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by Lalu Rudyat Telly Savalas C7

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The b'x Region of Yeast Protein Disulfide Isomerase is Not Essential for *Saccharomyces cerevisiae* Viability at 30 °C

PURKAN^{1,2}, LALU RUDYAT TELLY SAVALAS^{1,3}, MULIAWATI SINDUMARTA¹, AND DESSY NATALIA^{1*}

¹Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia, ²Chemistry Department, Faculty of Sciences and Technology, Universitas Airlangga, Jalan Mulyorejo, Surabaya 60115, Indonesia, ³Chemistry Department, Faculty of Science Education, Universitas Mataram, Jalan Majapahit 62, Mataram 85125, Indonesia

Protein disulfide isomerase (PDI) catalyzes thiol oxidation, reduction and isomerization of disulphide bond of cell surface and secreted proteins. Yeast PDI consists of two catalytic domains (a and a') which are separated by two non-catalytic domains (b and b'), and a x region linked the b' and a' domains. The b' domain is important for the non-covalent binding of partially folded protein. To understand the contribution of b' domain and x-linker of yeast PDI we have deleted the b'x and investigated its functional role *in vitro* and *in vivo*. Yeast PDI without b'x region retained only 50% activity and became more sensitive toward Proteinase K. Interestingly, yeasts containing full length PDI and *pdi1Δb'x* showed approximately the same growth rate. However, the yeast *pdi1Δb'x* mutant growth impaired severely at 37 °C compared to that of the full length PDI. Our results suggested that the a-b-a'-c domains of PDI seems to be sufficient to support the growth of yeast cells in normal condition, but the b'x region might be essential in assisting refolding of highly accumulated unfolded protein at high temperature (37 °C).

Key words: b' domain, protein disulfide isomerase, *Saccharomyces cerevisiae*, x-linker

Formation of correct disulfide bonds is essential for proper folding of the majority of cell surface and secreted proteins in eukaryotic cells. Protein disulfide isomerase (PDI), which is a resident of the endoplasmic reticulum (ER), catalyzes the formation, reduction and isomerization of disulfide bonds. It has also a chaperone role which mediates the folding and assembly of nascent peptides into mature proteins in the cells (Wang and Tsou 2003). The chaperone activity is independent of its catalytic activity as it has been demonstrated that PDI improves secretion of redox-inactive β-glucosidase (Powers and Robinson 2007). In addition, PDI is found to form a complex with other protein molecules, namely prolyl-4 hydroxylase and microsomal triglyceride transfer protein (Pihlajaniemi *et al.* 1987; Wetterau *et al.* 1991).

Protein disulfide isomerase is composed of four thioredoxin-like domains, a, b, b' and a', followed by an acidic C-terminal extension (named a'') harboring ER retention signal (Freedman *et al.* 1994). The a and a' domains each contain active site sequence CXXC which is required for disulphide bond formation. The b' domain of human PDI contributes significantly to protein substrate interactions (Klappa *et al.* 2000; Denisov *et al.* 2009).

In *Saccharomyces cerevisiae* PDI is encoded by an essential gene *PDI1* (Farquhar *et al.* 1991). The three dimensional structure of yeast PDI1 has a twisted U-shape in which the a and a' domain facing each other on the end of U while the b and b' domain forming the base (Tian *et al.* 2006). The b' and a' domains is connected by 17 residues which is referred as x-linker. The crystal structure shows that the b and b' domains have hydrophobic patch which together with hydrophobic areas surrounding the active sites, form a continuous hydrophobic surfaces across the a, b' and a' domain (Tian *et al.* 2006). These hydrophobic residues are involved in the interaction of PDI with its substrates.

In this paper we described that yeast *pdi1* without b'x region still maintains half of its insulin reductase activity, though it becomes more sensitive toward Proteinase K compared to that of full length PDI. Furthermore, we demonstrated that *pdi1Δb'x* can support yeast viability.

