

The *pydA*–*pydB* fusion gene produces an active dioxygenase–hydrolase that degrades 3-hydroxy-4-pyridone, an intermediate of mimosine metabolism

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Abstract The objective of this research was to construct a *pydA*–*pydB* hybrid gene that encodes a functional dioxygenase–hydrolase (PydA–PydB) fusion protein for degradation of 3-hydroxy-4-pyridone (HP). HP is an intermediate in both synthesis and degradation of mimosine, a toxic amino acid produced by the tree legume *Leucaena leucocephala*. Computer-generated models of the fusion proteins suggested that joining of PydA and PydB with 0, 3, or 7 glycine residues as a linker should produce a functional PydA–PydB fusion protein. Accordingly, three hybrid genes, G0, G3, and G7, were constructed in which *pydA* and *pydB* were connected with 0, 9, and 21 nucleotides, respectively, encoding the glycine residues of the linker region. When these hybrid genes were expressed in *Rhizobium* and *Escherichia coli*, only one of them, G3, produced a functional PydA–PydB fusion protein, having both the dioxygenase and hydrolase activities. The G3 hybrid gene could complement both *pydA* and *pydB* mutants of *Rhizobium*, and *E. coli* lysate containing the overexpressed G3 protein was able to degrade HP. This hybrid gene may be useful for developing mimosine-free *L. leucocephala* plants in the future.

Introduction

Mimosine is a toxic non-protein amino acid produced by the tree legume of *Leucaena leucocephala* (Brewbaker and Hylin 1965). It is toxic to animals, plants, and microorganisms because it arrests cell division by depriving cells from available iron in the surrounding. Mimosine is a strong chelator of iron with a binding constant of 10^{36} (Kato et al. 1992). A major degradation product of mimosine, 3-hydroxy-4-pyridone (HP), is also toxic to cells due to its iron-chelating property (Molenda et al. 1994). *Rhizobium* sp. strain TAL1145, which forms nitrogen-fixing nodules on the roots of *L. leucocephala*, can utilize mimosine as a source of nutrients (Soedarjo et al. 1994). TAL1145 has a specific transport system to uptake mimosine from the rhizosphere of *L. leucocephala*. Inside the *Rhizobium* cell, mimosine is first converted to HP by an aminotransferase encoded by the *midD* gene (Borthakur et al. 2003). HP is then degraded to produce pyruvate, formate, and ammonia by a dioxygenase and a hydrolase, encoded by the *pydA* and *pydB* genes, respectively (Awaya et al. 2005). The HP dioxygenase encoded by *pydA* is a class III meta-cleavage enzyme involved in an extradiol cleavage reaction that opens the aromatic ring of HP by incorporating two atoms of molecular oxygen to the carbons at positions 2 and 3 of the aromatic ring, resulting in a cleavage adjacent to the hydroxyl group. The *pydB*-encoded HP hydrolase acts on the cleavage product of HP by dioxygenase and degrades it further.

The foliage of *L. leucocephala* can be used as a fodder because of its high palatability and protein content, which is comparable to that of alfalfa (Jones 1979). However, mimosine present in the foliage causes harmful side effects, such as poor growth, hair loss, and development of goiter in animals (Jones 1979). Traditional breeding methods cannot

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develop *L. leucocephala* with reduced mimosine content because there is no mimosine-free germplasm for use in a breeding program. *L. leucocephala* is a polyploid species; therefore, developing a mimosine-free plant by disrupting a gene for biosynthesis of mimosine will be also difficult. Moreover, the enzymes and genes involved in the mimosine biosynthesis pathway have not yet been identified. An alternative approach for developing a mimosine-free *L. leucocephala* plant is through introducing the *pydA* and *pydB* genes for degradation of HP, which is also an intermediate in mimosine biosynthesis. Production of the dioxygenase (PydA) and hydrolase (PydB) enzymes in the *L. leucocephala* leaves should degrade HP, which is the precursor for the synthesis of mimosine. However, the transfer and expression of two genes to the same plant is difficult. One solution to this problem is to produce a functional PydA–PydB hybrid protein by fusing the *pydA* and *pydB* genes into a single gene. Combining two genes into a single hybrid gene may not always produce a functional protein because the resulting hybrid protein may not maintain the original structural conformation of the two proteins. Computer modeling may be useful to predict the possible conformation of a hybrid protein. In this report, we have demonstrated the successful production of a functional PydA–PydB hybrid protein, which has both dioxygenase and hydrolase activities.

