The N-Terminal α-Helix Domain of *Pseudomonas aeruginosa* Lipoxygenase Is Required for Its Soluble Expression in *Escherichia coli* but Not for Catalysis

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Lipoxygenase (LOX) is an industrial enzyme with wide applications in food and pharmaceutical industries. The available structure information indicates that eukaryotic LOXs consist of N terminus β-barrel and C terminus catalytic domains. However, the latest crystal structure of *Pseudomonas aeruginosa* LOX shows it is significantly different from those of eukaryotic LOXs, including the N-terminal helix domain. In this paper, the functions of this N-terminal helix domain in the soluble expression and catalysis of *P. aeruginosa* LOX were analyzed. Genetic truncation of this helix domain resulted in an insoluble *P. aeruginosa* LOX mutant. The active C-terminal domain was obtained by dispase digestion of the *P. aeruginosa* LOX derivative containing the genetically introduced dispase recognition sites. This functional C-terminal domain showed raised substrate affinity but reduced catalytic activity and thermostability. Crystal structure analyses demonstrate that the broken polar contacts connecting the two domains and the exposed hydrophobic substrate binding pocket may contribute to the insoluble expression of the C terminus domain and the changes in the enzyme properties. Our data suggest that the N terminus domain of *P. aeruginosa* LOX is required for its soluble expression in *E. coli*, which is different from that of the eukaryotic LOXs. Besides this, this N-terminal domain is not necessary for catalysis but shows positive effects on the enzyme properties. The results presented here provide new and valuable information on the functions of the N terminus helix domain of *P. aeruginosa* LOX and further improvement of its enzyme properties by molecular modification.

**Keywords:** Lipoxygenase, *Pseudomonas aeruginosa*, N-terminal domain, soluble expression, catalysis

Introduction

Lipoxygenases (LOXs) are dioxygenases that can oxidate the polyunsaturated fatty acids to generate Z, E conjugated hydroperoxides [8]. These hydroperoxide derivatives are widely employed in the production of flavor compounds, flour, and pharmaceuticals [9, 12, 15, 24, 27]. LOXs are monomeric proteins found in animals, plants, fungi, and bacteria. The eukaryotic LOXs are single peptides containing the N terminus β-barrel and the C terminus catalytic domains (Fig. 1A) and this organization is highly conservative [3, 23]. Even though these N-terminal domains share a similar structure, their functions on enzyme folding and characteristics appear different. In most eukaryotic LOXs, such as rabbit 12/15-LOX [25], Macaca 12/15-LOX [10], and human platelet 12-LOX [1], genetic removal of the N-terminal β-sheet domains had no influence on the soluble expression of these LOXs in an *E. coli* system. For several
mammalian LOX species, truncation of the N-terminal domains led to impaired catalytic properties [1, 25, 26]. However, improvement of the catalytic activity after this truncation was described for soybean LOX-1 [21].

Most recently, the structure of prokaryotic LOX from *Pseudomonas aeruginosa* was illustrated. This crystal structure indicates that the LOX folded into a unique structure consisting of the N terminus helix and the C terminus catalytic domains [13] (Fig. 1B). Although the C terminus of *P. aeruginosa* and eukaryotic LOXs share a similar overall structure, the N-terminal domains from these sources show significant differences both in amino acid sequence (data not shown) and structure (Fig. 1), suggesting potentially different functions. A previous report showed that genetic truncation of the partial N-terminal sequence led to the insoluble expression of *P. aeruginosa* LOX in *E. coli* [18]. However, the native LOX purified from *P. aeruginosa* 42A2 is an N terminus truncated active enzyme [7], suggesting that this N terminus is not necessary for catalysis but may be required for its soluble expression. The protease responsible for this truncation and the N-terminal amino acid sequence of this native truncated enzyme are still ambiguous, impeding the possibility to obtain the active truncated enzyme artificially.

In our previous study, we overexpressed *P. aeruginosa* LOX in *E. coli* with a titer of 3.89 U/ml [20], providing the potential for a large-scale production of this valuable enzyme. Its enzyme characteristics were then improved by genetic deletion, fusion with self-assembling peptides, and point mutations [18, 19]. In order to further improve the enzyme characteristics, revealing the functions of this N terminus helix domain seems imperative. In this study, the functions of this N terminus domain on the soluble expression and catalysis of *P. aeruginosa* LOX were analyzed and the potential mechanism involved was also discussed.