MATERIALS AND METHODS

Materials. Plasmid pRS314-*PDI1* (a generous gift from Prof. W. J. Lennarz, Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, USA) containing *PDI1* was used as a template for amplification of a DNA fragment encoding for *pdi1* without b'x. *Escherichia coli* DH5 α was used for plasmid propagation and BL21 (DE3) (ara, *leu7967*, *lacX74*, *phoA*, PvuII, *phoR*, *malF3*, F' [*lac*, *lacP*], *pro*], *trxB::kan(DE3)*) was used for expression host of PDI proteins. Yeast *S. cerevisiae* strain 2736 (*MATa ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15 pdi1::HIS3*) containing pCT37-*PDI1* [*URA3*] (obtained from Prof. T. H. Steven, Institute of Molecular Biology, University of Oregon, USA) and pUKC639 (kindly provided by Prof. M. F. Tuite, School of Biosciences, University of Kent, United Kingdom) were used for plasmid shuffling in yeast cell. Plasmid pT7.7 (Novagen, Madison, WI) was used for expression of a *pdi-Δb'x* mutant in *E. coli*. Plasmid pGEM[®]-T, *Vent* and *Taq* DNA polymerases were obtained from Promega. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. All synthetic DNA oligomers were synthesized by Pro-Oligo Singapore. Yeast cells were grown in minimal medium [0.7% (w/v) yeast nitrogen base (YNB) without amino acid, 2% (w/v) glucose or 2% (w/v) galactose] supplemented with appropriate amino acids and bases, and YEPD/Gal yeast extract [(1% (w/v), (w/v) bacto peptone 1%, (w/v) glucose 2%, (w/v) galactose 2%)] media. *Escherichia coli* was grown in Luria-Bertani (LB) (Sambrook *et al.* 1989).

Construction of *pdi1Δb'x* Mutant. A yeast PDI lacking b'x region coded by nucleotides at position 739-1149 in *PDI1* gene was constructed through 3 steps of PCR (Fig 1) using

*Corresponding author, Phone: +62-22-2502103, Fax: +62-22-2504154, E-mail: dessy@chem.itb.ac.id

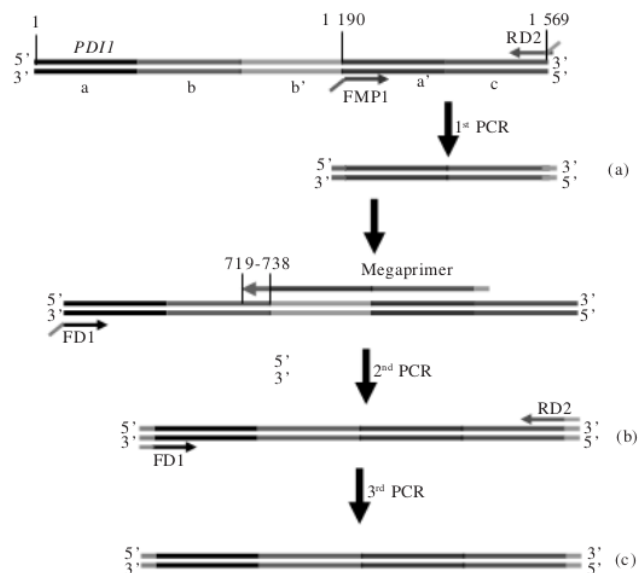


Fig 1 Strategy of deletion of DNA fragment encoding b'x region of yeast *PDII* using three steps PCR. The first PCR was performed to produce a 447 bp DNA fragment encoding a'c domain (a). The resulted fragment was used as a mega primer together with FD1 primer in the second PCR to produce a *pdi1Δb'x* mutant. A low concentration of *pdi1Δb'x* mutant (b) was obtained at this step. The third PCR was conducted to increase the concentration of *pdi1Δb'x* mutant using FD1 and RD1 primers. This step gave a high concentration of *pdi1Δb'x* mutant (c).

pRS314-*PDII* as a template. Oligonucleotide primers for PCR were designed based on published sequences of *PDII* gene (Accession number 850314). Primers FMP1 (5'-ACT TTGGTGAAATCGACGGTAAGAACCATGACGAA-3') and RD2 (5'-GAGGAGGATCCTTACAATTTCATCGTGAATGG-3') were used to amplify a DNA fragment corresponding to coding region 1150-1569 of *PDII*. The FMP1 contained 20 nucleotides corresponding to the coding region 719-738 of *PDII*, while RD2 primer had a *Bam*HI restriction site.

The resulted DNA fragment from the first round PCR as a reverse mega primer together with primer FD1 (5'-GAG GAC ATA TGA AGT TTT CTG CTG GTG-3') and a template of pRS314-*PDII* were used in the second PCR. The third PCR was carried out using primers forward FD1 and reverse RD2 (5'-GAGGAGGATCCTTACAATTTCATCGTGAATGG-3'), and the second PCR results as a template. All PCR products were analyzed by agarose gel electrophoresis and purified by GFX purification kit.

