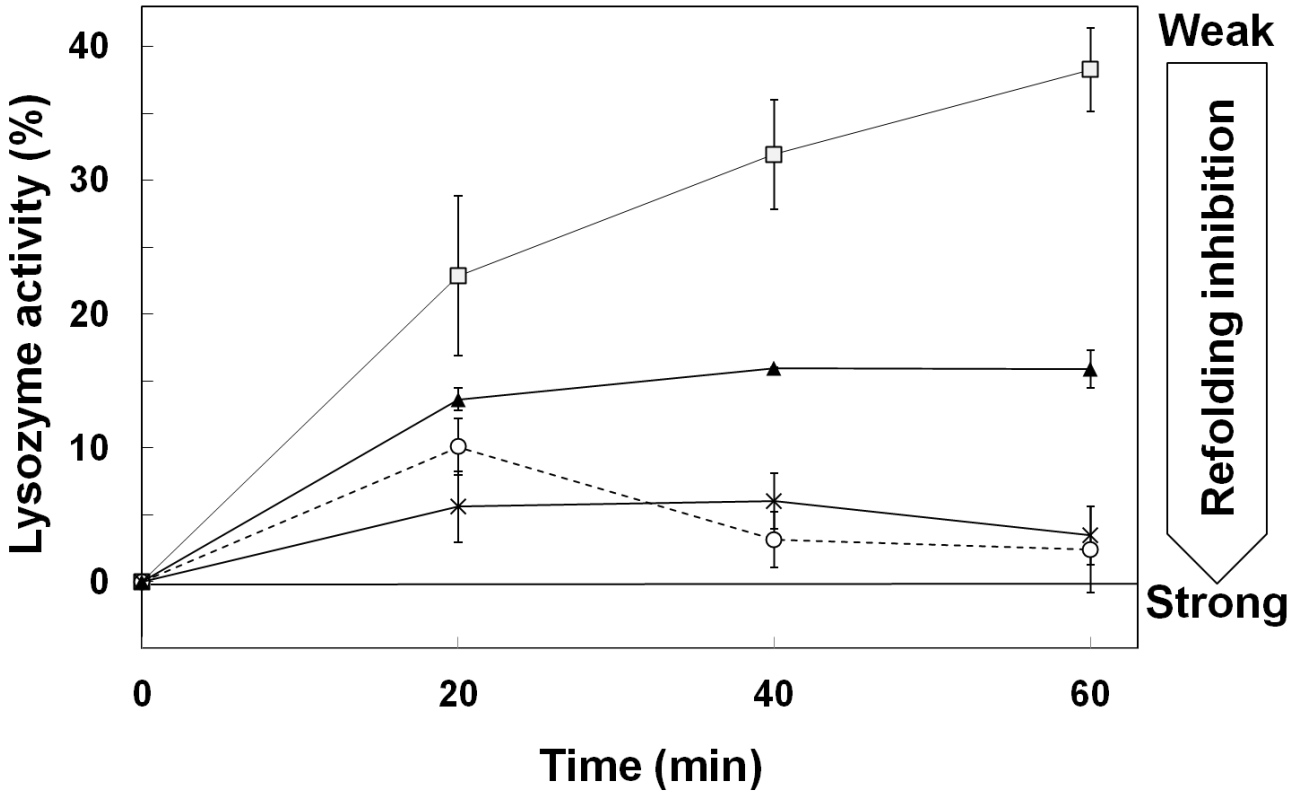


Fig. 10. Effects of C36/39/171/174A and *b* domain on the refolding of lysozyme at 0.7 μM.

C36/39/171/174A and *b* domain inhibited the refolding even without catalytic cysteines.

□, lysozyme only; ▲, *b*; ×, C36/39/171/174A; ○, P5.

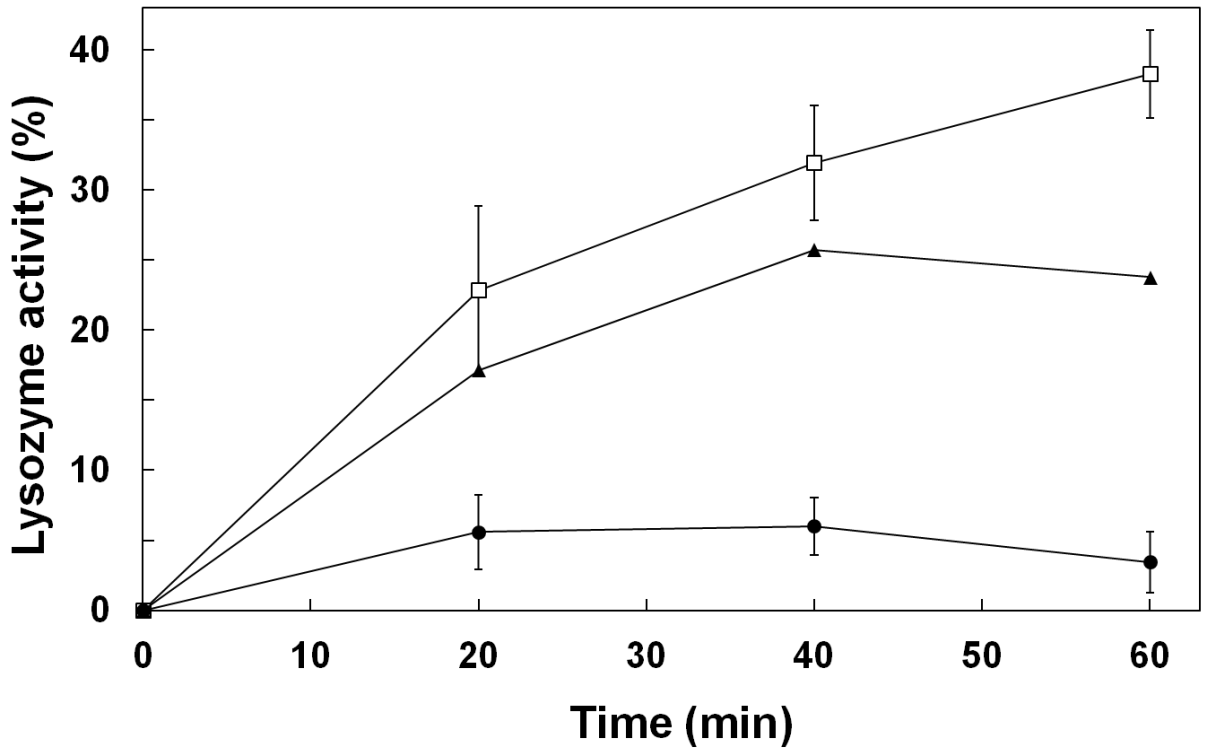


Fig. 11. Dose-dependent effects of C36/39/171/174A on lysozyme refolding.

Lysozyme activities in the presence of C36/39 /171/174A at 0.7 and 0.15 μM are shown. □, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM.

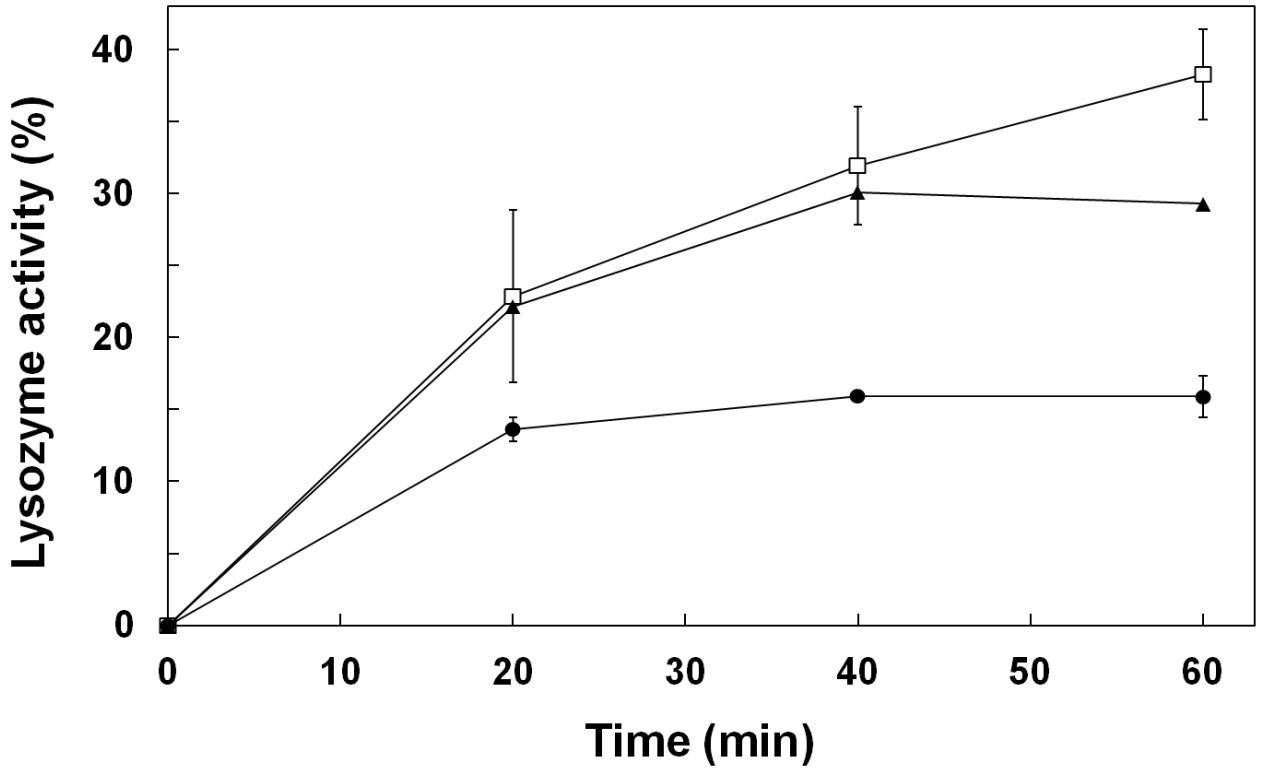


Fig. 12. Dose-dependent effects of *b* on lysozyme refolding.

Lysozyme activities in the presence of *b* at 0.7 and 0.15 μM are shown. □, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM.

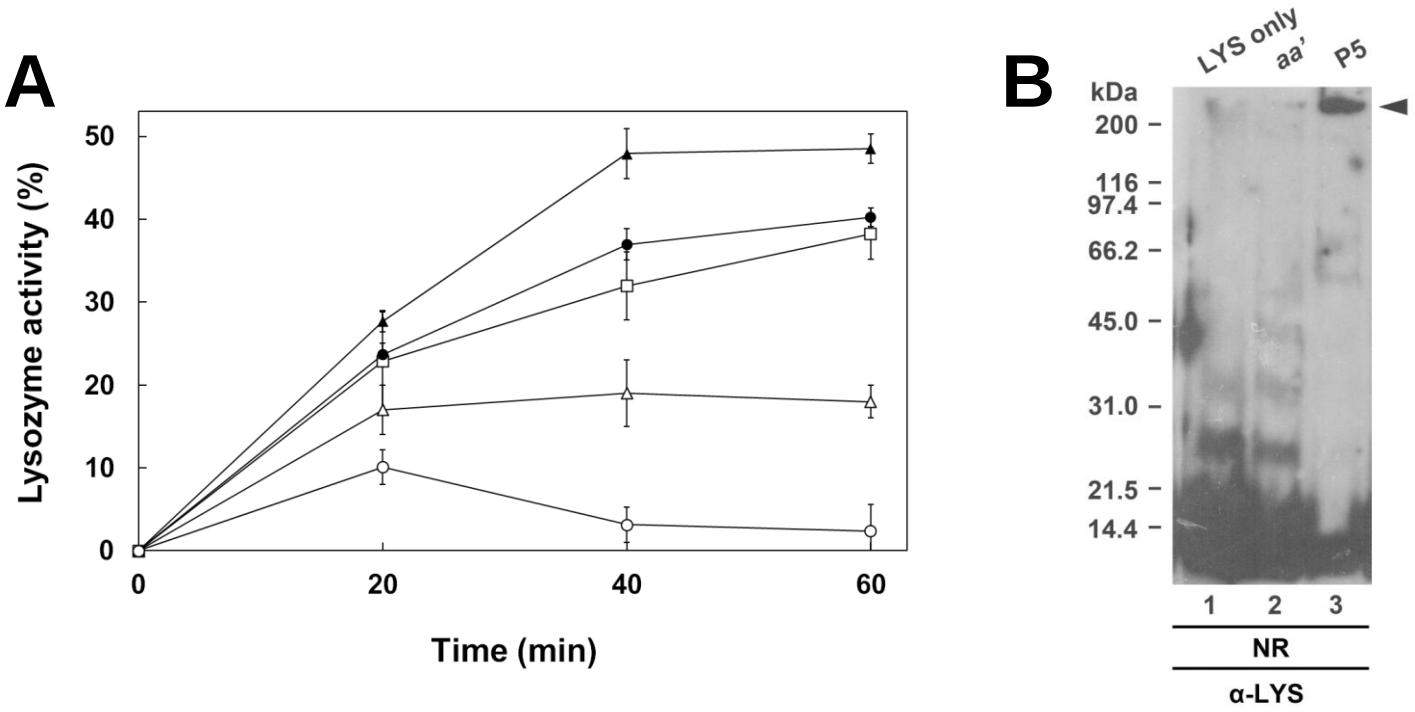


Fig.13. Effects of the *aa'* domain on lysozyme refolding.