### Materials and Methods

#### Chemicals and Kits

The substrate linoleic acid was supplied by Sigma-Aldrich (Germany). Antibiotics, dispase (neutral protease), isopropyl β-D-1-thiogalactopyranoside (IPTG), primers, and DNA and protein sequencings were supplied by Sangon (China). All genetic manipulation kits were supplied by Takara (China). The AKTA purifier, HiLoad 16/60 Superdex 200 pg, Histrap FF crude, and Histrap Desalting were supplied by GE Healthcare (GE, Sweden).

#### Plasmid Construction

The plasmid pET-22b(+)/nLOX(His) contains the gene of native LOX fused with an N terminus 6×His tag without its endogenous signal peptide [18]. The N terminus truncated mutants with and without N-terminal 6×His tag (pET-22b(+)/lLOX(6His) and pET-22b(+)/lLOX), the insertion mutant with His-tag (pET-22b(+)/mLOX(6His)), and the wild-type LOX without His-tag (pET-22b(+)/nLOX) were constructed by using the pET-22b (+)/nLOX(6His) as the template (Fig. 1). The insertion mutant without His-tag (pET-22b(+)/mLOX) was constructed by using pET-22b(+)/nLOX as the template (Fig. 2). All these plasmids were constructed according to the manual of the MutanBEST kit (Takara). The mutagenesis primers are listed in Table 1.

The DNA encoding the N-terminal region (residues 1 to 114) of rabbit reticulocyte 15-LOX (GI:157831856) with and without N-terminal His-tag was chemically synthesized and fused with the N terminus truncated LOX mutant by Sangon, generating the hybrid enzymes rLOX and rLOX(6His) (Fig. 2). All plasmids constructed here are illustrated in Fig. 2 and were all transformed into *E. coli* Rosetta (DE3) for protein expression.

#### Protein Expression, Purification, and Disparse Digestion

Luria-Bertani medium was applied for the cultivation of these recombinant *E. coli* Rosetta (DE3) strains. The cultivated cells were
then incubated in Terrific-Broth medium at 37°C. The temperature was shifted to 20°C and 1 mM (final concentration) IPTG was added to induce the protein expression for 50 h when the OD$_{600}$ reached 0.6. Ampicillin and chloramphenicol were added to the final concentration of 100 and 34 μg/ml, respectively, if necessary.

The culture supernatant was centrifuged at 10,000 × g for 20 min. The nLOX(His) and mLOX(His) were purified on an Ni$^{2+}$ affinity column. The imidazole in these obtained LOX fragments was removed on a HiTrap Desalting column with buffer A (25 mM KH$_2$PO$_4$-K$_2$HPO$_4$, pH 7.5, 10% glycerol, 100 mM (NH$_4$)$_2$SO$_4$).

Dispase digestion of the purified mLOX(His) was performed by mixing mLOX(His) and dispase at 37°C for 30 min. Then, the mixture was purified by gel filtration with buffer A. All purification procedures were performed at 4°C.

**Enzymatic Analysis**

The substrate solution (0.3 mM linoleic acid in 0.15 M KH$_2$PO$_4$-K$_2$HPO$_4$ (pH 7.5)) was freshly prepared and aerated to saturate oxygen before analysis. One unit of activity was defined as the enzyme required to synthesize 1 μmol of hydroperoxide in 1 min.
at 25°C [14]. The activity analysis was performed by a spectrophotometer at 234 nm. The initial linear part of the absorbance curve was used to evaluate enzyme activity.

The thermostability was defined as the half-life time of enzyme activity at 50°C in buffer A and calculated as literature reported [22]. Apparent $K_m$ was analyzed in substrate solutions containing different concentrations of linoleic acid (from 0.02 to 0.1 mM), and the kinetic parameters were calculated by plotting the data on a double reciprocal graph.

**Statistical Analysis**

All the data presented here are the mean values of at least triplicates. ANOVA with multiple comparisons was performed on SigmaStat 3.1 (Systat Software Inc., USA). A $P$ value of <0.05 was taken as the standard of statistical difference.

**SDS-PAGE and Protein Assay**

SDS-PAGE analysis was performed on a 12% running gel [16] and visualized by staining with Coomassie Brilliant Blue R-250. The Bradford method [6] was used to analyze protein concentration by using bovine serum albumin as the standard.

### Results

**Genetic Truncation of P. aeruginosa LOX**

The LOX fragment without endogenous signal peptide was fused with the N terminus 6×His tag, generating the recombinant enzyme nLOX(His) (Fig. 2). The mutant tLOX(His) encoding the C-terminal catalytic domain was also constructed (Fig. 2). The enzyme activity and SDS-PAGE analyses (Fig. 3A, Lane 1) showed that LOX activity was detected in the culture supernatant (3.61 U/ml) of the cells harboring the plasmid pET-22b(+)/nLOX(His). However, a molecular mass of about 55 kDa band, which appears to correspond to the C-terminal domain, was only observed in the intracellular insoluble fractions of the cells harboring the plasmid pET-22b(+)/nLOX(His) (Fig. 3C, Lane 5), indicating insoluble expression of the C-terminal domain. These results indicate that the presence of the N terminus domain is necessary for the soluble expression of P. aeruginosa LOX in E. coli, which is significantly different.