The resulted *pdi1Δb'x* DNA fragment was ligated with pGem[®]-T vector and the the ligation product was transformed into *E. coli* DH5α using CaCl₂ method (Sambrook *et al.* 1989). The pGem[®]-T-*pdi1Δb'x* was digested with *Nde*I and *Bam*HI restriction enzymes and the resulted 1400 bp *pdi1Δb'x* fragment was isolated and then ligated with an expression vector previously digested with *Nde*I and *Bam*HI. The resulted pT7-7-*pdi1Δb'x* was confirmed by restriction analysis. The pT7-7-*pdi1Δb'x* was subjected to DNA sequence analysis.

Construction of *pdi1Δb'x* in Yeast Expression Plasmid. Plasmid pT7-7-*pdi1Δb'x* as a template, and primers PDIF1 (GCTAGCATGAAGTTTCTGCTGGTGC) and DIR1 (GCTAGCTTACAATTTCATCGTGAATGG) were used to amplify *pdi1Δb'x*. The PCR program was 94 °C for 4 min,

25 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C and 5 min at 72 °C. *Nhe*I sites were included in both forward and reverse primers. The PCR products were gel purified, subcloned into pGem[®]-T vector. A *Nhe*I fragment of *pdi1Δb'x* taken out from pGem[®]-T-*pdi1Δb'x*/*Nhe*I was then inserted into pUKC639 previously digested with similar restriction enzyme. The resulted pUKC639-*pdi1Δb'x* was sequenced to confirm the presence of the deletion.

Transformation of Yeast. The resulted pUKC639-*pdi1Δb'x* was used to transform *S. cerevisiae* 2736 using a modified lithium acetate method (Ito *et al.* 1983). The transformed cells were plated onto minimal medium supplemented with 0.001% (w/v) adenine and 0.002% (w/v) tryptophan and grown at 30 °C for 4-7 d.

Plasmid-Shuffling. The yeast transformants carrying pUKC639-*pdi1Δb'x* were grown in solid minimal medium containing 2% (w/v) galactose, 1 mg mL⁻¹ 5'fluoroorotic acid (5'FOA), 0.002% (w/v) tryptophan, 0.001% (w/v) adenine and 0.001% (w/v) uracil at 30 °C for 4-7 d. The yeast cells designated as 2736D were then transferred into a minimal medium supplemented with similar amino acid and bases.

Protein Expressions. *Escherichia coli* strain BL21 (*DE3*) carrying *PDII* or *pdi1Δb'x* genes on plasmid pT7-7 were grown in 10 mL LB media containing 100 g mL⁻¹ ampicillin at 37 °C for 16 h. One mL of overnight culture was transferred into a fresh 50 mL LB/ampicillin media and grown until the culture OD₆₀₀ reached 0.5. Isopropyl-α-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM and the culture was grown for further 2.5 h. Cell cultures were precipitated by centrifugation at 2100 x g for 30 min at 4 °C. The pellet cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 0.2 mM NaCl dan 10% glycerol) containing 1 mg mL⁻¹ lysozyme. Cells were lysed using

sonication with 10x 1 min burst at maximum frequency and cooled on ice for 1 min after each 1 min burst. The lysed cells were then centrifuged at 9500 x g for 30 min at 4 °C.

Assay of PDI Protein Activities. The ability of PDI1 to reduce disulphide bond was determined as described by Sutter *et al.* (1994). The assay mixture contained 1.67 mM insulin in 100 mM potassium phosphate buffer, pH 7.0, 20 mM ethylene diamine tetraacetic acid, crude bacterial cell extract PDI, 10 mM dithiothreitol. The increase of OD at 650 nm from precipitation of insulin B chain was measured. One unit activity was defined as an amount of PDI to reduce disulfide bonds of insulin in order to produce ΔA650 of 0.001 per min at experimental condition.

Stability Test of Mutant pdi1Δb'x. Stability of mutant PDI toward proteinase K was conducted based on method described by Klappa *et al.* (2000). PDI was mixed with various concentration of Proteinase K (1; 3; 10 mg mL⁻¹) in PBS buffer (NaCl 140 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.8 mM, pH 8) for 30 min at 4 °C. The reaction was stopped with 5 mM phenyl methyl sulphonyl fluoride (PMSF) for 5 min.