(A) *aa'* accelerated lysozyme refolding at 0.3 μM. □, lysozyme only; ▲, *aa'* (0.3 μM); ●, *aa'* domain (0.7 μM) ; △, P5 (0.3 μM); ○, P5 (0.7 μM). In the presence of 0.7 μM of *aa'*, the acceleration was decreased. Even in the presence of P5 at 0.3 μM, P5 inhibited lysozyme refolding (refolding activity; 10%, relative to lysozyme alone [17]). These results indicated that the *aa'* domain did not show inhibitory effect on the refolding. (B) Lysozyme product after 60 min refolding in the presence of *aa'* domain. Lysozyme refolding product in the presence of *aa'* at 0.7 μM was detected by anti-lysozyme antibody (α-LYS) under the non-reducing conditions (NR) as described in Fig. 18 to ensure *aa'* did not form hyLYS at 0.7 μM; arrowhead, hyLYS, resulting in lysozyme refolding inhibition.

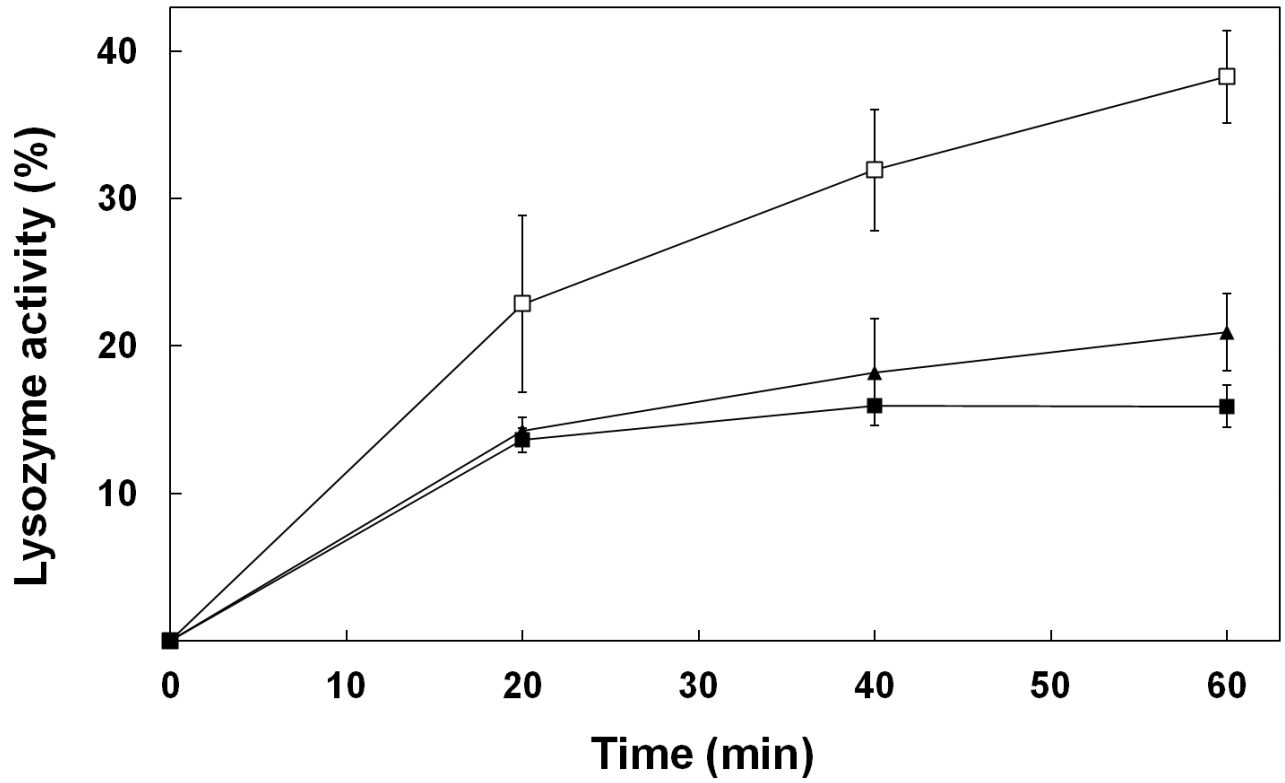


Fig. 14. Effects of equimolar *aa*'and *b* on the refolding of lysozyme.

Lysozyme refolding in the presence of *aa'*, *b* mixture was monitored. □, lysozyme only; ▲, *aa'* and *b* (0.7 μM); ■, *b* domain (0.7 μM).

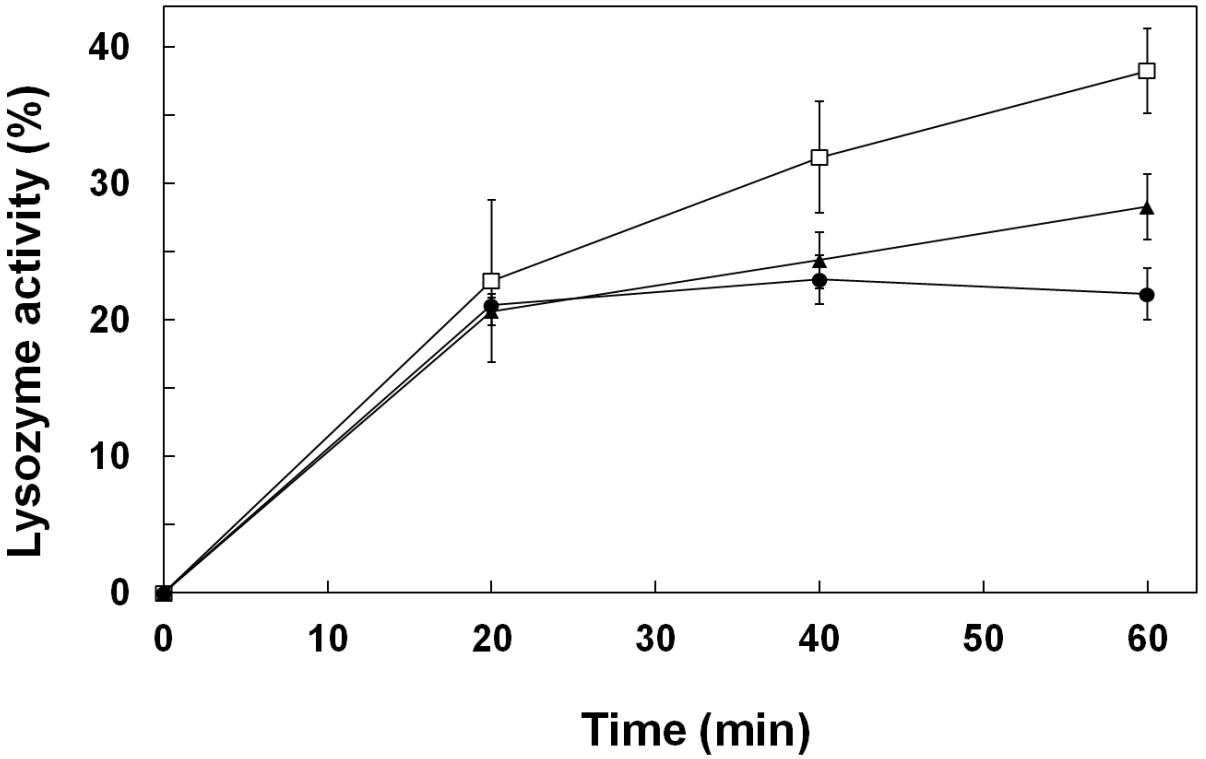


Fig. 15. Dose-dependent effects of *a'b*.

Lysozyme activities in the presence of *a'b* at 0.7 and 0.15 μM are shown.

□, lysozyme only; ▲, 0.15 μM; ●, 0.7 μM

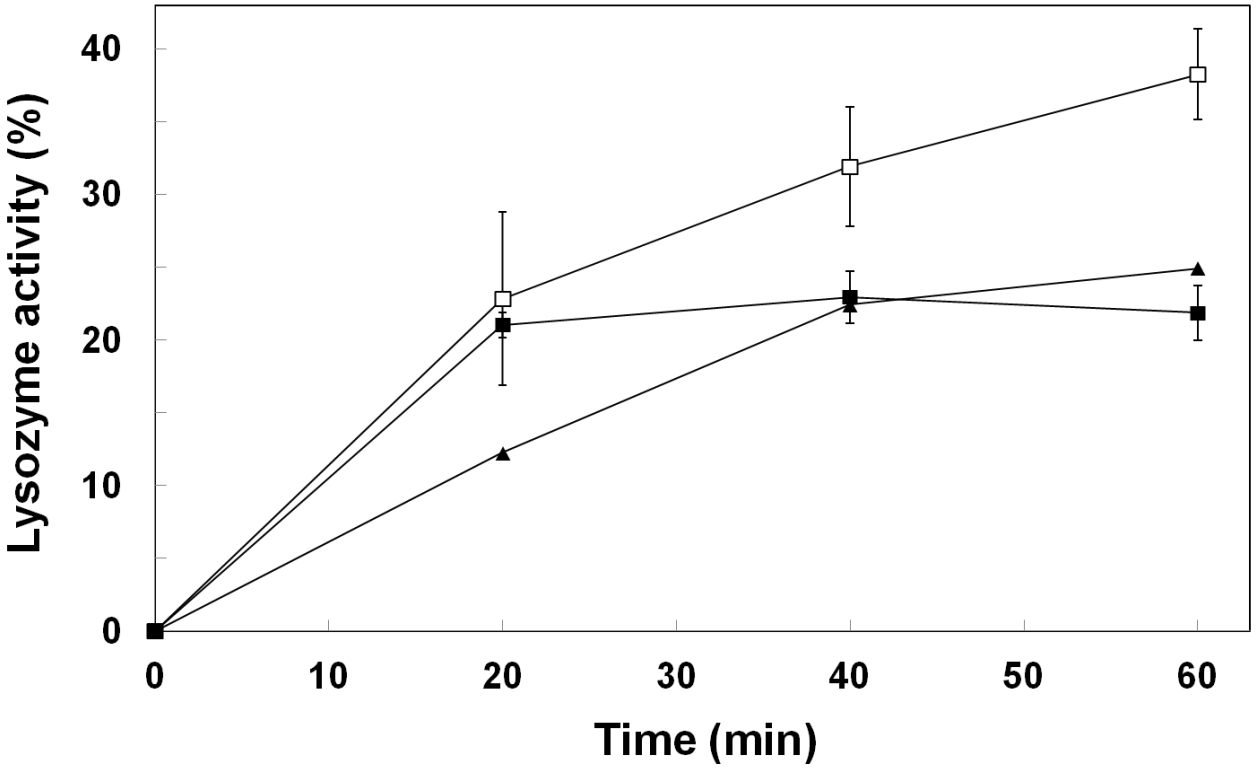


Fig. 16. Effects of equimolar *a* and *a'b* on the refolding of lysozyme.