**Fig. 3.** SDS-PAGE analysis of protein expression, digestion, and purification in this paper. (A) Culture supernatant; (B) intracellular soluble protein; (C) intracellular soluble protein; (D) purified enzymes; and (E) dispase digested mLOX. Lane M, molecular weight marker; Lane con, control (pET-22b(+)); Lane 1, nLOX(His); Lane 2, nLOX; Lane 3, mLOX(His); Lane 4, mLOX; Lane 5, tLOX(His); Lane 6, tLOX; Lane 7, rLOX(His); Lane 8, rLOX; Lane 9, purified nLOX(His); Lane 10, purified mLOX(His); and Lane 11, purified mini-LOX. The tLOX(His) and tLOX expressed as inclusion bodies are indicated by arrows.
from that of eukaryotic LOXs. Mutants without the N-terminal His-tag (nLOX and tLOX) (Fig. 2) were also constructed. The expression level of nLOX was reduced and showed lower activity (3.01 U/ml) (Fig. 3, Lane 2). However, the tLOX was still only observed in intracellular insoluble fractions (Fig. 3). These results indicate that the N-terminal fused His-tag has no effect on the solubility of tLOX but increases the expression level of nLOX.

### Preparation of the Recombinant LOX with Introduced Dispase Recognition Sites

Previous reports indicate that the N terminus helix domain may be not necessary for catalysis but affects the enzyme properties of *P. aeruginosa* LOX [7, 20]. Besides the aforementioned genetic truncation, protease digestion is another strategy to obtain an active truncated LOX mutant. The soybean LOX-1 can be tryptic digested into an active fragment without its N-terminal β-barrel domain [21]. Nevertheless, dispase and trypsin had no effect on nLOX(His). Although the native LOX can be digested into an ~35kDa active enzyme, the protease responsible for this digestion and the N-terminal amino acid sequence of this native digested LOX are still ambiguous [7]. The crystal structure of *P. aeruginosa* LOX suggests that its N- and C-terminal domains are connected by six highly flexible residues (Thr²⁰¹, Gln²⁰², Arg²⁰³, Gly²⁰⁴, Gln²⁰⁵, and Gly²⁰⁶) [13]. To obtain the LOX fragment without its N-terminal helix domain by protease digestion, the dispase recognition sites (LF) were introduced between Arg²⁰³ and Gly²⁰⁶ to generate the LOX mutant mLOX(His) (Fig. 2). SDS-PAGE and enzyme activity analyses revealed that the mLOX(His) was solubly expressed extracellularly (2.72 U/ml) (Fig. 3A, Lane 3) in the *E. coli* system. Similarly, the mLOX without the N terminus fused His-tag showed reduced expression level and activity (Fig. 3, Lane 4). For further dispase digestion, mLOX(His) was purified by affinity chromatography (Fig. 3D, Lane 10).

### Dispase Digestion of mLOX(His)

The purified mLOX(His) was digested with dispase at 37°C for 30 min. SDS-PAGE analysis of the digested mixture showed three main bands of approximately 55, 40, and 20 kDa, which were similar to the theoretic molecular masses of the C terminus domain, dispase, and the N terminus domain, respectively (Fig. 3E). The 55 kDa protein was purified by gel filtration (Fig. 3D). Subsequent N terminus amino acid sequencing of this fragment presented the sequence GQGDGLNRFR. This result suggests that mLOX(His) was correctly digested by dispase to generate the target protein, referred to as “mini-LOX,” without the N-terminal helix domain. The enzyme activity analysis showed that mini-LOX had the ability to catalyze linoleic acid to form hydroperoxide, indicating that this N terminus helix domain is not essential for catalysis.

### Enzyme Properties

As shown in Table 2, the $K_m$ value of mini-LOX decreased from 0.0489 mM to 0.0155 mM, suggesting increased substrate affinity. The $k_{cat}$ value of mini-LOX was about 20% of that of the nLOX(His). As expected, the specific activity of mini-LOX was only half that of nLOX(His). Compared with nLOX(His), the half-life time of mini-LOX at 50°C decreased by 66.7%, indicating that the thermostability was reduced after truncation of the N-terminal domain (Table 2). These results demonstrate that the N terminus helix domain has a negative effect on substrate binding but is beneficial to its catalytic efficiency and thermostability.