Materials and methods

Bacterial strains, plasmids, and culture conditions Bacterial strains and plasmids used in this study are described in Table 1. The *Escherichia coli* and *Rhizobium* strains were grown as described previously (Soedarjo et al. 1994; Vincent 1970). Media samples were analyzed through

high-performance liquid chromatography (HPLC) as described below.

Detection of HP Cell cultures were centrifuged (6,000×g), and the supernatants were filtered through 0.2-μm nylon filters. Analysis was done on a Dionex HPLC with Chromeleon software and performed with reverse-phase C₁₈ column (250×4.6 mm; Phenomenex, Torrance, CA) at room temperature with a 0.2% orthophosphoric acid mobile phase at a flow rate of 1 ml/min. The wavelength for the detection of HP was determined at 275 nm, and HP had a retention time of 4.8 min.

DNA manipulation, analysis, and sequencing Plasmid DNA isolation and manipulations were performed by standard procedures (Maniatis et al. 1982). Nucleotide sequence was determined by automated sequencing at the Molecular Biology and Biotechnology Facilities, University of Hawaii at Manoa.

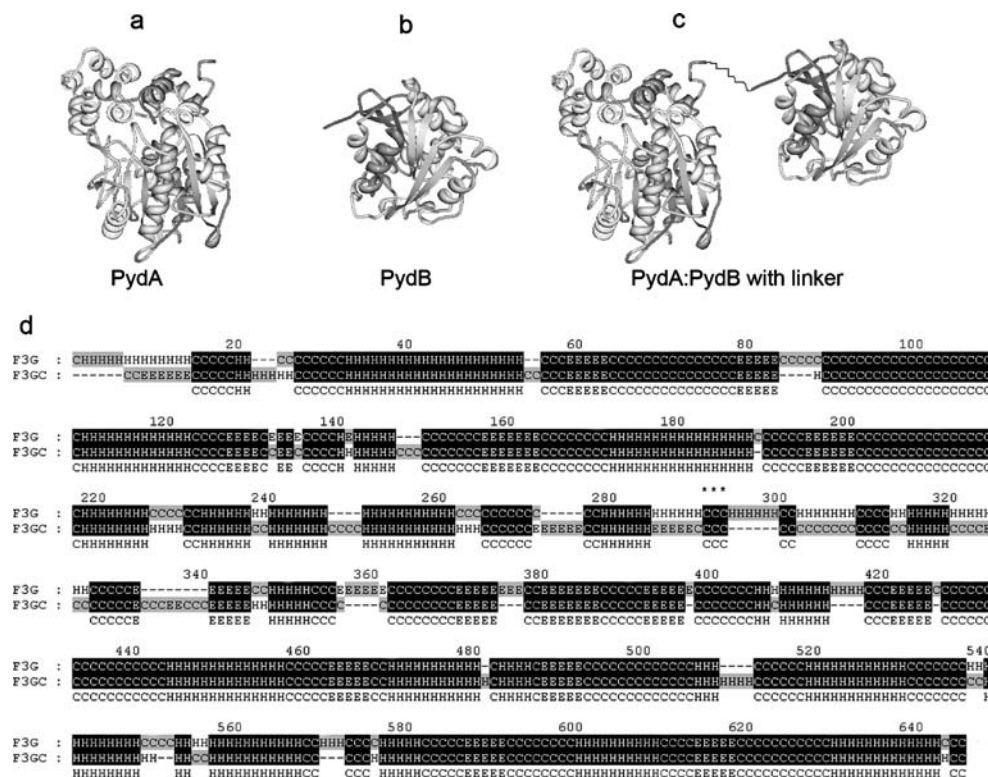
Design of fusion protein Due to the low homology with known proteins, sequence alignment was done based on secondary structure to determine the conservation of the purposed active sites via Clustal X (Thompson et al. 1997). GeneDoc was used to visualize and shade the alignments (Nicholas and Nicholas 1997). Secondary structure predictions were made using SCRATCH (Pollastri et al. 2002), and homology to known structures was made using the Conserved Domain Database (CDD via NCBI; Marchler-Bauer et al. 2005). Deepview Pdb viewer was used to visualize the known domains and determine relative length of purposed linkers (Guex and Peitsch 1997).

