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The human lysyl oxidase-like 2 protein functions as an amine oxidase toward collagen and elastin

Young-Mi Kim · Eun-Cheol Kim · Youngho Kim

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Abstract The lysyl oxidase-like 2 (LOXL2) protein is a human paralogue of lysyl oxidase (LOX) that functions as an amine oxidase for formation of lysine-derived cross-links found in collagen and elastin. In addition to the C-terminal domains characteristic to the LOX family members, LOXL2 contains four scavenger receptor cysteine-rich (SRCR) domains in the N-terminus. In order to assess the amine oxidase activity of LOXL2, we expressed a series of recombinant LOXL2 proteins with deletions in the SRCR domains, using an *Escherichia coli* expression system. All of the purified recombinant LOXL2 proteins, with or without the SRCR domains in the N-terminus, showed significant amine oxidase activity toward several different types of collagen and elastin in in vitro amine oxidase assays, indicating deletion of the SRCR domains does not interfere with amine oxidase activity of LOXL2. Further, amine oxidase activity of LOXL2 was not susceptible to inhibition by β-aminopropionitrile, an irreversible inhibitor of LOX, suggesting a different enzymatic mechanism between these two paralogues.

Keywords Lysyl oxidase-like 2 · Amine oxidase · β-Aminopropionitrile · Scavenger receptor cysteine-rich domain

Young-Mi Kim, Eun-Cheol Kim equally contribute to this work.

Y.-M. Kim · Y. Kim (✉)
Department of Biochemistry, School of Medicine, Wonkwang University, Iksan, Jeonbuk 570-749, Korea
e-mail: youngkim@wku.ac.kr

E.-C. Kim
Department of Oral and Maxillofacial Pathology, School of Dentistry, Wonkwang University, Iksan, Jeonbuk 570-749, Korea

Introduction

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that regulates formation of lysine-derived cross-links in elastin and collagen fibers. LOX catalyzes oxidative deamination of ε-amino groups of peptidyl lysine residues to α-aminoadipic δ-semialdehyde groups which, in turn, undergo spontaneous condensation to form the intra- and inter-molecular cross-linkages in collagen and elastin fibers [1]. In addition to the cross-linking activity toward extracellular matrix proteins, several novel functional roles of LOX have been identified in tumor suppression, hypoxia-induced metastasis, and chemotaxis [2–4].

The lysyl oxidase-like 2 gene (*LOXL2*) is a member of the *LOX* family that is composed of five paralogues (*LOX*, *LOXL*, *LOXL2*, *LOXL3*, and *LOXL4*) in humans. Each LOX family protein contains a copper-binding motif, lysyl-tyrosyl-quinone (LTQ) residues, and a cytokine receptor-like (CRL) domain in its highly conserved carboxyl (C)-terminus [5–8]. In contrast to the characteristic C-terminal domains, the LOX family members show sequence divergence in their amino (N)-terminal regions. In particular, LOXL2, LOXL3, and LOXL4 contain four scavenger receptor cysteine-rich (SRCR) domains in their N-terminal regions [6–8]. The functional role of the SRCR domains in these three LOX paralogues has not yet been characterized; however, SRCR domains are involved in protein–protein interactions in several secreted or receptor proteins [9]. While the regulation of the LOX family gene expression has been well characterized in various cells and tissues [10–13], the specific mechanisms by which these members exert the enzymatic activity are yet to be further elucidated.

LOXL2 was first isolated as an overexpressed gene in senescent fibroblasts [6], and *LOXL2* upregulation has been reported more often in invasive metastatic tumors than