Lysozyme refolding in the presence of *a*, *a'b* mixture was monitored. □, lysozyme only; ▲, *a* and *a' b* (0.7 μM); ■, *a' b* domain (0.7 μM).

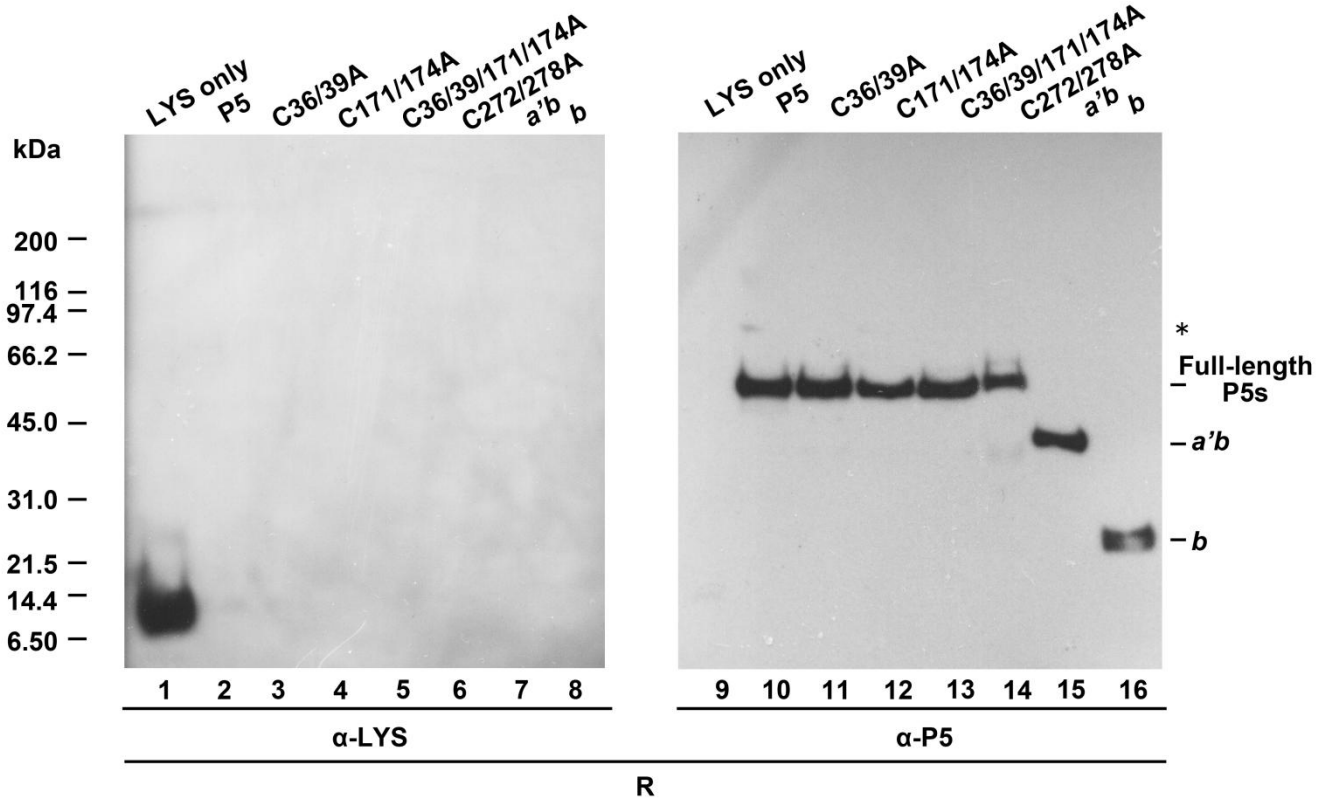


Fig. 17. Western blotting analysis of the denatured lysozyme, P5, and its variants under the reducing conditions.

The denatured lysozyme, P5, and its variants were detected by anti-lysozyme antibody or anti-P5 antibody. α-LYS, anti-lysozyme antibody; α-P5, anti-P5 antibody; R, under reducing conditions. *, nonspecific bands detected by α-P5

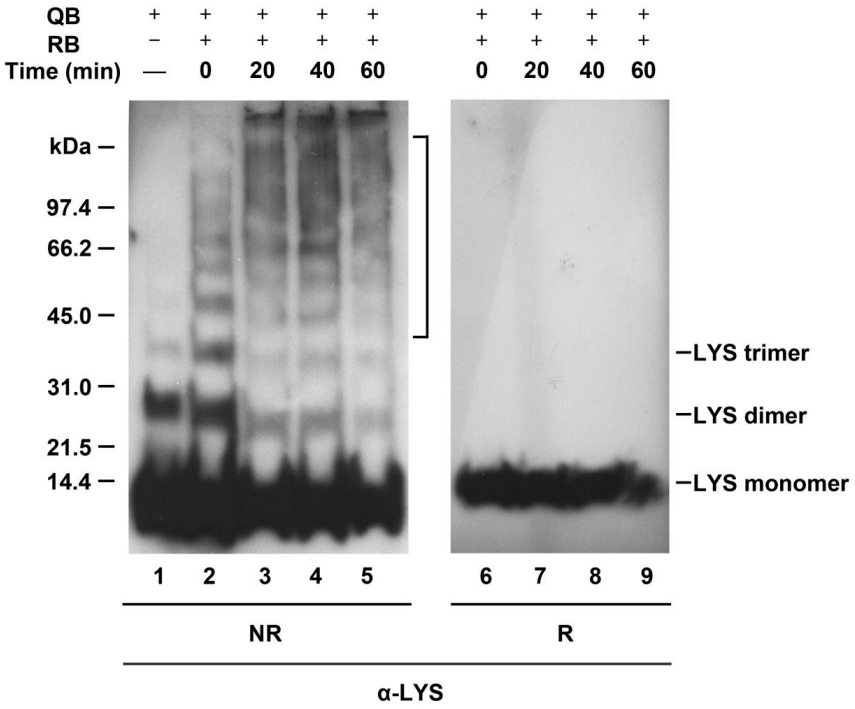


Fig. 18. Western blotting analysis of the products obtained by oxidative refolding of lysozyme.

After refolding for 60min (Fig. 6), the refolding products were treated with an equal volume of 2% SDS sample buffer containing 5 mM NEM or 3 mM 2-Me at 100℃ for 3 min, and subjected to SDS-PAGE in a Tris-Tricine buffer system on a 7.5% gel. The membranes were probed with α-LYS. Refolding products of the denatured lysozyme alone, were sampled every 20 min for 1hr. QB, quenching buffer; RB, refolding buffer; LYS, lysozyme. NR, non-reducing conditions; R, reducing conditions; Lysozyme intermediates with intermolecular disulfide cross-linkages are indicated by the square bracket.

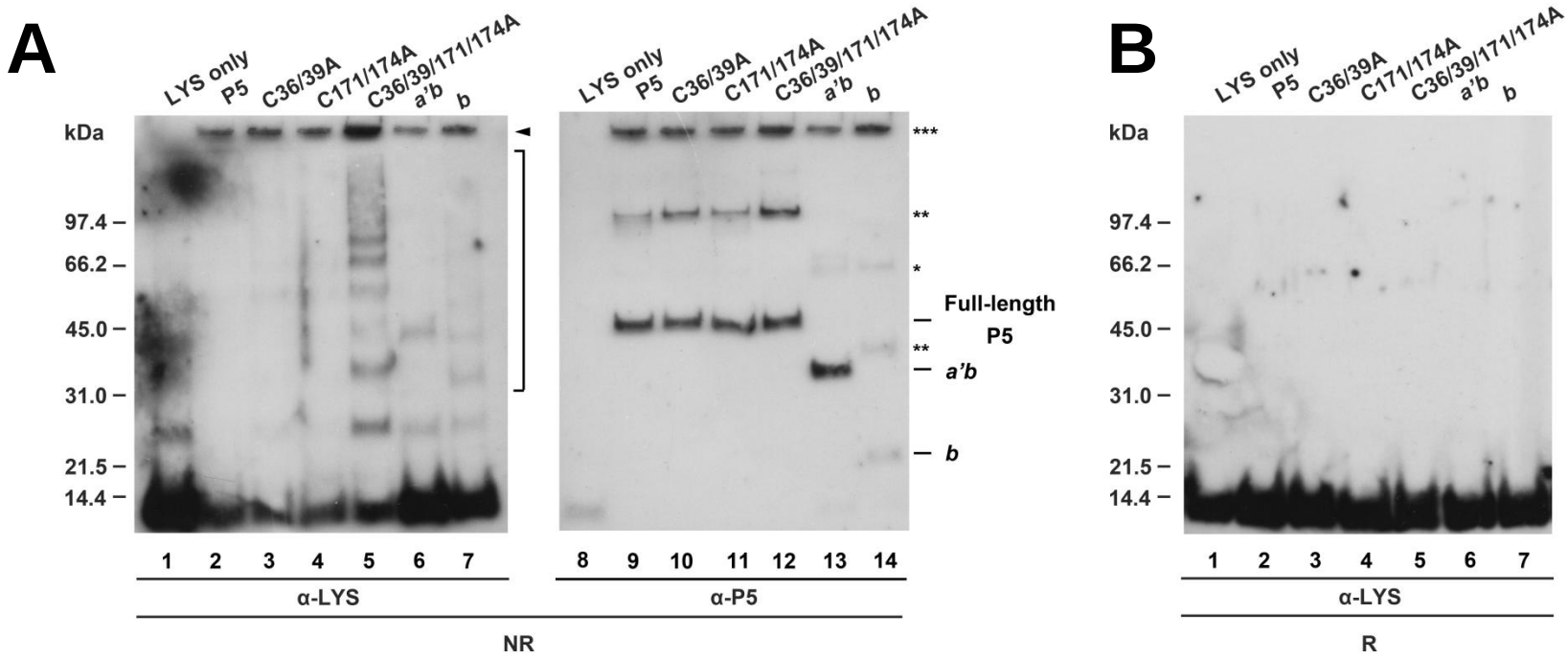


Fig. 19. Lysozyme refolding products in the presence of P5 variants.

Lysozyme refolding products after incubation for 60min in the presence of P5 variants at 0.7 μM were prepared in the same manner described in Figs. 6 and Fig. 18. The membranes were probed with α-LYS or α-P5. (A) In the presence or absence of P5 and its variants under the non-reducing conditions. (B) Under the reducing conditions. NR, non-reducing conditions; R, reducing conditions; *, non-specific bands; **, dimers of full-length P5 and *b* domain; ***, P5 and its variants at the hyper molecular region; arrowheads, lysozyme multimers at the hyper molecular region. Lysozyme intermediates with intermolecular disulfide cross-linkages are indicated by the square bracket.