Expression of *PydA*, *PydB*, *G0*, *G3*, and *G7* in *E. coli* Expression constructs were polymerase chain reaction (PCR)

Table 1 Plasmids and strains used in this study

Strain/plasmid	Characteristics	Reference
Bacterial strain		
TAL1145	Wild-type <i>Rhizobium</i> sp. strain that nodulates <i>Leucaena</i> , Mid ⁺ HP ⁺	George et al. (1994)
PF9	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydA</i> , Kan ^R , Mid ⁺ HP ⁻	Fox and Borthakur (2001)
JA140	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydB</i> , Kan ^R , Mid ⁺ HP ⁻	Awaya et al. (2005)
Plasmid		
pNAM	Low copy expression vector, Apr ^R	Hoang et al. (1999)
pRK404	Wide-host range P1-group cloning vector, Tet ^R	Ditta et al. (1985)
pUHR500	G0 cloned in pRK404	This study
pUHR501	G3 cloned in pRK404	This study
pUHR502	G7 cloned in pRK404	This study
pUHR503	G0 cloned in pNam	This study
pUHR504	G3 cloned in pNam	This study
pUHR505	G7 cloned in pNam	This study

Fig. 1 Structural and sequence analysis for fusion protein. **a** PydA from CDD, **b** PydB from CDD, **c** fusion protein PydA–PydB with three glycine linker, and **d** secondary structure alignment via Clustal X (litbio server). The three asterisks indicate the three glycine repeat and represent the junction between the PydA and PydB proteins. C, H, and E represent all other helix and strand, respectively. F3G represents the sequence of the experimental fusion protein, and F3GC represents a fusion of the two highest homology proteins with a three glycine linker sequence



amplified using primer pairs pydA-UP (5'-CGCA CATA-TGAGGAGAAAGTTAATGGCTG 3') and pydB-DWN (5'-GTTGGAATTCATATTGTGGTTGCGGGAAGAGC-3'). Underlining indicates restriction sites. The pydA-UP contained an *NdeI* site, and the pydB-DWN primer contained an *EcoRI* site. Amplified products were digested with *NdeI* and *EcoRI* and ligated into pNam (Hoang et al. 1999). G0, G3, and G7 constructs into pNam used pydAB-junction (5'CTCGGTCTTCAAATGAGGCATTGCTGCTGCGTCGTAGGCGATG3'), pydAB-junction3 (5'CTCGGTCTTCAAATGAGGCATAACCACACCTGCTGCTGCGTCGTAGGCGATG3'), and pydAB-junction7 (5'CTCGGTCTTCAAATGAGGCATAACCACACCACCACCACCTGCTGCTGCGTCGTAGGCGATG3') primers, respectively. Each hybrid gene was constructed using a PCR megaprimer technique. Expression of each protein was done by inoculating *E. coli* expression cells SA1503 (DE3) and grown in Luria broth at 37°C up to an optical density of 0.5. Cells were induced with 0.5-mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then grown at room temperature for an additional 6–8 h.