SDS-Polyacrylamide Gel Electrophoresis and Western blotting. Protein electrophoresis was performed as described by Laemmli (1970). Protein blotting was done by using semi-dry blotter (BioRad). Protein in the gel was transferred onto nitrocellulose membrane (Hybond ECL). The membrane was incubated with an antibody anti PDI and subsequently with the peroxidase-conjugated anti rabbit Ig antibody. The signal was developed using ECL detection reagent (Amersham).

RESULTS

Yeast pdi1Δb'x has been Generated by Three Steps PCR.

Yeast *pdi1Δb'x* mutant was constructed by deletion of a 739-1146 DNA region of *PDI1* corresponding to Ser247-Gly383 using three steps of PCR (Fig 2B). The first PCR was performed to amplify the DNA fragment at nucleotide position of 1150 to 1569 corresponding to the a'c domains. The resulted DNA fragment with the size of 447 bp also contained 20 oligonucleotides of b domain (Fig 3, lane 3). The oligonucleotide of a'c domain was used as a reverse mega primer in combination with FD1 primer to generate a low yield of 1190 bp DNA fragment corresponding to aba'c domain in the second PCR (Fig 3, lane 4). To increase the amount of the 1190 bp *pdi1Δb'x* DNA fragment, a third PCR using FD1 primer and RD2 primer was performed. The resulted DNA fragment (Fig 3, lane 5) was first ligated with pGem[®]-T plasmid and then subcloned into pT7.7 expression vector. The sequence of the 1190 bp was confirmed by dideoxynucleotide sequence analysis and found to be free of mutation. A full length *PDI1* generated by PCR was also subcloned into pT7-7 plasmid.

Mutant pdi1Δb'x Produced as a Soluble Protein in E. coli.

The full length *PDI1/pdi1Δb'x* gene is placed under the control of ϕ10 promoter which requires T7 RNA polymerase to activate the promoter. The host *E. coli* strain used had already been transfected with phage DE3 containing the T7 RNA polymerase gene. The T7 RNA polymerase was under the control of of inducible *lacUV5* promoter and thus addition

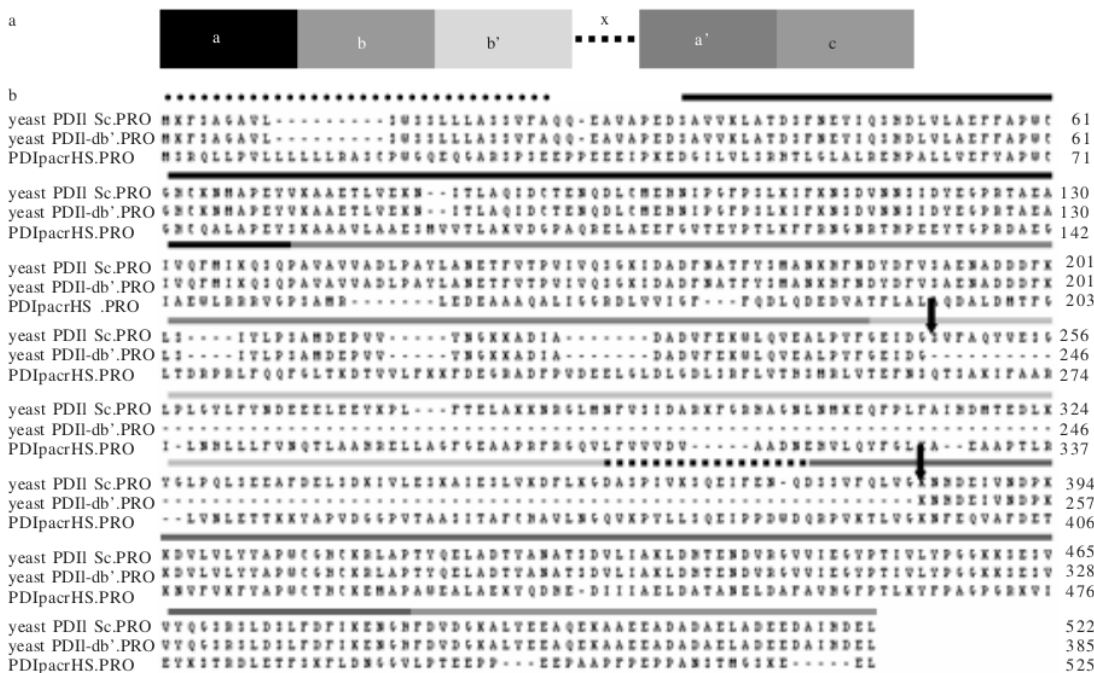


Fig 2 Primary structure of yeast PDI, pdi1Δb'x and human pancreas PDI. a domain organization of PDI based on the yeast three dimensional structure (Tian *et al.* 2006), b multiple sequences alignment of *Saccharomyces cerevisiae* PDI1 (yeast PDI1Sc.PRO), pdi1Δb'x (yeast PDI1 db'.PRO) and human pancreas PDI (PDIpancrHS.PRO).