noninvasive tumors [14, 15]. Further, LOXL2 has been shown to mediate induction of epithelial-mesenchymal transition by repression of *E-cadherin* expression, suggesting a functional role for LOXL2 in tumor progression [16]. Based on conservation of the C-terminal domains, LOXL2 was predicted to function as an amine oxidase. However, the amine oxidase activity of LOXL2 has not yet been thoroughly studied. In order to assess the amine oxidase activity of LOXL2, we expressed the LOXL2 protein as a series of recombinant forms, with deletions in the SRCR domains. We then tested amine oxidase activity of the purified recombinant proteins toward physiological substrates of LOX, including elastin and several different types of collagen. All of the recombinant LOXL2 proteins, in the presence or absence of the SRCR domains, showed significant amine oxidase activity toward collagen and elastin in in vitro peroxidase-coupled fluorometric assays. However, the amine oxidase activity of the recombinant LOXL2 proteins was not significantly inhibited by β-aminopropionitrile (BAPN), a specific inhibitor of LOX, suggesting that LOX and LOXL2 may be subject to potentially different biochemical mechanisms for modulation of the amine oxidase activity.

Materials and methods

Construction of LOXL2 expression plasmids

A full-length LOXL2 cDNA was PCR-amplified from human placental total RNA (BD Biosciences) with *Pfu* turbo polymerase (Stratagene), according to the manufacturer's suggestions. Subsequently, LOXL2 cDNAs, with a series of deletions in the SRCR domains, were generated by PCR amplification of the full-length LOXL2 cDNA using the following primers:

LOXL2-fl forward primer: 5′-GCGGCTAGCATGGAGAGGCCTCTGTGC-3′
LOXL2-p1 forward primer: 5′-GCGGCTAGCGTGCGACTGAGAGGCGG-3′
LOXL2-p2 forward primer: 5′-GCGGCTAGCCTGCGCCTGAACGGCG-3′
LOXL2-p3 forward primer: 5′-GCGGCTAGCGATGTCAACAGCAACAAAG-3′
Reverse primer: 5′-GCGAAGCTTCTGCGGGGACAGCTGGTTG-3′

Underlined letters in the primer sequences indicate a unique *Nhe*I or *Hin*dIII site introduced into the primers for convenient subcloning. The reverse primer was commonly used for construction of all LOXL2 expression plasmids. Thermocycling consisted of 30 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 60 s, with predenaturation at 94°C for 2 min and final extension at 72°C for 7 min. PCR-amplified DNA fragments were gel-purified and then ligated into pET21a (Novagen) at *Nhe*I and *Hin*dIII restriction sites in frame with the C-terminal hexa-histidine tag. All resulting expression constructs were confirmed by DNA-sequencing analysis to contain the desired LOXL2 sequences.

Expression and purification of the recombinant LOXL2 proteins

Expression, purification, and refolding of recombinant LOXL2 proteins were carried out as we previously described for other LOX family proteins [17, 18]. Briefly, the *Escherichia coli* strain BL21 (DE3) (Novagen) was transformed with pET21a-derived expression constructs of LOXL2. After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), the transformed bacterial cells were lysed in a buffer containing 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1% lysozyme, Triton X-100, and 0.1 mg/ml DNase. Inclusion bodies were isolated by centrifugation and homogenized in a buffer containing 6 M urea, 10 mM K_2HPO_4, pH 8.2, and 3 mM β-mercaptoethanol. Solubilized inclusion bodies were then purified using Ni-NTA agarose resins (Qiagen) according to the manufacturer's suggestions. For refolding of the recombinant LOXL2 proteins denatured by urea during the purification steps, stepwise dialysis was performed in a buffer of 10 mM K_2HPO_4, pH 9.6, 200 μM $CuCl_2$, and 2% sodium *N*-lauroylsarcosinate, and then in a buffer of 10 mM K_2HPO_4, pH 9.6 and 5 μM $CuCl_2$. The protein samples were further dialyzed twice in 10 mM K_2HPO_4, pH 9.6. After the final dialysis, the protein solutions were lyophilized in the presence of 10 mM trehalose using a freeze dryer (Labconco). All purification procedures were carried out at 4°C. Purity and sizes of the recombinant proteins were assessed using SDS-PAGE.