Enzyme assay The cells were centrifuged (5,000×g) at 4°C and washed with 0.2 M NaCl–50 mM sodium phosphate buffer (pH 7) and resuspended in 0.2 M NaCl–50 mM sodium phosphate buffer. On ice, 0.5-mM phenylmethanesulfonyl fluoride was added to the cells and lysed with 1-mM lysozyme. The cells were sonicated and centrifuged (30,000×g) at 4°C for 30 min. The soluble protein concentration was

adjusted to 1 mg/ml, and 0.1 mM of HP was added to the crude *E. coli* extract. Samples were incubated at 28°C, and incubating the samples at 75°C for 10 min stopped the reaction. Control samples were performed using denatured protein samples before incubation with HP. The supernatant was filtered through a 0.2-μm nylon filter and subjected to HPLC analysis.

Protein analysis *E. coli* and *Rhizobium* proteins were separated with sodium dodecyl sulfate–10% polyacrylamide gels. Protein gels were transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA), and Western blotting was performed with an electrochemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech). Polyclonal antibodies were developed from purified PydA and PydB and prepared by Alpha Diagnostic International (San Antonio, TX, USA). The polyclonal antibodies were used at a concentration of 1:3,000.

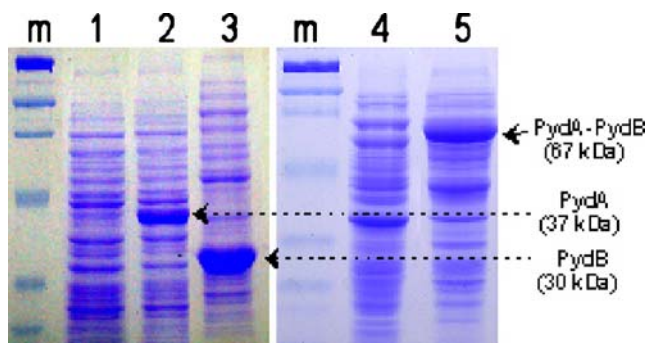
Results

Computer modeling of PydA, PydB, and PydA–PydB fusion proteins The three-dimensional structures of the PydA and PydB proteins are not known. The deduced amino acid sequence of PydA has 338 residues and shows 19.5% identity and 38% similarity with the dioxygenase beta subunit (LigB) of *Sphingomonas paucimobilis*, the three-

Table 2 The ability of *pydA* and *pydB* mutants of *Rhizobium* TAL1145 containing G0, G3, and G7 to degrade HP

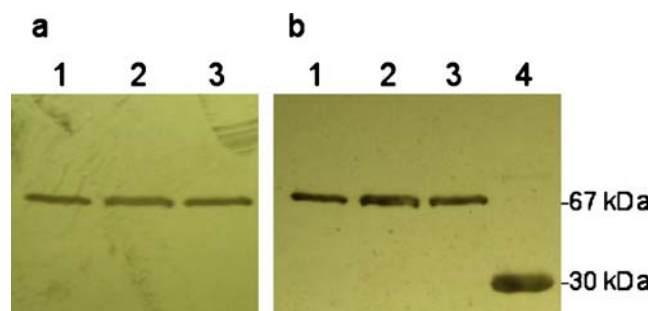
<i>Rhizobium</i> Strains	Degraded HP (%)
TAL1145	100
<i>pydA</i> mutant	0
<i>pydB</i> mutant	0
<i>pydA</i> mutant::G0	4
<i>pydB</i> mutant::G0	41
<i>pydA</i> mutant::G3	81
<i>pydB</i> mutant::G3	78
<i>pydA</i> mutant::G7	4
<i>pydB</i> mutant::G7	44