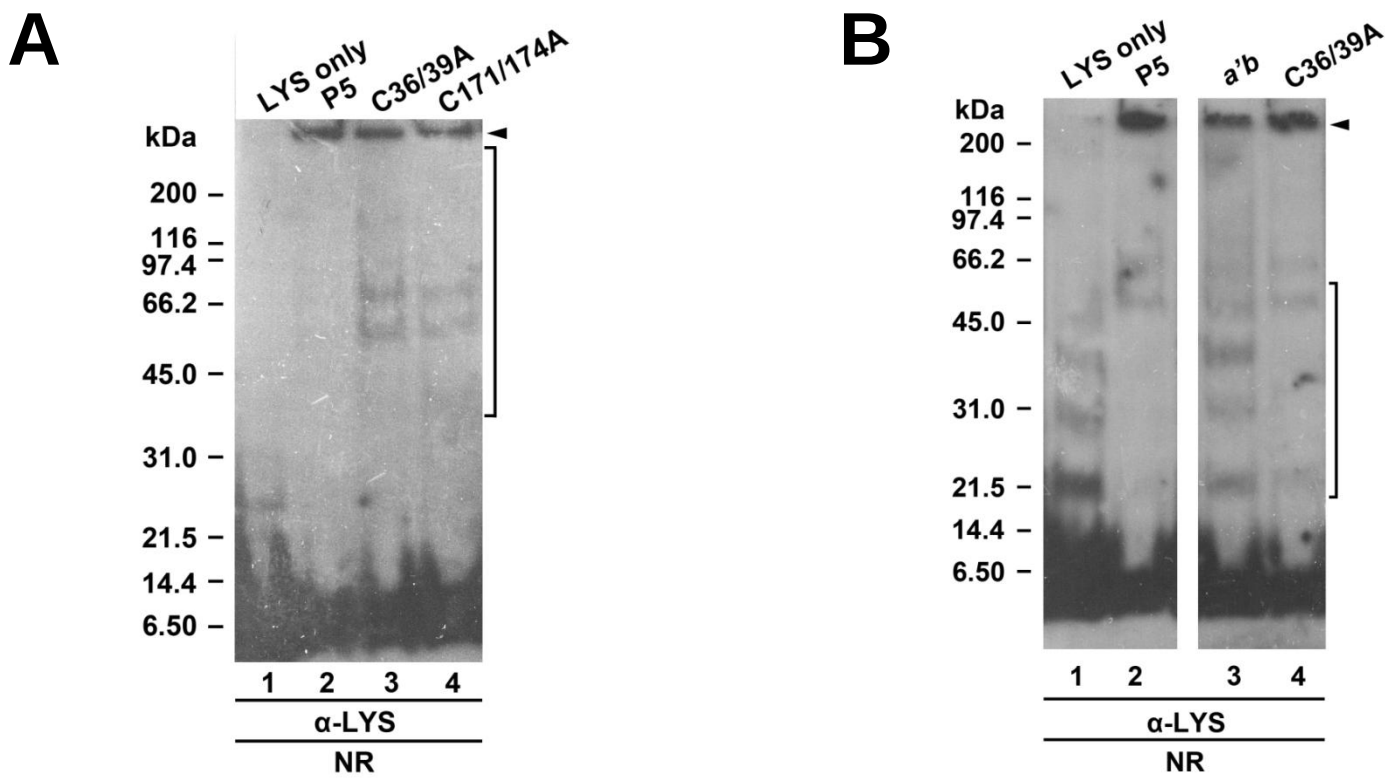


Fig. 20. Comparison of lysozyme products in the presence of variants of catalytic domains.

The refolding products twice as much as those of Fig. 19 in the presence or absence of P5 and the double mutant(s) or *a'b* were subjected to Western blotting and the amount of the lysozyme intermediates in the presence was compared. (A) In the presence of the double mutants. (B) In the presence of C36/39A and *a'b*. Arrowhead; hyLYS. Lysozyme intermediates were indicated by the square bracket. The lanes necessary were split from a single membrane and compared.

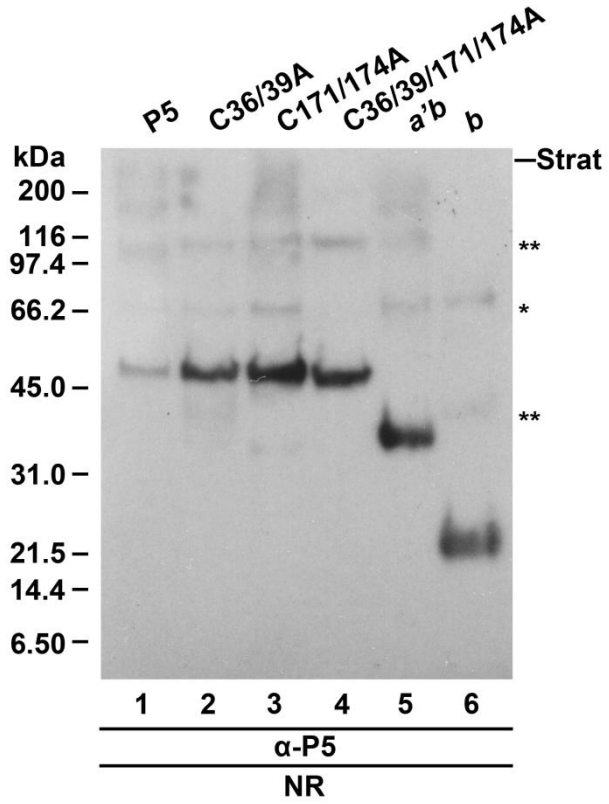


Fig. 21. Refolding buffer did not cause migration of P5 and its variants to the hyper molecular region.

P5 and its variants in the refolding buffer in the absence of denatured lysozyme were incubated for 60 min under the same conditions as Fig. 6 and subjected to Western blotting under the non-reducing conditions. *, non-specific bands; **, dimers.

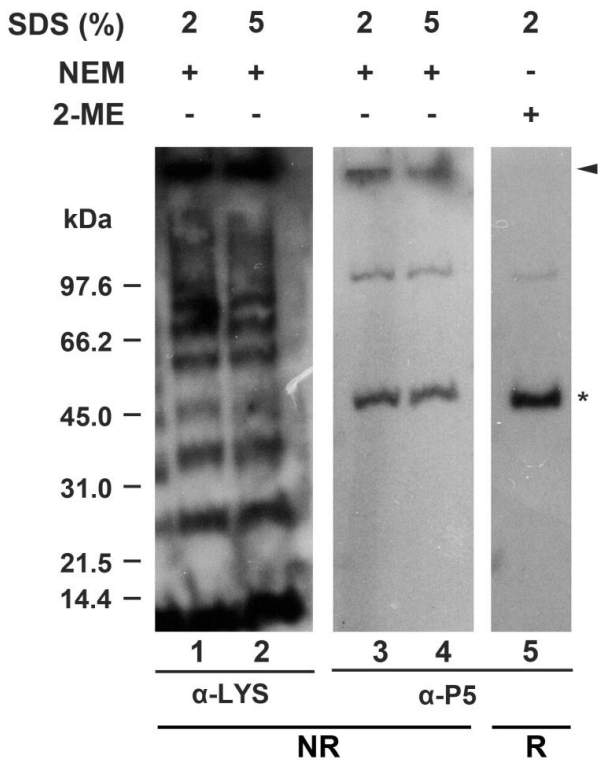


Fig. 22. Lysozyme multimers with hyper molecular mass independent of SDS concentration.

Lysozyme refolding products after incubation for 60 min in the presence of C36/39/171/174A at 0.7 μM were treated with 2% and 5% SDS sample buffer under the non-reducing and reducing conditions and detected by α-LYS or α-P5. The lanes necessary were split from a single membrane and compared. Arrowhead, lysozyme multimers with hyper molecular mass; *, C36/39/171/174A.

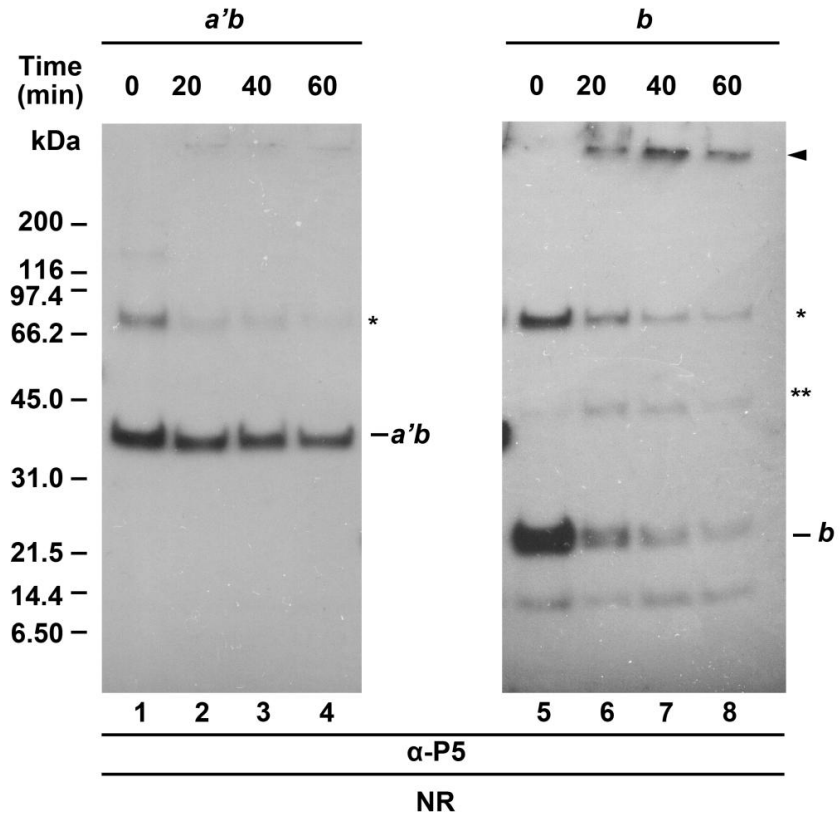


Fig. 23. Migration of *a'b* and *b* domains to the hyper molecular mass in lysozyme refolding.

Lysozyme refolding products in the presence of *a'b* and *b* at 0.7 μM were sampled every 20 min for 60 min and detected by α-P5. *, non-specific bands; **, dimmers of *b* domain; arrowhead, *b* domain at the hyper molecular region.

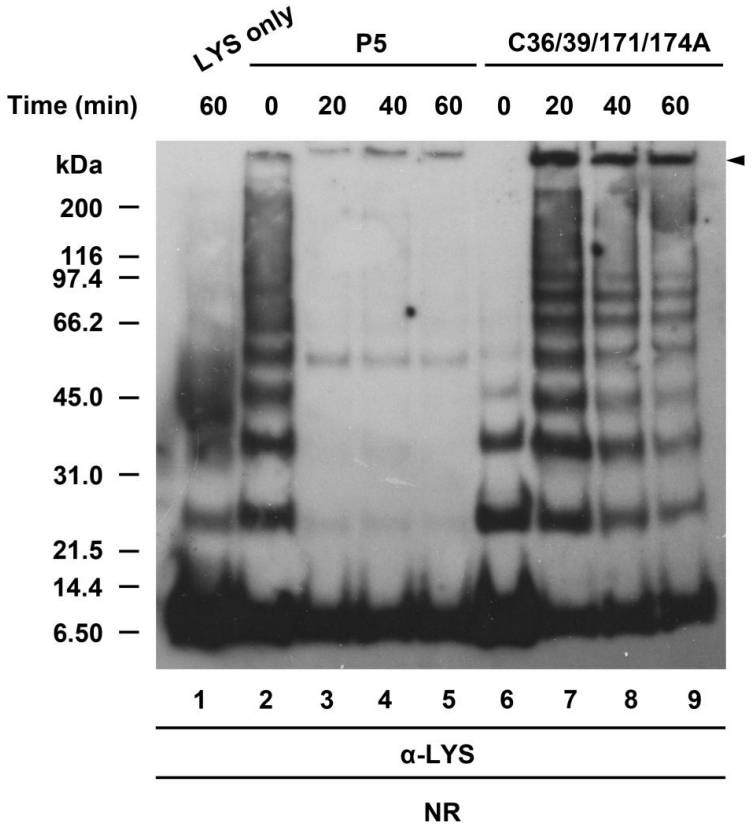


Fig. 24. Formation rates of lysozyme multimers with hyper molecular mass in the presence of P5 and C36/39/171/174A.