of IPTG induced the *lacUV5* promoter to produce T7 RNA polymerase thereby allowing transcription of the *PDII* gene. Proteins were isolated from the *E. coli* cultures as described in Material and Methods. The full length PDII appeared as a protein with molecular weight of approximately 60 kDa on SDS-PAGE while the molecular weight of *pdi1Δb'x* was 45 kDa (Fig 4). These are in agreement with the predicted molecular weight from deduced amino acids in which full length PDII consists of 522 amino acid residues while *pdi1Δb'x* contains 386 amino acid residues.

Mutant *pdi1Δb'x* has Lower Reductase Activity and is Proteinase K Sensitive. To determine whether deletion of *b'x* region affected PDI activity, the ability of both *pdi1Δb'x* mutant and full length PDII to catalyze the reductive cleavage of insulin was determined. The *in vitro* assay for PDI activity in the crude bacterial cell extract showed that specific activity of the *pdi1Δb'x* was $1.4 \times 10^3 \text{ U mg}^{-1}$ which is approximately 50% of that of the full length PDII ($2.8 \times 10^3 \text{ U mg}^{-1}$).

To know the stability of the yeast PDI lacking of *b'x* toward Proteinase K, the *pdi1Δb'x* and full length PDI were treated with Proteinase K. Proteolysis of crude bacterial cell extracts was carried out using different concentration of proteinase K. Western blot analysis using anti yeast PDI antibody showed that a significant amount of *pdi1Δb'x* were digested upon the increase of Proteinase K concentration. There is hardly *pdi1Δb'x* left after treatment with 10 mg mL^{-1} of proteinase K (Fig 5, lane 8). The full length PDII remains stable upon Proteinase K treatment (Fig 5). A lower band observed in the full length PDI could be the degradation product during protein preparation. This results suggest that *pdi1Δb'x* mutant is more susceptible to Proteinase K degradation.

Mutant *pdi1Δb'x* can Support Yeast Viability. Since *PDII* is an essential gene, a plasmid-shuffle procedure to observe the function of mutant *pdi1Δb'x* on supporting yeast viability was conducted. The recombinant plasmid pUKC639-*GALI-pdi1Δb'x* containing a *LEU2* selectable marker was introduced into a yeast strain 2736. The strain 2736 containing *pdi1::HIS3* null mutation carried a plasmid-borne *GALI-PDII* with a *URA3* marker (Tachibana and Steven 1992). After transformation, the cells were grown in a medium containing 5 FOA which allow cells to lose its *URA* based plasmid-containing full length *PDII*. Cells grown in the FOA medium indicated that the newly introduced *pdi1Δb'x* mutant is able to complement the chromosomal *pdi1* null mutation.

The Growth of *pdi1Δb'x* Yeast is Almost the Same as the Full Length *PDII* Yeast. The effect of *b'x* deletion on the growth rate of yeast cell was investigated by growing the yeast 2736 carrying full length *PDII* and 2736D containing *pdi1Δb'x* in rich media containing galactose at 30 °C. The growth rate of yeast carrying full length *PDII* was 0.13 U OD₆₀₀ per h, while 2736D had a growth rate of 0.12 U OD₆₀₀ per h. The results show that *pdi1Δb'x* and full length *PDII* can support the yeast growth at nearly the same growth rate 30 °C.

The *pdi1Δb'x* Yeast Impaired Severely at High Temperature. Protein disulphide isomerase is one of the main cellular chaperones in the ER. Under temperature stress, the amount of unfolded proteins accumulated in the ER will increase. We further investigated the effect of *b'x* deletion

on the yeast growth at temperature stress (37 °C). As shown in Fig 6 both 2736 and 2736D grew well at 30 °C. However, reduced growth were observed for both yeast strains at 37 °C in which 2736D growth impaired severely.