Amine oxidase assays of the recombinant LOXL2 proteins

Amine oxidase activity of the recombinant LOXL2 proteins was assessed using a peroxidase-coupled fluorometric assay with the Amplex red hydrogen peroxide assay kit (Molecular Probes), as previously described [19]. Each reaction contained 5 μg of a purified LOXL2 protein (0.48 μM for LOXL2-p1, 0.61 μM for LOXL2-p2, and 0.74 μM for LOXL2-p3) and 20 pmol of a substrate in a reaction volume of 200 μl. Parallel assays were performed in the absence or presence of 1 mM BAPN. Bovine neck ligament elastin (Sigma), calf skin collagen type I (Sigma), calf skin collagen type III, and human placenta collagen type IV were used as substrates for the assays. Fluorescence was measured using a fluorescence spectrophotometer (Molecular

Devices) with excitation and emission wavelengths of 571 and 585 nm, respectively.

Results and discussion

Expression and purification of the recombinant LOXL2 proteins

The recombinant LOXL2 proteins, LOXL2-fl, LOXL2-p1, LOXL2-p2, and LOXL2-p3 were designed to start at different residues, with a serial deletion of the repeated SRCR domains located in the N-terminus, beginning at Met^{1}, Val^{326}, Leu^{435}, and Asp^{498}, respectively. The characteristic C-terminal region containing the copper-binding motif, LTQ residues (Lys^{653} and Tyr^{689}), and the CRL domain was included in all recombinant LOXL2 proteins (Fig. 1a). After induction with 1 mM IPTG, all recombinant LOXL2 proteins, with the exception of LOXL2-fl, showed high levels of expression. The apparent sizes of the hexa-histidine tagged recombinant LOXL2 proteins were in good agreement with the deduced molecular mass; 52 kDa for LOXL2-p1, 41 kDa for LOXL2-p2, and 34 kDa for LOXL2-p3 (Fig. 1b). Recombinant LOXL2-proteins were more than 95% pure on SDS-PAGE gels. The reason that the LOXL2-fl protein was not expressed at detectable levels is currently unknown, but is possibly due to the presence of rare codons clustered in the N-terminal region of LOXL2. Clusters of rare codons have been shown to frequently impede translation of heterologous genes in *E. coli* [20]. The rare codons, found in the N-terminal region of LOXL2, include CCC (Pro^{21}, Pro^{31}, Pro^{34}, Pro^{47}, Pro^{50}, Pro^{120}), AGG (Arg^{3}, Arg^{65}, Arg^{162}, Arg^{240}, Arg^{254}), and CGG (Arg^{71}, Arg^{98}, Arg^{187}, Arg^{253}).

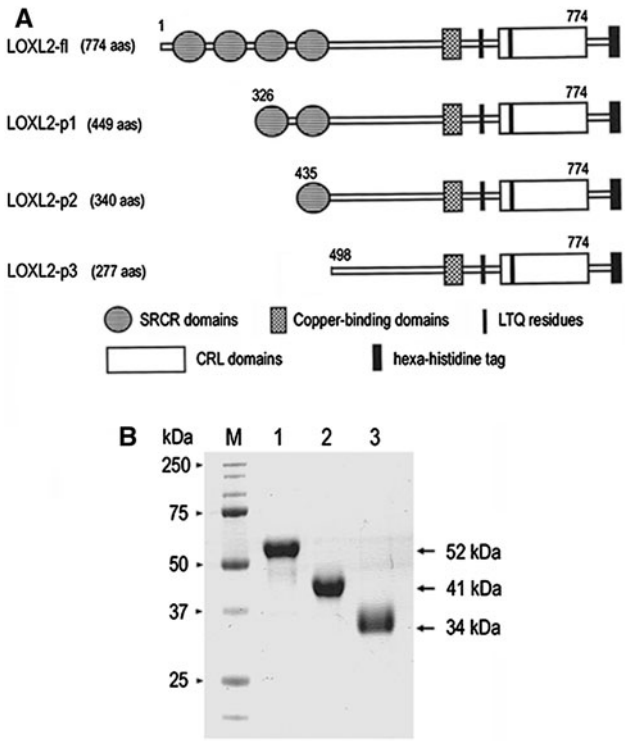

Fig. 1 Expression and purification of LOXL2 in *E. coli*. **a** Schematic diagrams of the recombinant LOXL2 proteins. All recombinant LOXL2 proteins were tagged with an additional Met-Ala-Ser sequence at the N-terminus and six histidine residues at the C-terminus. **b** purification of the recombinant LOXL2 proteins. Each lane contains approximately 5 μg of a purified recombinant LOXL2 protein. *Lane M*, protein standard markers; *lane 1*, LOXL2-p1; *lane 2*, LOXL2-p2; *lane 3*, LOXL2-p3