Lysozyme refolding products in the presence of P5 and C36/39/171/174A at 0.7 μM were sampled every 20 min for 60 min and detected by α-LYS. Arrowhead, hyLYS formed by P5 and lysozyme multimers with hyper molecular mass formed by C36/39/171/174A.

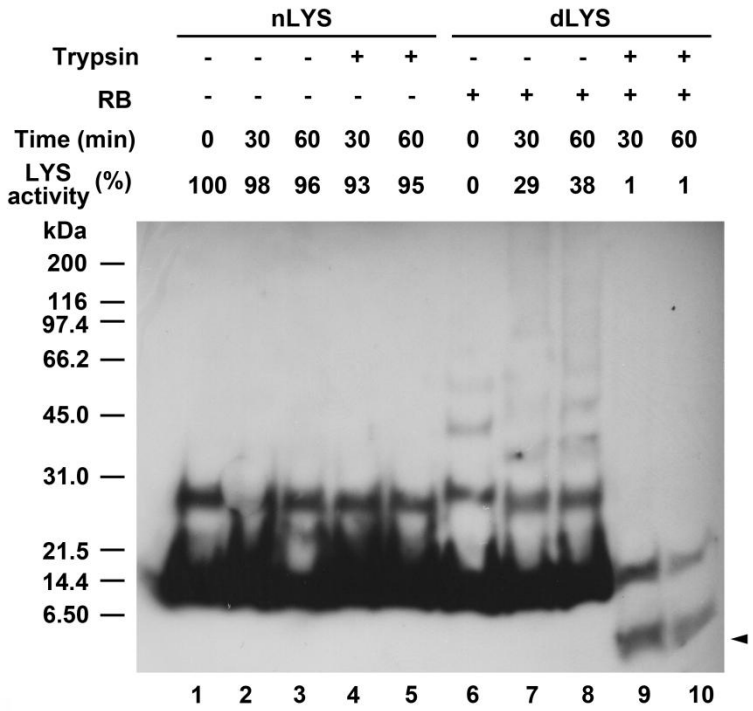


Fig. 25. Comparison of trypsin sensitivities of native lysozyme, and reduced denatured lysozyme.

Native lysozyme (nLYS), and reduced denatured lysozyme (dLYS) incubated for 60min in the presence or absence of trypsin were sampled every 30min, followed by treatment with 2% SDS sample buffer containing 5 mM NEM. Samples were subjected to SDS-PAGE as described in Fig. 18. RB, refolding buffer; Time, refolding and digestion time; Arrowhead, digested fragment of lysozyme.

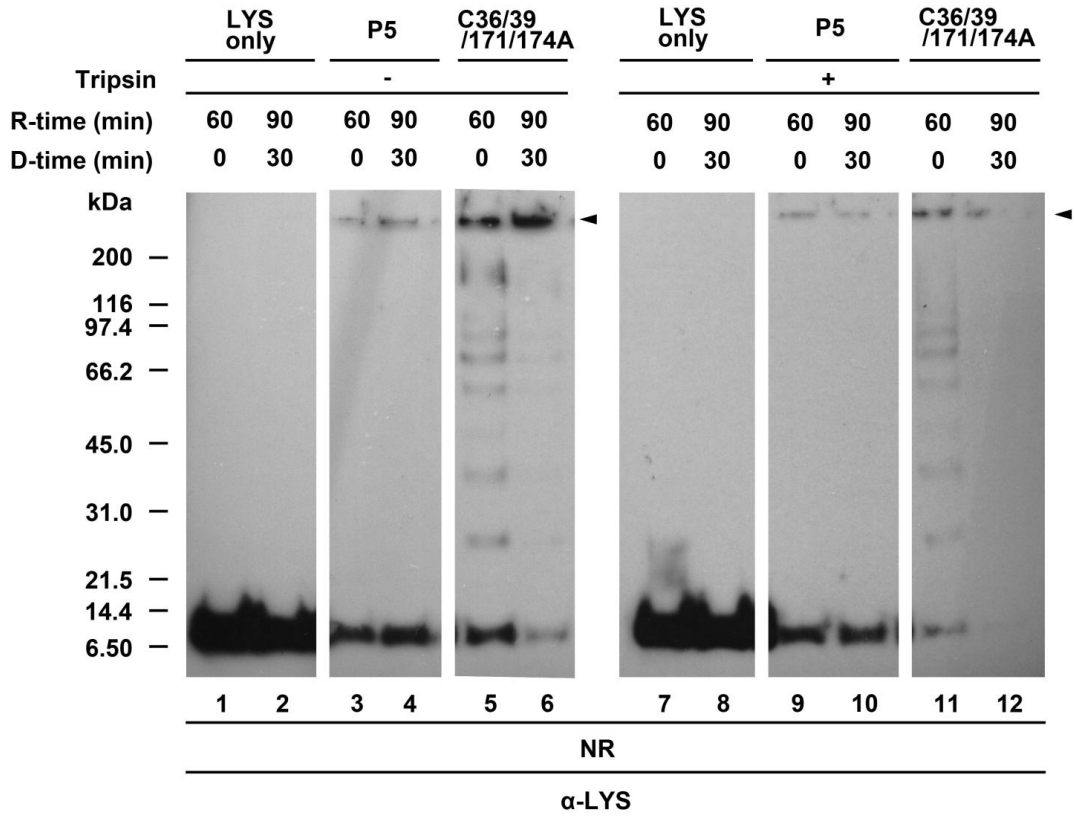


Fig. 26. Lysozyme multimers at the hyper molecular region formed by P5 more resistant to trypsin than those formed by C36/39/171/174A.