The *pdi1Δb'x* Yeast Shows No Cell Wall Defect. To study whether the *b'x* region is responsible in the folding of cell wall forming protein, the *pdi1Δb'x* mutant was grown in a medium containing calcofluor white. Defect on the cell wall forming protein leads to calcofluor sensitive phenotype. The *pdi1Δb'x* mutant seems to grow in a similar manner as the full length PDII (Fig 7) which indicates that there is no defect on the synthesis of cell wall in *pdi1Δb'x*.

DISCUSSION

Protein disulphide isomerase as a key player in the formation of correct disulphide bond in protein was discovered 40 years ago by Anfinsen and co workers (Goldberger *et al.* 1964). It is a member of thioredoxin superfamily which consists of five consecutive domains a, b, b', a' and c (Fig 2A). There have been many studies reported on the contribution of each domain or domain combinations in PDI activities both as an enzyme or as a chaperone. We have constructed a yeast PDI without b' domain and x-linker in which the N-terminals of 19 amino acid residues of the b' domain and 7 amino acid residues of the a' domain were still included (Fig 2B).

The *pdi1Δb'x* was expressed as a soluble protein in *E. coli* and retained only 50% of its activity in the reductive cleavage of insulin B chain. This represented that b' domain is required to PDI activity. However, other domains is also capable of interacting with insulin. Other study has shown that deletion of a,b and b'a'c domains of bovine PDI resulted in a decrease of insulin reductase activity of 94% and 78%, respectively (Sun *et al.* 2000). Since *pdi1Δb'x* still has two a and a' active site domains which are responsible in the reduction of disulphide bond of insulin, hence it possess higher activity compared to the other two bovine PDI variants. Several researchs have shown that multi domain fragments had enhanced catalytic activities compared with individual a or a' domains, that the b' domain had a particularly important role in this enhancement (Darby *et al.* 1998; Klappa *et al.* 1998; Tian *et al.* 2006).

To analyze whether there is a correlation between stability as defined by protease-resistance, crude bacterial cell extract containing *pdi1Δb'x* mutants was treated with Proteinase K. It appeared that *pdi1Δb'x* is protease sensitive while the full length PDII was only slightly affected at high Proteinase K concentration (Fig 4). The stability of full length human PDI toward Proteinase K had also been reported (Klappa *et al.* 2000). From the crystal structure of PDII, it was found that the two flexible catalytic a and a' domains are attached to more rigid b' and b domains (Tian *et al.* 2006), hence deletion of *b'x* region will decrease conformational stability.

Furthermore, we had investigated how the function of mutant yeast PDI lacking of *b'x* to support yeast viability. Plasmid shuffling experiment showed that *pdi1Δb'x* can rescue the *pdi1::HIS3* null mutation in 2736 yeast strain. LaMantia and Lennarz (1993) have demonstrated that *pdi1* mutant containing a, b and b' domains can support yeast

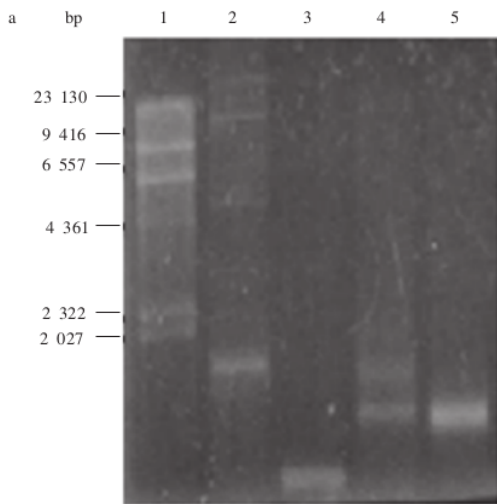


Fig 3 PCR product: Lane 1, DNA λ /Hind III; lane 2, full length *PDI1* (1569 bp); lane 3, *c'-a* fragment (447 bp); lane 4 and 5, *pdi1Ab'x* (1190 bp).