Effect of SRCR domains on amine oxidase activity of LOXL2

In an attempt to convert the purified recombinant LOXL2 proteins into enzymatically active forms, the purified proteins were subjected to stepwise dialysis in the presence of *N*-lauroylsarcosinate and Cu^{2+}. Following refolding, the recombinant LOXL2 proteins were assessed for amine oxidase activity toward collagen and elastin using an in vitro peroxidase-coupled fluorometric assay. Elastin and collagen types I, III, and IV were used as substrates for the amine oxidase assays. All recombinant LOXL2 proteins showed significantly increased levels of fluorescence over negative controls in the amine oxidase assays, with or without the SRCR domains in the N-terminus (Fig. 2). Further, the recombinant LOXL2 proteins showed no

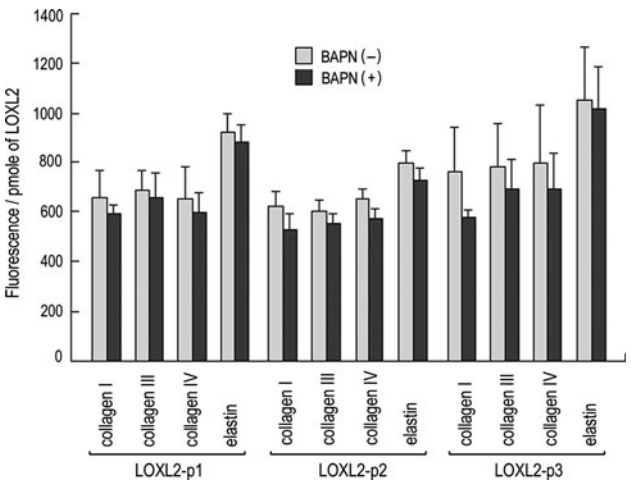

Fig. 2 Amine oxidase activity of the LOXL2 proteins toward collagen and elastin. Reactions that did not contain any of the recombinant LOXL2 proteins but the substrates were used as negative controls for both BAPN (−) and (+) reactions. After subtraction of the negative control value from the value of each reaction, each relative activity was expressed as the fluorescence value per pmol of the recombinant LOXL2 used for the assay. Amine oxidase activity assays were repeated in quadruplicate. Standard deviations are indicated on the graph

noticeable differences in substrate specificity toward collagen and elastin substrates, indicating that deletion of the SRCR domains did not affect amine oxidase activity of LOXL2 (Fig. 2). Previously, we showed that the conserved C-terminal domains of the LOX family proteins were sufficient to confer amine oxidase activity [17]. We also reported that neither deletion nor truncation of the N-terminal SRCR domains affected amine oxidase activity LOXL4, a close paralogue of LOXL2 [18]. Our current findings with the recombinant LOXL2 proteins provide further support that deletion of the SRCR domains does not interfere with amine oxidase activity of the LOX family proteins toward physiological substrates such as collagen and elastin, at least in in vitro assays.