Trypsin sensitivities of lysozyme multimers at the hyper molecular region formed by P5 and C36/39/171/174A at 0.7 μM were compared. Lysozyme products refolded for 60 min in the presence or absence of P5 and C36/39/171/174A, followed by trypsin treatment for 30 min were detected by α-LYS. R-time, refolding time; D-time, digestion time; arrowheads, hyLYS formed by P5 and lysozyme multimers with hyper molecular mass formed by C36/39/171/174A. Lanes 1-6 and lanes 7-12 were split from a single membrane and compared, respectively.

```
P5_BOAR                 SEDVAKKSCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYKKKMWGWLWTEAGAQ 342
P5_BOVINE               NEDVAKKTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYKKKMWGWLWTEAGAQ 342
P5_MOUSE                NEDIAKKTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYKKKMWGWLWTEAGAQ 347
P5_RAT                  NEDIAKKTCEEHQLCVVAVLPHILDTGATGRNSYLEVLLKLADKYKKKMWGWLWTEAGAQ 342
P5_HUMAN                NEDIAKRTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYKKKMWGWLWTEAGAQ 342
P5_CHICKEN              NEDVLKTTCDAHQLCIISVLPHILDTGASGRNSYLDVMLKMAEKYKKKMWGWLWTEAGAQ 348
P5_XENOPUS              NGDIVKKTCDEHQLCIVAVLPHILDTGASGRNSYLDVMMKMADKYKKKMWGWLWAEAGAQ 343
P5_DANIO                NEGILKKTCEDYQLCIIAVLPHILDTGASGRNSYLEVMKTMAEKYKKKMWGWLWTEAGAQ 342
P5_LANCELET             GSEVMKA-CAEKQLCVVSFLPHILDTGASGRNQYLEQLRMMGEKYKKKVWGWVWAEAGAQ 342
P5_NEMATOSTELLA         SNEVLKEGCNEHPICVIAFLPHILDSGASGRNTYLANLKELGEKYKKNRWGWLWSEAAAQ 330
P5_CAENORHABDITIS       NQQVVEDACKEKQLCIFAFLPHILDCQSECRNNYLAMLKEQSEKFKKNLWGWIWVEGAAQ 345
P5_DROSOPHILA           NESTFETACEGKPLCVVSVLPHILDCDAKCRNKFLDTLRTLGEKFKQKQWGWAWAEGGQQ 338
P5_CIONA                SQEVFDENCGTH-LCILAFLPDIADDGKDGRNRYIDLLKSLGDRFKKQRWGWAWLPANAN 341
P5_ORIZA                GPDAMEEKCASAAICFVSFLPDILDSKAEGRNKYLELLLSVAEKFKKSPYSFVWTAAGKQ 344
P5_ZEA                  GPDVMEEKCASAAICFVSFLPDILDSKAEGRNKYLELLLSVAEKFKKSPYSFVWTAAGKQ 342
P5_TRITICUM             SADVMEEKCASAAICFVSFLPDILDSKAEGRNKYLELLLSVAEKFKKSPYSFVWAGAGKQ 343
P5_GLYCINE              SPDVLEEKCGSAAICFVAFLPDILDSKAEGRNRYLQQLLSVAEKFKRSPYSYVWVAAGKQ 341
P5_ARABIDOPSIS          GPDVMEKKCGSAAICFISFLPDILDSKAEGRNKYLEMLLSVAEKFKKQPYSFMWVAAVTQ 341
P5_PHYSCOMITRELLA       GQDVLDKECGSAAICFVSFLPDILDSKAEGRNKYLATLRNVAEKYKRN--------AYRQ 336
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Fig. 27. Alignment of sequences around the conserved cysteines in *b* domain of P5 subfamily.