dimensional structure of which is known from X-ray crystallography (Sugimoto et al. 1999). PydB contains 274 amino acids and shows homology with hydrolases involved in degradation of aromatic compounds. It shows 30% identity and 52% similarity with CumD of *Pseudomonas fluorescens* and 26% identity and 48% similarity with BphD of *Rhodococcus* sp., the three-dimensional structures of which are known (Fushinobu et al. 2002; Nandhagopal et al. 2001). Based on the X-ray crystallographic structures of known dioxygenases and hydrolases, the possible structures of PydA and PydB were predicted (Fig. 1a,b). The possible three-dimensional structures of the PydA–PydB fusion proteins were also developed in which PydA and PydB were spaced with 0, 3, or 7 glycine residues (Fig. 1c). Glycine-rich linkers have been shown to provide flexibility to fusion proteins (Evers et al. 2006; Chen et al. 2000). However, varying the length of a glycine linker may also increase fusion protein stability, thereby optimizing the protein's functional activity. Close observation of the structures of these proteins showed that the conformations of the PydA and PydB segments did not

**Fig. 2** a Overexpression of *pydA*, *pydB*, and *pydA*–*pydB* hybrid gene (G0) in *E. coli*. Fifty micrograms of total protein was loaded for each sample. Lane *m*, broad range molecular weight ladder; lanes 1 and 4, uninduced *E. coli*; lane 2, IPTG-induced *E. coli* containing *pydA*; lane 3, IPTG-induced *E. coli* containing *pydB*; and lane 5, IPTG-induced *E. coli* containing G0. PydA, PydB, and PydA–PydB proteins are approximately 37, 30, and 67 kDa, respectively

change significantly from those of PydA and PydB alone. The three histidine residues at positions 17, 66, and 198 in PydA are conserved among many aromatic ring opening dioxygenases (Mendel et al. 2004). PydB contains an alpha/beta hydrolase fold, a characteristic conserved domain, present in several hydrolytic enzymes (Nandhagopal et al. 2001). The structural features such as the relative positions of the conserved histidine residues in PydA and the alpha/beta sheet in PydB were maintained in the three fusion proteins. When homology to known structures was made using the CDD (CDD via NCBI), domains 1BOU_B and NP_294515 were identified for PydA and PydB, respectively. Three, seven, and no glycine residues were evaluated for maintaining the secondary structure alignments and for the low amount of additional structure with glycine repeats and their ability to keep the two domains separate (Fig. 1d). By fusing the proteins with a three-glycine linker, we were able to maximize the secondary structure and purposed function within the PydA and PydB proteins. The G0 and G7 secondary structure alignments were not as conserved as the G3, indicating that the G3 construct was optimal.

Construction of *pydA*–*pydB* fusion genes In *Rhizobium* TAL1145, there is a 38-bp gap between the stop codon of *pydA* and the start codon of *pydB*. The *pydA* and *pydB* genes were amplified through PCR to produce a single gene product, which encodes a protein with the function of both the ring-cleaving dioxygenase and hydrolase enzymes. The stop codon of *pydA* and the 38-bp intergenic region were removed and replaced with a linker sequence to connect *pydA* to *pydB* in a translational fusion. Three different fusion genes, G0, G3, and G7, were constructed, which contained different lengths of glycine linkers between *pydA* and *pydB*. The G0 construct contained no glycine linker between *pydA* and *pydB*, while G3 and G7 contained 9 and 21 nucleotides as linkers encoding three and seven glycines, respectively. The G0, G3, and G7 hybrid gene

**Fig. 3** G0, G3, and G7 proteins in *Rhizobium* TAL1145 *pydA* mutant detected by Western blot analysis using polyclonal antibodies specific for PydA (a) and PydB (b). Fifty micrograms of total protein was loaded for each sample. Lane 1, TAL1145 *pydA* mutant::G0; lane 2, TAL1145 *pydA* mutant::G3; lane 3, TAL1145 *pydA* mutant::G7; and lane 4, TAL1145 induced with HP

constructs were cloned into the TOPO cloning vector for sequencing and other analyses.