The SRCR superfamily consists mainly of secreted or membrane proteins often associated with the innate immune system. The SRCR domain is a highly conserved domain of approximately 110 residues, containing six (group A) or eight (group B) cysteines that are presumably important to tertiary structural conformation [21]. LOXL2 contains four repeated copies of the group A SRCR domain in its N-terminus. In several different types of SRCR superfamily proteins, including CD6, the Mac-2 binding protein, and the macrophage receptor with collagenous structure (MARCO), SRCR domains have been shown to play essential roles in interaction with ligands or other regulatory proteins for cell signaling or adhesion [22–24]. Therefore, in more in vivo circumstances, it may be possible that the repeated SRCR domains modulate the amine oxidase activity of LOXL2, possibly through interacting with regulatory ligands or proteins that may exist in the extracellular matrix. Detailed functional analysis on interactive property of LOXL2 will be helpful for further characterizing the role of SRCR domains in regulation of the amine oxidase activity of LOXL2.

Effect of BAPN on amine oxidase activity of LOXL2

In our in vitro assays with physiological substrates, BAPN did not inhibit the amine oxidase activity of all recombinant LOXL2 proteins at 1 mM that corresponded to a greater than 1000 M excess of the recombinant LOXL2 proteins (Fig. 2). BAPN is a well known irreversible inhibitor of LOX [25]. It was recently reported that amine oxidase activity of LOXL2 could not be inhibited by BAPN, although the assays were performed in less direct ways without any purified LOXL2 protein [26]. Our current results with the recombinant LOXL2 proteins provide more straightforward evidence that amine oxidase activity of LOXL2 is not susceptible to inhibition by BAPN, suggesting that the structural conformation and binding affinity of LOXL2 may be different from those of LOX.

BAPN was initially identified as a lathyrogen found in plants of the genus *Lathyrus,* and its administration to animals resulted in increased fragility of connective tissues [25]. Our results indicate that LOXL2 may not be involved in the lathyrism caused by BAPN, implying a different functional role of LOXL2 in maintenance of the structural integrity of connective tissues. BAPN contains the same CH_2–CH_2–NH_2 group as lysyl residues in the substrates of LOX, such as collagen and elastin. It has been previously proposed that BAPN covalently binds to LOX in a substrate-like manner, irreversibly inhibiting the amine oxidase activity [27]. However, the active or catalytic site that BAPN binds to has not yet been identified in LOX, while cofactor sites, such as the copper-binding motif and LTQ residues, have been well characterized in the conserved C-terminal region. Further detailed structural analysis will be helpful for elucidation of enzymatic differences between LOX and LOXL2.

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References

1. Csiszar K (2001) Lysyl oxidases: a novel multifunctional amine oxidase family. Prog Nucleic Acid Res Mol Biol 70:1–32
2. Peyrol S, Raccurt M, Gerard F, Gleyzal C, Grimaud JA, Sommer P (1997) Lysyl oxidase gene expression in the stromal reaction to in situ and invasive ductal breast carcinoma. Am J Pathol 150:497–507
3. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 440:1222–1226
4. Li W, Liu G, Chou IN, Kagan HM (2000) Hydrogen peroxide-mediated, lysyl oxidase-dependent chemotaxis of vascular smooth muscle cells. J Cell Biochem 78:550–557
5. Kim Y, Boyd CD, Csiszar K (1995) A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase. J Biol Chem 270:7176–7182
6. Saito H, Papaconstantinou J, Sato H, Goldstein S (1997) Regulation of a novel gene encoding a lysyl oxidase-related protein in cellular adhesion and senescence. J Biol Chem 272:8157–8160
7. Jourdan-Le Saux C, Tomsche A, Ujfalusi A, Jia L, Csiszar K (2001) Central nervous system, uterus, heart, and leukocyte expression of the LOXL3 gene, encoding a novel lsysl oxidase-like protein. Genomics 74:211–218
8. Asuncion L, Fogelgren B, Fong KS, Fong SF, Kim Y, Csiszar K (2004) A novel human lysyl oxidase-like gene (LOXL4) on chromosome 10q24 has an altered scavenger receptor cysteine rich domain. Matrix Biol 20:487–491
9. Hohenester E, Sasaki T, Timpl R (1999) Crystal structure of a scavenger receptor cysteine-rich domain sheds light on an ancient superfamily. Nat Struct Biol 6:228–232
10. Boyd CD, Mariani TJ, Kim Y, Csiszar K (1995) The size heterogeneity of human lysyl oxidase mRNA is due to alternate polyadenylation site and not alternate exon usage. Mol Biol Rep 21:95–103