A part of the sequences around the conserved cysteines in *b* domain of P5 subfamily are shown. Arrows, the conserved cysteines. The conserved non- catalytic cysteines at C272 and C278 of boar mature P5, used in this study, are positioned at C291 and C297 in the precursor form. The listed 19 sequences are from BOAR (*Sus scrofa*, BAJ11758), BOVINE (*Bos taurus*, NP_001193274), MOUSE (*Mus musculus*, NP_082235), RAT (*Rattus norvegicus*, PDIA6_RAT), HUMAN (*Homo sapiens*, AAH01312), CHICKEN (*Gallus gallus*, XP_419952), XENOPUS (*Xenopus tropicalis*, CAJ82905), DANIO (*Danio rerio*, NP_922915), LANCELET (*Branchiostoma floridae*, EEN42176), NEMATOSTELLA (*Nematostella vectensis*, XP_001629218), CAENORHABDITIS (*Caenorhabditis elegans*, PDIA6_CAEEL), DROSOPHILA (*Drosophila melanogaster*, NP_609792), CIONA (*Cionaintestinalis*, XP_009857523), ORIZA (*Oryza sativa Japonica Group*, PDI23_ORYSJ), ZEA (*Zea mays*, NP_001105804), TRITICUM (*Triticum aestivum*, CBG91909), GLYCINE (*Glycine max*, NP_001236576), ARABIDOPSIS (*Arabidopsis thaliana*, AEC08762) and PHYSCOMITRELLA (*Physcomitrella patens*, EDQ54468). The sequences were aligned using the CLUSTALW program.

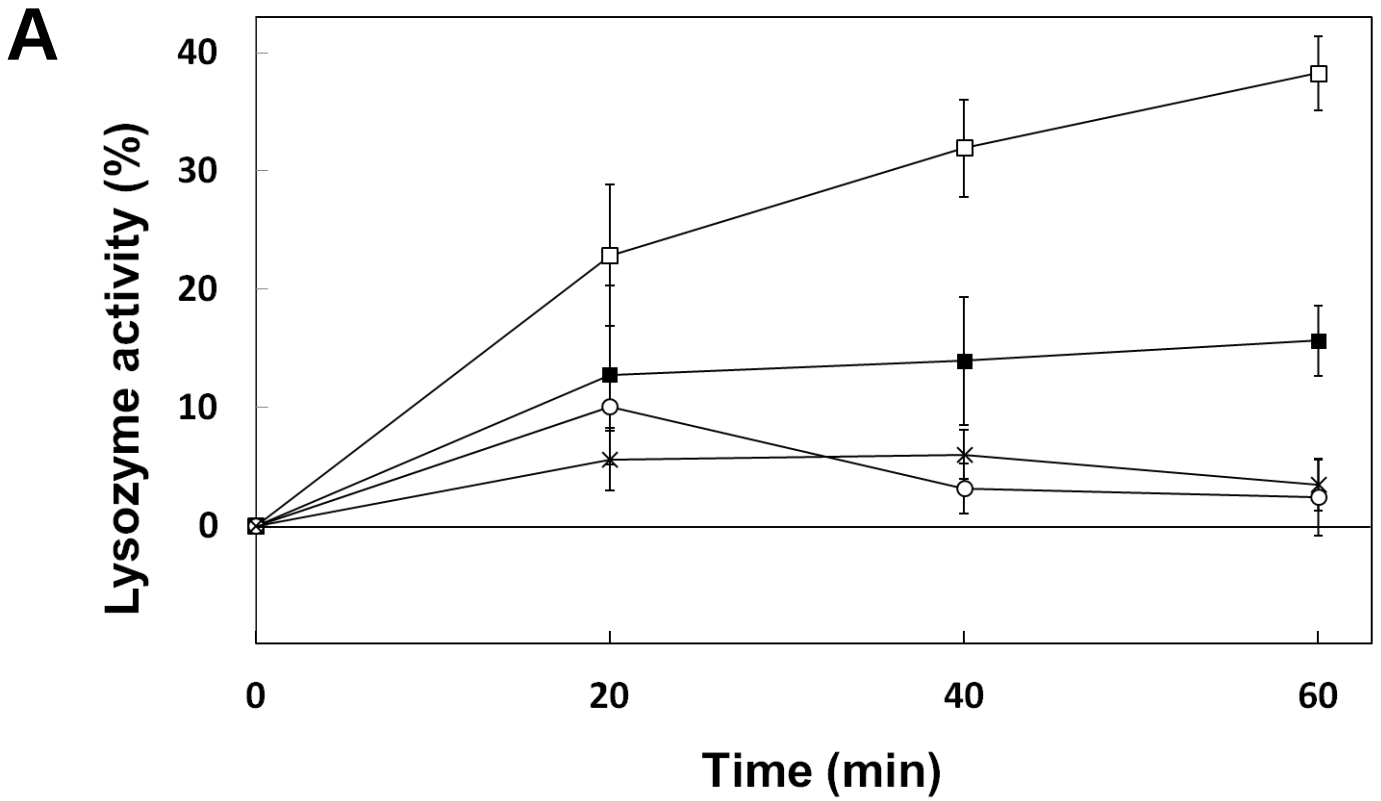


Fig. 28. Effect of C272/278A on lysozyme refolding

(A) Lysozyme activity in the presence of C272/278A were examined to find moderate inhibition of lysozyme activity in the presence of C272/278A

□, lysozyme only; ■, C272/278A; ○, P5; ×, C36/39/171/174A.

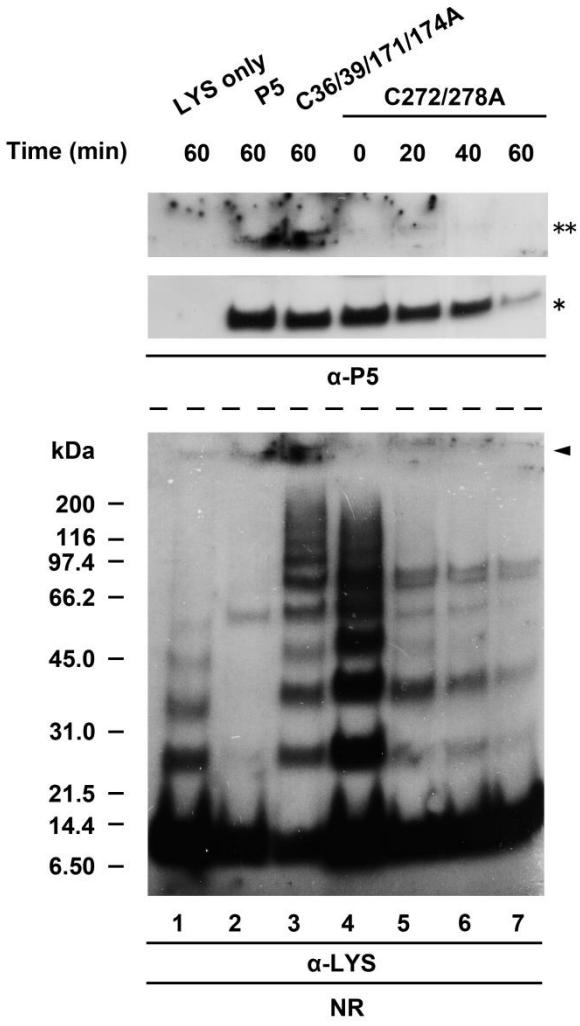


Fig. 28. Effect of C272/278A on lysozyme refolding

(B) Western blotting analysis of lysozyme refolding products in the presence of C272/278A, P5 and C36/39/171/174A at 0.7 μM. Refolding products in the presence of C272/278A were sampled every 20 min. *, P5, C36/39/171/174A and C272/278A in monomeric forms; **, P5 and C36/39/171/174A at the hyper molecular region. Arrowheads, hyLYS formed by P5 and lysozyme multimers with hyper molecular mass formed by C36/39/171/174A.

C

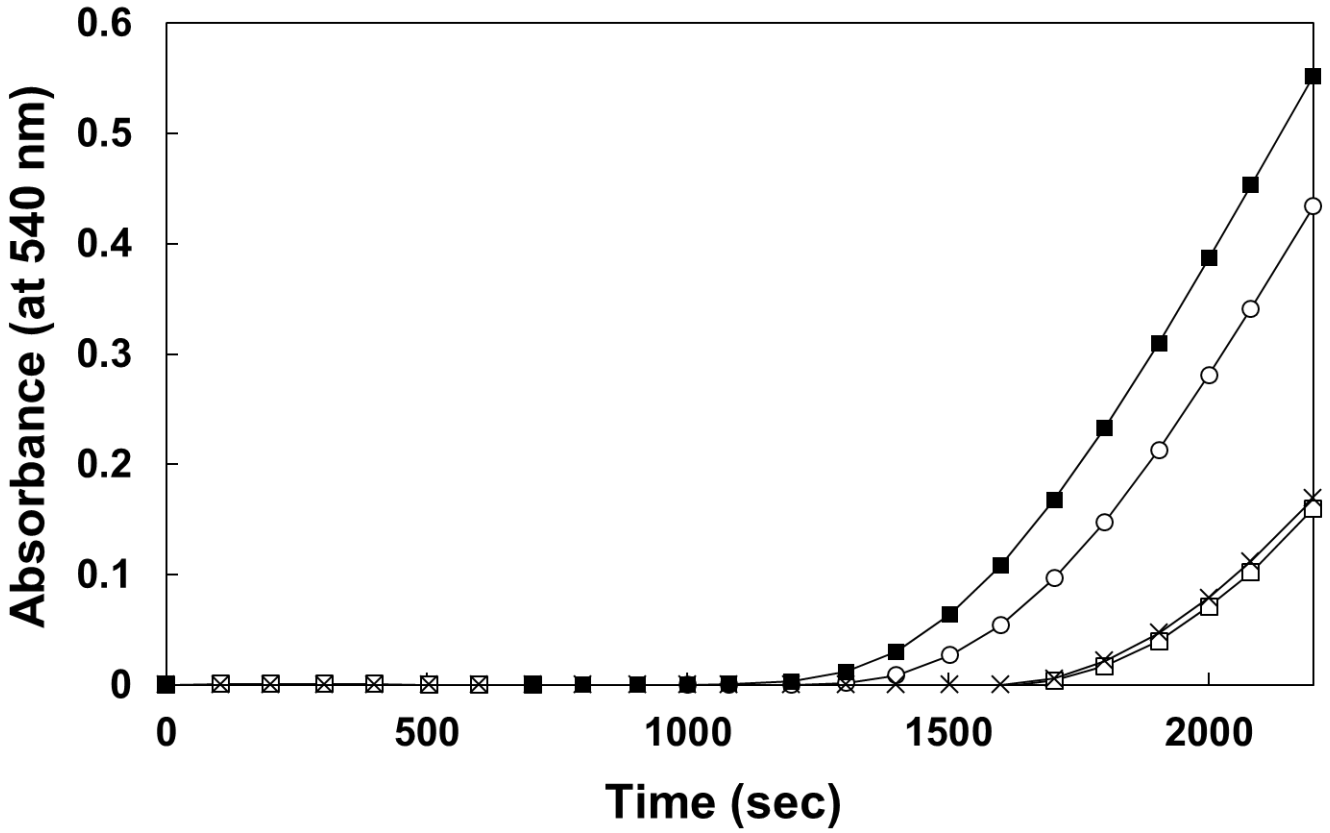
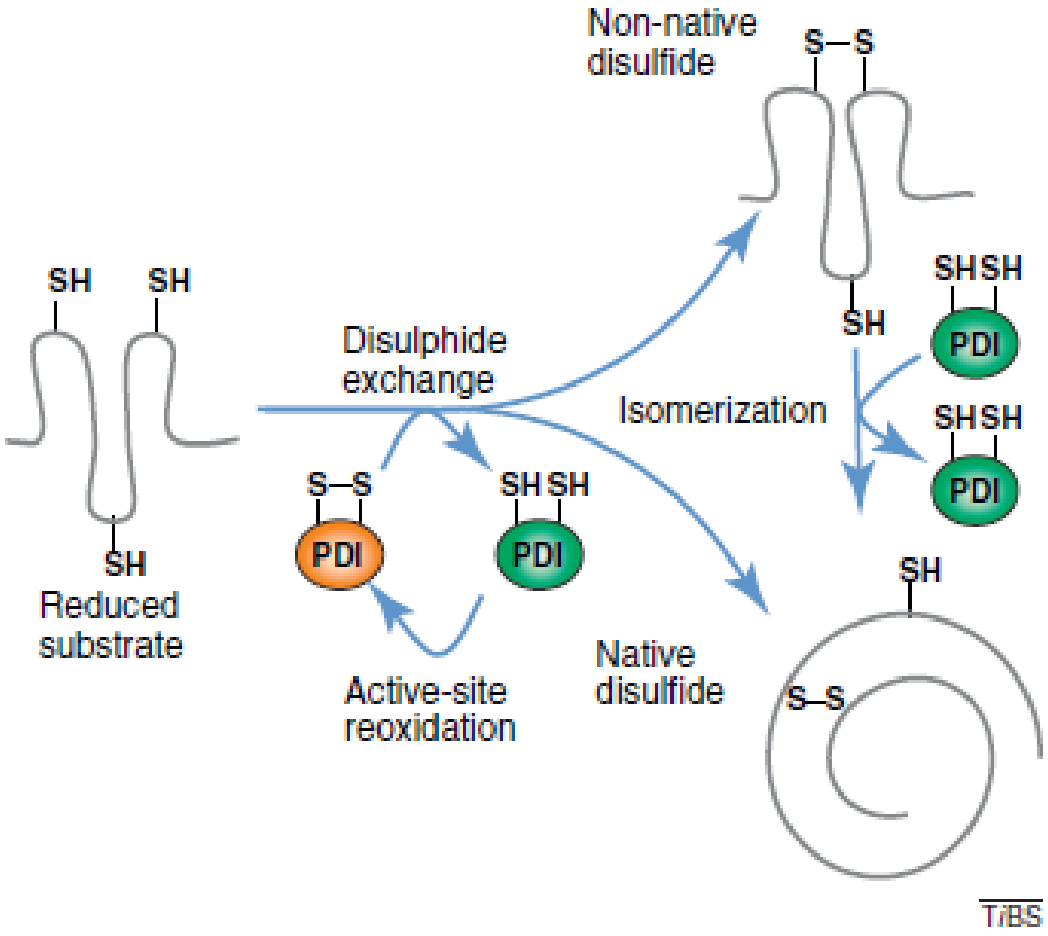


Fig. 28. Predominant reductase activity of C272/278A.

(C) Comparison of insulin reductase activities in the in the presence of C272/278A, P5 and C36/39/171/174A at 0.19 μM.

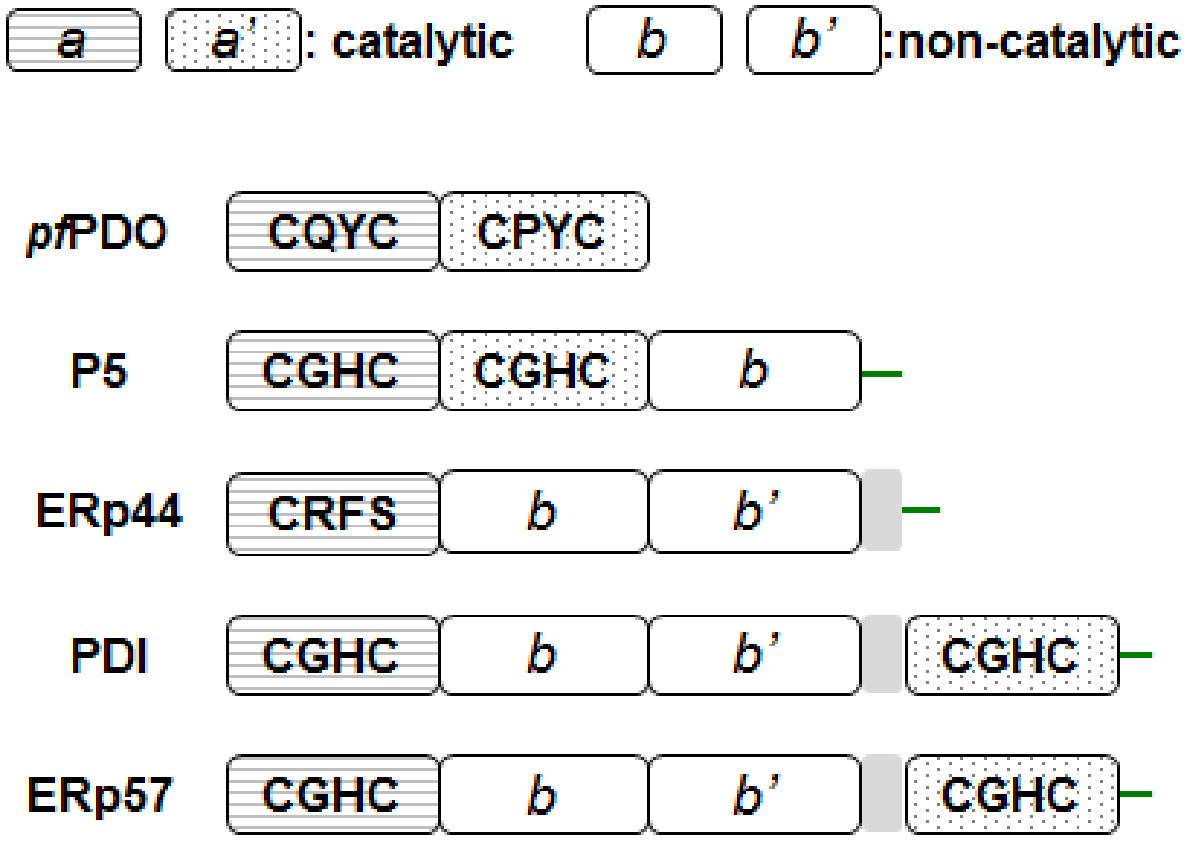
○, P5; ■, C272/278A; ×, C36/39/171/174A; □, DTT only.

APPENDICES



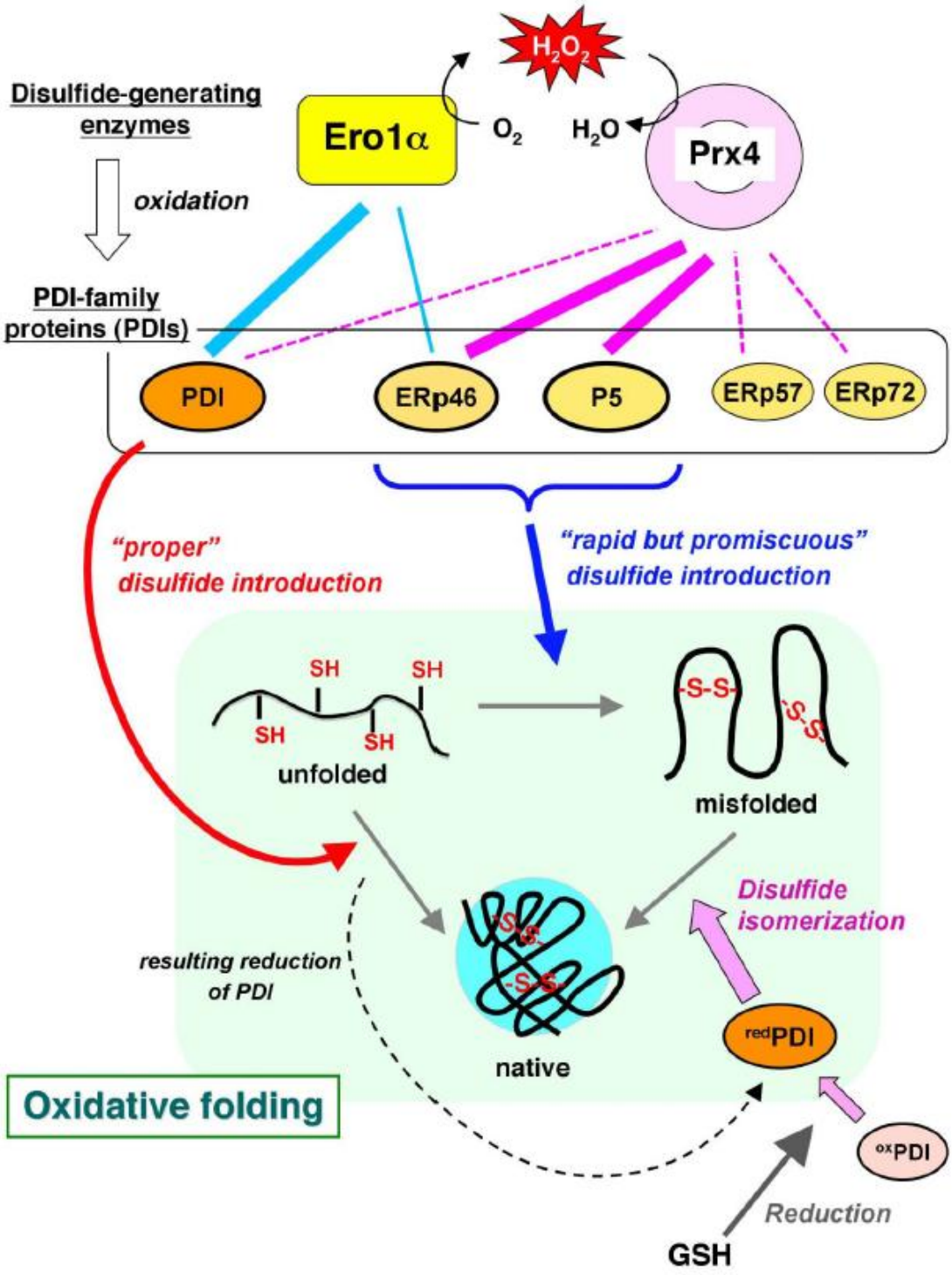
App. 1. PDI catalyzes the formation, isomerization, and reduction of disulfide bonds of proteins.

PDI plays the centering role for protein folding. Picture from Multiple ways to make disulfides, N. Bulleid and L. Ellgaard, Cell Press 36 (2011) 485-492.



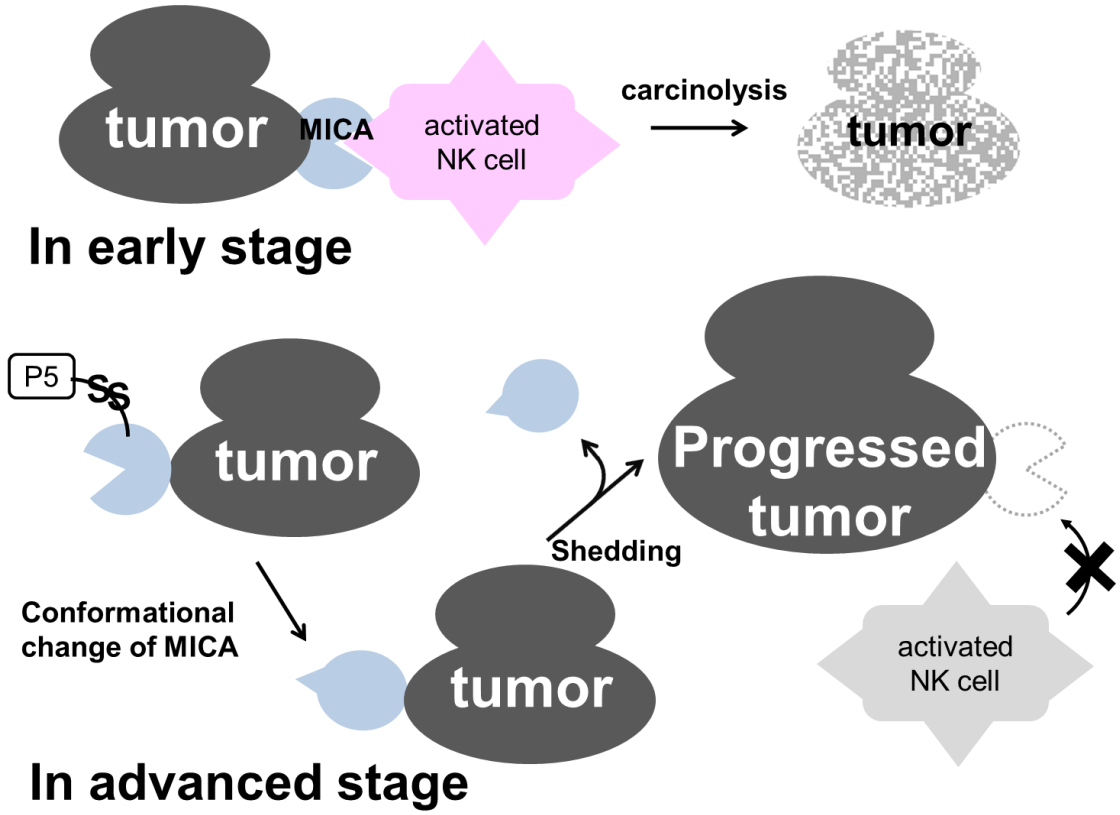
App. 2. Domain structures of PDI family members.