Complementation of *Rhizobium* sp. strain TAL1145 *pydA* and *pydB* mutants by the G0, G3, and G7 The *pydA* and *pydB* mutants of *Rhizobium* sp. strain TAL1145 are defective in HP degradation (Awaya et al. 2005). The G0, G3, and G7 hybrid genes were cloned into the wide-host-range cloning vector pRK404 and introduced to both *pydA* and *pydB* mutants through triparental mating, and the transconjugants were grown in yeast extract–mannitol media containing 3 mM HP. The cultures were grown until stationary phase, and the culture supernatants were analyzed by HPLC to measure the concentration of HP that had been degraded. G3 could complement both *pydA* and *pydB* mutants and degraded 78–81% of the HP in the media. G0 and G7 did not complement either of these mutants. Ninety-six percent of the HP added to the media remained in the culture supernatant of the *pydA* mutant containing either G0 or G7. Similarly, 58% of HP was detected in the culture supernatants of the *pydB* mutant containing G0 or G7 (Table 2).

Expression of G0, G3, and G7 in *E. coli* G0, G3, and G7 hybrid genes were cloned into a low copy number expression vector pNAM, and overexpressed in *E. coli* by IPTG induction to determine if the constructs were expressed as fusion proteins and or two separate proteins. The three proteins were each expressed as a fusion protein with a molecular weight of 67,000 Da, which is the size of PydA and PydB combined (Fig. 2). Western blot analyses using antibodies specific to PydA and PydB showed that either of these antibodies could detect all three fusion proteins (Fig. 3). G0, G3, and G7 proteins were also measured for enzyme activity towards HP. IPTG-induced crude *E. coli* lysates containing the overexpressed G3 protein were able to degrade HP. HP added to the *E. coli* G3 lysate disappeared completely within 4 h, while HP added to the lysate of the control *E. coli*, which did not contain the G3 plasmid, was not degraded.

The overexpressed G0 and G7 did not degrade HP. Therefore, it was determined that only G3 expressed as an active enzyme in *E. coli*.

Discussion

The aim of this research was to construct a hybrid protein that produces an active dioxygenase–hydrolase protein in both *Rhizobium* and *E. coli*. Computer modeling software was used to determine a linker sequence that would produce the most stable hybrid protein. However, the PydA

and PydB have only low similarities to known three-dimensional protein structures. Therefore, analysis of the G0, G3, and G7 fusion constructs showed little or no differences in structure when fused with different linker sequences. The glycine linkers were used because computer modeling showed that they would not produce conformational change in PydA and PydB in the fusion proteins, and this type of rational design has been employed successfully in other fusion proteins (Park et al. 2001). On the basis of computer-generated structures, we expected all three fusion proteins to be functional, with the G3 fusion conserving the most amount of secondary structure when compared to the known homologous domains. Upon experimentation, only the G3 fusion protein was found to be functional for both PydA and PydB activities, supporting our *in silico* discoveries. In the constructs, G0 and G7, the PydA activity was completely inhibited while the PydB activity was reduced to about half. This shows that the C terminus of PydA and the N terminus of PydB cannot be too close or too far apart to constitute a functional fusion protein.

The G3 fusion protein has properties of both PydA and PydB. When the G3 fusion gene was cloned into a wide-host-range cloning vector, it complemented both *pydA* and *pydB* mutants of *Rhizobium* sp. strain TAL1145 for defects in mimosine degradation. All three fusion genes expressed as single transcriptional and translational units. In *Rhizobium*, *pydA* and *pydB* are co-transcribed from a common promoter located upstream of *pydA*. The fusion genes did not contain the native *pydA* promoter and were transcribed from a vector promoter in *Rhizobium*. When the fusion genes were cloned into an *E. coli* expression vector, they were transcribed from a T7 promoter. For expression in both *Rhizobium* and *E. coli*, the ribosome-binding site was provided by the cloning vector. In both *Rhizobium* and *E. coli*, all three fusion proteins were detected as 67-kDa bands. The G0 and G7 fusion genes were transcribed and translated and produced stable proteins in both *Rhizobium* and *E. coli*, although these proteins were not functional like G3 fusion protein. The G3 fusion gene constructed in this research will be useful in transforming *L. leucocephala* for developing a transgenic line with reduced mimosine content.

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