11. Csiszar K, Entersz I, Trackman PC, Samid D, Boyd CD (1996) Functional analysis of the promoter and first intron of the human lysyl oxidase gene. Mol Biol Rep 23:97–108
12. Rodríguez C, Alcudia JF, Martínez-González J, Raposo B, Navarro MA, Badimon L (2008) Lysyl oxidase (LOX) down-regulation by TNFalpha: a new mechanism underlying TNFalpha-induced endothelial dysfunction. Atherosclerosis 196:558–564
13. Wu G, Guo Z, Chang X, Kim MS, Nagpal JK, Liu J, Maki JM, Kivirikko KI, Ethier SP, Trink B, Sidransky D (2007) LOXL1 and LOXL4 are epigenetically silenced and can inhibit ras/extracellular signal-regulated kinase signaling pathway in human bladder cancer. Cancer Res 67:4123–4129
14. Fong SF, Dietzsch E, Fong KS, Hollosi P, Asuncion L, He Q, Parker MI, Csiszar K (2007) Lysyl oxidase-like 2 expression is increased in colon and esophageal tumors and associated with less differentiated colon tumors. Genes Chromosomes Cancer 46:644–655
15. Kirschmann DA, Seftor EA, Fong SF, Nieva DR, Sullivan CM, Edwards EM (2002) A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 62:4478–4483
16. Peinado H, del Carmen Iglasias-de la Cruz M, Olmeda D, Csiszar K, Fong KS, Vega S, Nieto MA, Cano A, Portillo F (2005) A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. EMBO J 24:3446–3458
17. Jung ST, Kim MS, Seo JY, Kim HC, Kim Y (2003) Purification of enzymatically active human lysyl oxidase (LOX) and lysyl oxidase-like protein (LOXL) from *Escherichia coli* inclusion bodies. Protein Expr Purif 31:240–246
18. Kim MS, Kim SS, Jung ST, Park JY, Yoo HW, Ko J, Csiszar K, Choi S, Kim Y (2003) Expression and purification of enzymatically active forms of the human lysyl oxidase-like protein 4. J Biol Chem 278:52071–52074
19. Palamakumbura AH, Trackman PC (2002) A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. Anal Biochem 300:245–251
20. Nakamura Y, Gojobori T, Ikemura T (2000) Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res 28:292
21. Sarrias MR, Grønlund J, Padilla O, Madsen J, Holmskov U, Lozano F (2004) The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. Crit Rev Immunol 24:1–37
22. Patel DD, Wee SF, Whichard LP, Bowen MA, Pesando JM, Aruffo A, Haynes BF (1995) Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. J Exp Med 181:1563–1568
23. Sasaki T, Brakebusch C, Engel J, Timpl R (1998) Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds beta1 integrins, collagens and fibronectin. EMBO J 17:1606–1613
24. Chen Y, Sankala M, Ojala JR, Sun Y, Tuuttila A, Isenman DE, Tryggvason K, Pikkarainen T (2006) A phage display screen and binding studies with acetylated low density lipoprotein provide evidence for the importance of the scavenger receptor cysteine-rich (SRCR) domain in the ligand-binding function of MARCO. J Biol Chem 281:12767–12775
25. Kagan HM (1986) Characterization and regulation of lysyl oxidase. In: Mecham RP (ed) Biology of extracellular matrix. Academic Press, Orlando, pp 281–304
26. Vadasz Z, Kessler O, Akiri G, Gengrinovitch S, Kagan HM, Baruch Y, Izhak OB, Neufeld G (2005) Abnormal deposition of collagen around hepatocytes in Wilson's disease is associated with hepatocyte specific expression of lysyl oxidase and lysyl oxidase like protein-2. J Hepatol 43:499–507
27. Kagan HM (1994) Lysyl oxidase: mechanism, regulation and relationship to liver fibrosis. Path Res Pract 190:910–919