Domain structures of some PDI family are shown. PDI family is characterized with one or more thioredoxin domain(s). Catalytic domains include typically CXXC active site(s), involved in the formation, isomerization, and reduction of disulfide bonds. Some catalytic motives include Ser as shown in ERp44. *pf*PDO, protein disulfide oxidoreductase from *Pyrococcus furiosus*.



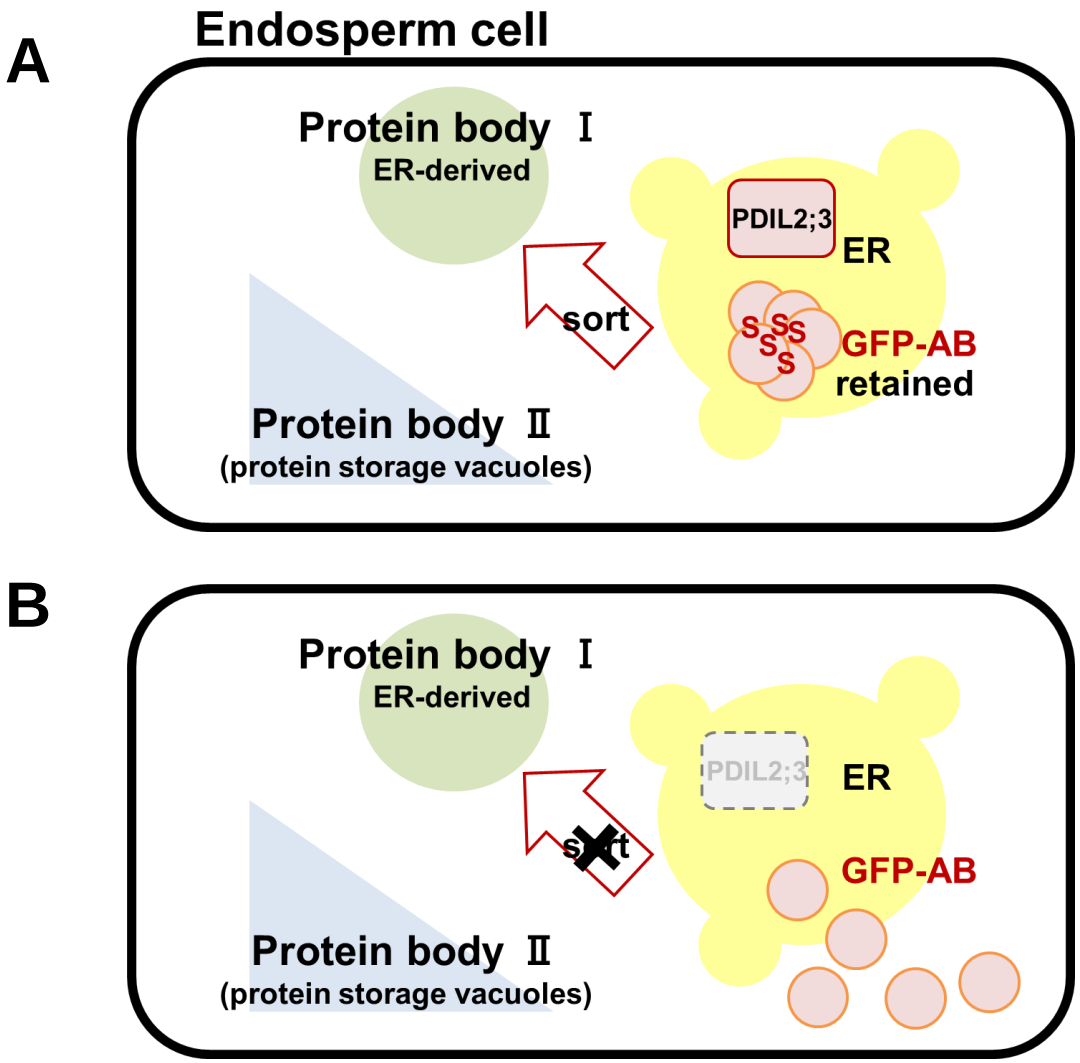
App. 3. A cooperative quality control network in the redox pathway.

A hierarchical electron transfer network centering on the PDI-Ero1α complex, including ERp57, ERp46, and P5 [7].



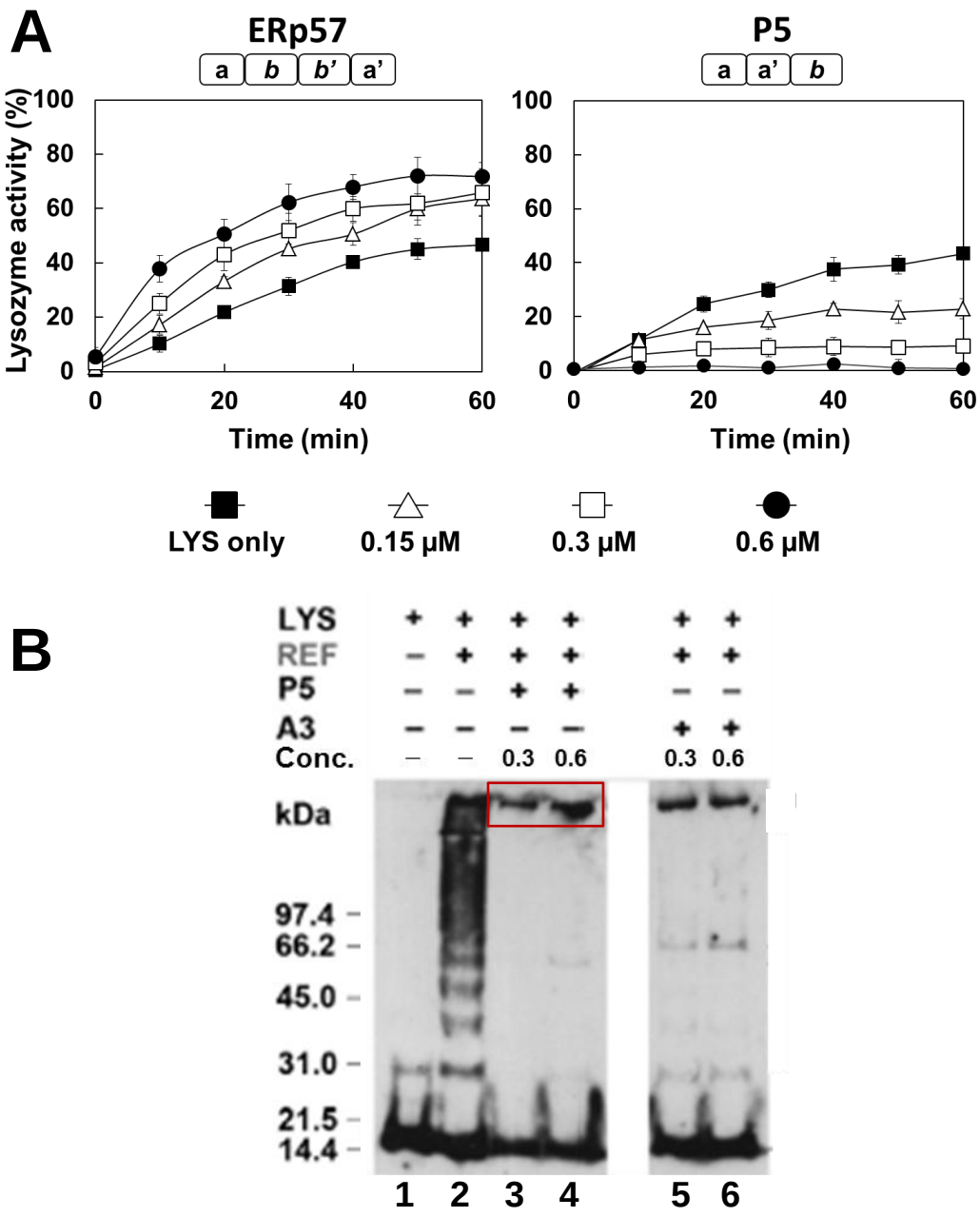
App. 4. Tumor immune evasion triggered by disulfide reduction.

While tumors in early stage undergo carcinolysis, those in advanced stage form transient mixed disulfide complexes via MICA, followed by the disulfides reduction, causing its conformational change. The change enables proteolytic cleavage of MICA from membrane surface, resulting in tumor progression. MICA, major histocompatibility complex class I-related ligand [13].



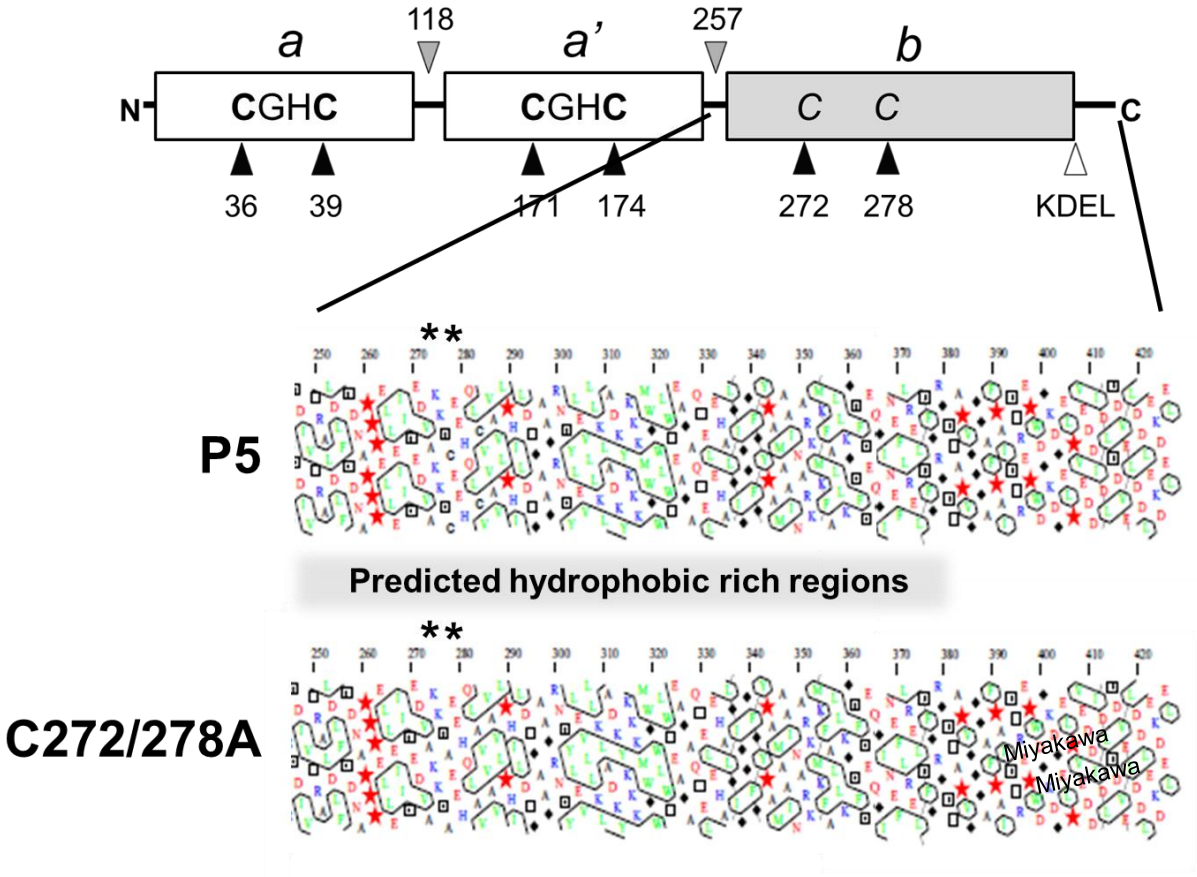
App. 5. Sorting promotion of GFP-AB by PDIL2;3.

Sorting promotion of GFP-AB, the N-terminal region of α-globulin fused to GFP, by PDIL2;3 (a rice orthologue of human P5) is illustrated. (A) PDIL2;3 retains GFP-AB within the ER by accumulating it via intermolecular disulfide cross-linkages, and promote sorting of the protein to protein body Ⅰ (PB-Ⅰ). (B) The knockdown of PDIL2;3 inhibits the efficient localization of GFP-AB in PB-Ⅰ and promotes its exit from the ER. [16]



App. 6. P5 inhibits oxidative refolding of denatured lysozyme to form lysozyme multimers with intermolecular disulfides and hyper molecular mass.

(A) Effects of ERp57 and P5 on oxidative refolding. (B) Western blotting of the refolding products after incubation for 60 min in the presence of ERp57 and P5. P5 forms hyLYS in dose dependent manner. Box, hyLYS.
Note that lysozyme in multimeric forms (lane 2) refolded and detected as a monomer in the presence of ERp57 (lanes 5 and 6), while those migrated to the hyper molecular region and detected as the hyLYS in the presence of P5 (lanes 3 and 4). Conc., concentration (μM) [17].



App. 7. The hydrophobic-rich regions around C272/278 in *b* domain.

The hydrophobic regions predicted by Luc CANARD are colored in green. Asterisks, the positions of cysteines. Even with cysteine mutations, the hydrophobic region same as P5 are shown.

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Hope that Science will make the universe happy to see blue immensity of the sky.