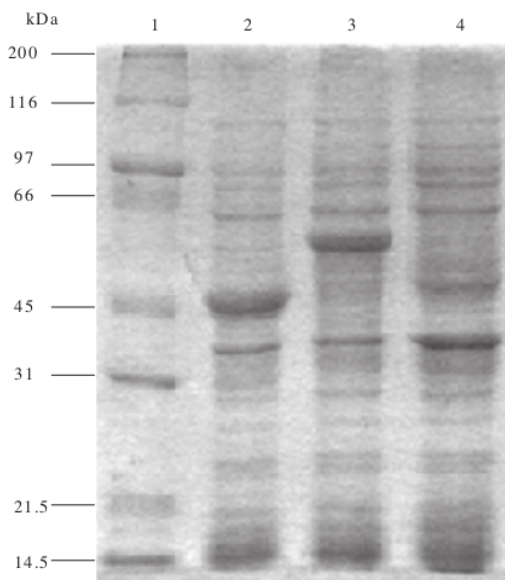


Fig 4 SDS-PAGE analysis of *pdi1Ab'x* and full length *PDI1*. Lane 1, protein marker; lane 2, *pdi1Ab'x*; lane 3, full length *PDI1*.

growth, while the *ab* domain has lost its essential function in yeast viability. We speculate that in the *pdi1Δb'x*, the *abc* domains might adopt the modular U shape structure which presumably able to interact with polypeptide substrate to carry out its activity *in vivo*. Crystal structure of *pdi1Δb'x* would be able to reveal the three dimensional structure of the mutant.

Protein disulfide isomerase can act both as a catalyst and also as a chaperone. Deletion of the *b'* domain significantly slows down the refolding rate of misfolded RNase (Darby *et*

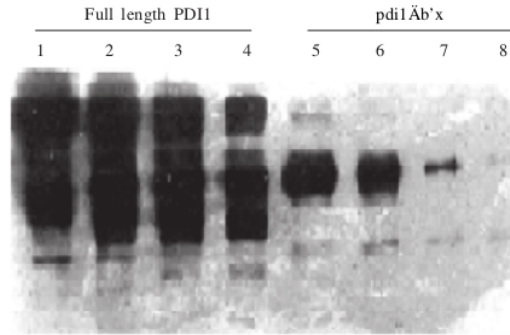


Fig 5 Western blot analysis of full length *PDI1* and *pdi1Ab'x* after treatment with Proteinase K. Lane 1 and 5, 0 $\mu\text{g mL}^{-1}$; lane 2 and 6, 1 $\mu\text{g mL}^{-1}$; lane 3 and 7, 3 $\mu\text{g mL}^{-1}$; lane 4 and 8, 10 $\mu\text{g mL}^{-1}$.

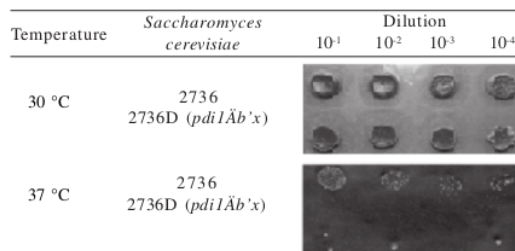


Fig 6 The effect of temperature stress on yeast growth. The parent 2736 and mutant *pdi1Ab'x* 2736D yeasts were first grown in minimal media at 30 °C for 2 d. The cultures were then diluted to obtain OD_{600} of 1.5. The yeasts were spotted in serial dilutions from 10^{-1} to 10^{-4} on YEPGal at 30 and 37 °C. Growth was scored after 4 d incubation.

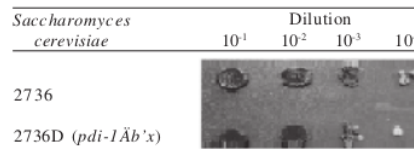


Fig 7 The effect of calcofluor on yeast growth. The parent 2736 and mutant *pdi1Ab'x* 2736D yeasts were first grown in minimal media at 30 °C for 2 d. The cultures were then diluted to obtain OD_{600} of 1.5. The growth rates of yeasts strains were evaluated by spotting 5 μL of serial dilutions of yeast cells on YEPGal containing calcofluor (50 $\mu\text{g mL}^{-1}$). The cells were incubated at 30 °C for 4 d.

*al.*1998; Tian *et al.* 2006). Furthermore, it has also been demonstrated that the *b'* domain is generally important for folding of proteins, whereas the *b* domain might contribute to the refolding rate in selected cases (Tian *et al.* 2006). Our data showed that the growth of *pdi1Δb'x* yeast strain producing the *a,b,a',c* protein was reduced dramatically at non permissive temperature. At high temperature the rate of protein unfolding increases, hence the chaperone role of *PDI* becomes predominant. Taken together, we propose that the presence of *b'x* region together with *b* domain is required in assisting refolding of misfolded protein at non-permissive temperature.

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