### Discussion

Previous studies showed that the N-terminal domains of eukaryotic LOXs are not essential for their soluble expression in *E. coli* but impact the enzyme properties [1, 25, 26]. Here, the roles of the N-terminal domain in the soluble expression and catalysis of *P. aeruginosa* LOX in *E. coli* were observed. The nLOX(His), nLOX, mLOX(His), and mLOX were detected in both culture supernatant and intracellular soluble fractions, suggesting that the cell lysis due to the prolonged cultivation (50 h) may be responsible for the detection of extracellular target enzymes that are not fused with the signal peptide. Previously, deletion of the N-terminal residues IPPSLPQYDN, which form 4 polar contacts.

### Table 2. Enzyme properties of nLOX and mini-LOX.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (1/s)</th>
<th>$k_{cat}/K_m$ (l/(mmol·s))</th>
<th>Specific activity (U/mg)</th>
<th>$T_{1/2}$ (50°C) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nLOX</td>
<td>0.0489</td>
<td>23.5</td>
<td>481</td>
<td>28.5</td>
<td>10</td>
</tr>
<tr>
<td>mini-LOX</td>
<td>0.0155</td>
<td>4.9</td>
<td>313</td>
<td>14.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Note: the variation about the mean is below 5%.
with the C-terminal catalytic domain (Fig. 4), resulted in the insoluble expression of LOX [18]. Further crystal structure analyses showed that the N-terminal helices of \emph{P. aeruginosa} LOX form 20 polar contacts with its C-terminal catalytic domain. In comparison, there are only 6, 9, and 7 polar contacts connecting the two domains of rabbit reticulocyte 15-LOX (PDB code: 2P0M), soybean LOX-1 (PDB code: 3PZW), and human 5-LOX (PDB code: 3O8Y), respectively, indicating more interactions between the domains in \emph{P. aeruginosa} LOX than those of eukaryotic LOXs. Since the N terminus covers the substrate binding pocket [13], the removal of this N terminus domain may also expose this hydrophobic pocket to the water phase, leading to folding problems. The importance of polar contacts and surface hydrophobicity in protein folding has been reported [4, 17]; therefore, the broken polar contacts and the exposed hydrophobic residues may be responsible for the insoluble expression of the C terminus catalytic domain. To further analyze the potential roles of these two factors in enzyme expression, we tried to cover this hydrophobic pocket by fusion of the N-terminal region of rabbit reticulocyte 15-LOX to the N terminus truncated LOX mutant, generating the hybrid enzymes rLOX and rLOX(His). However, SDS-PAGE and activity analyses showed that rLOX and rLOX(His) were both expressed as inclusion bodies, indicating that the polar contacts may play a more important role in the soluble expression of LOX than the exposed hydrophobic residues.

The high flexibility of the residues (Glu201-Tyr206) may make this region exposed to solvent and freely accessible for dispase digestion to generate the active mini-LOX. The removal of the N terminus domain exposed the substrate binding pocket and therefore reduced the steric hindrance of substrate binding, leading to a decreased \( K_m \) value. In eukaryotic LOXs, the Trp in the conserved sequence FPCYRW in the N-terminal \( \beta \)-sheets forms a \( \pi \)-cation interaction with a Lys (variable) in the C-terminal catalytic domain, and this \( \pi \)-cation bridge binds and regulates the N- and C-terminal domains during catalysis [11]. In \emph{P. aeruginosa} LOX, the conserved Trp, and the variable Lys was not found by sequence alignment (Fig. 5). However, there are two polar contacts connecting the Trp\(_{105}\) and the ASN\(_{684}\), which is next to one of the active sites (His\(_{685}\)) (Fig. 4), indicating the potential importance of this Trp\(_{105}\). In the enzyme structure, the covered hydrophobic regions and polar interactions are important to enzyme properties [2, 5]. Thus, the exposed hydrophobic amino acids and the disrupted polar contacts and other potential noncovalent interactions created by removing the N-terminal domain may contribute to the decreased \( k_{cat} \) value, specific activity, and thermostability of the mini-LOX.

In summary, genetic truncation of the N-terminal domain generated the insoluble expression of \emph{P. aeruginosa} LOX in \emph{E. coli}. In contrast, the active C-terminal catalytic domain was obtained by dispase digestion of the mutant with introduced dispase recognition sites, and showed raised substrate affinity but reduced catalytic activity and thermostability. These results indicate that this N terminus helix domain is not necessary for catalysis but is essential for its soluble expression in \emph{E. coli}.

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References


