Published in final edited form as:

Connect Tissue Res. 2011 ; 52(6): 479–486. doi:10.3109/03008207.2011.564337.

A novel proteolytic processing of prollysyl oxidase

Phimon Atsawasuwan, DDS, MSc, PhD,

CB#7454, NC Oral Health Institute, University of North Carolina at Chapel Hill, Chapel Hill, USA

Yoshiyuki Mochida, DDS, PhD,

Department of Periodontology and Oral Biology, Boston University, Boston, USA

Michitsuna Katafuchi, DDS, PhD,

Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, Japan

Kentaro Tokutomi, DDS, PhD,

Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, Japan

Viorel Mocanu, PhD,

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, USA

Carol E. Parker, PhD, and

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, USA

Mitsuo Yamauchi, DDS, PhD

CB#7454, NC Oral Health Institute, University of North Carolina at Chapel Hill, Chapel Hill, USA

Abstract

Lysyl oxidase (LOX) is an amine oxidase that is critical for the stability of connective tissues. The secreted proLOX is enzymatically quiescent and is activated through proteolytic cleavage between residue Gly₁₆₂ and Asp₁₆₃ (residue numbers according to the mouse LOX) by bone morphogenetic protein (BMP)-1 gene products. Here we report a novel processing of proLOX identified *in vitro* and *in vivo*. Two forms of mature LOX were identified and characterized by their immunoreactivity to specific antibodies, amine oxidase activity and mass spectrometry. One form was identified as a well characterized BMP-1 processed LOX protein. Another was found to be a truncated form of LOX (tLOX) resulting from the cleavage at the carboxy terminus of Arg₁₉₂. The tLOX still appeared to retain amine oxidase activity. The results from the proLOX gene deletion and mutation experiments indicated that the processing occurs independent of the cleavage of proLOX by BMP-1 gene products and likely requires the presence of LOX propeptide. These results indicate that proLOX could be processed by two different mechanisms producing two forms of active LOX.

Keywords

Lysyl oxidase; Protein processing; Amine oxidases; Extracellular matrix

INTRODUCTION

The formation of covalent intermolecular cross-linking is the final post-translational modification of collagen, and is crucial for the stability and maintenance of connective tissues [1–2]. The process is initiated with the conversion of the specific telopeptidyl lysine (Lys) and hydroxylysine (Hyl) residues to aldehyde (Lys^{ald} and Hyl^{ald}, respectively) by a copper-dependent amine oxidase, lysyl oxidase (EC 1.4.3.13, protein-lysine 6-oxidases, LOX). The aldehyde produced then spontaneously interacts with the juxtaposed Lys^{ald} or ε-amino groups of Lys or Hyl to form intra- and intermolecular cross-links [3]. LOX protein is synthesized as a catalytically quiescent N-glycosylated ~50 kDa proLOX [4] by various cell types and is processed extracellularly by bone morphogenetic protein (BMP)-1/mammalian Tolloid-like proteins to produce an active mature ~30 kDa LOX [4]. The cleavage occurs between residues Gly₁₆₂ and Asp₁₆₃ in mouse LOX (Gly₁₆₈ and Asp₁₆₉ in human LOX) [5–6] possibly at the cell surface involving cellular fibronectin [7]. In this study, we report a novel processing site of proLOX between Arg₁₉₂ and Pro₁₉₃ (numbered according to the mouse sequence) resulting in a further truncated but active form of LOX (tLOX) that was identified in a cell culture system as well as in bovine aorta.

MATERIALS & METHODS

All experiments were performed in accordance with the guidelines of the University of North Carolina at Chapel Hill.

Cell Culture

Human embryonic kidney (HEK) 293 cells (Clontech) were maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) in a 5% CO₂ atmosphere at 37 °C. The medium was changed twice a week.

Generation of Stable Clones Overexpressing LOX

HEK 293 cells were stably transfected with a plasmid containing LOX coding sequences, cultured and selected as described [8]. Briefly, PCR products were amplified using the normal mouse kidney cDNA (BD Bioscience) as a template, purified and ligated into the pcDNA3.1/V5-His-TOPO mammalian expression vector (Invitrogen), sequenced and the plasmid containing LOX cDNA (pcDNA3.1/LOX/V5-His) was obtained. The sequences of the primers were; forward primer, 5'-CCCGGTCTTCCTTTTCTCCTAGCC-3' and reverse primer, 5'-ATACGGTGAAATTGTGCAGCCTGA-3'. HEK293 cells were transfected with pcDNA3.1/LOX/V5-His as described [9] and cultured in the presence of 400 µg/mL of G418 (Gibco) for 3–4 weeks. The positive clones derived from single G418-resistant cells were selected, cultured and the cultured media were subjected to immunoprecipitation (IP) followed by Western blot (WB) analysis with anti(α)V5 antibody (Invitrogen) [9] to verify the LOX-V5/His protein (LOX-V5).

Purification and Identification of LOX-V5 Protein

The 293-derived clones synthesizing the highest level of LOX-V5 protein were plated onto 15 cm plates, cultured for 6 days and the cultured media containing a cocktail of protease inhibitors (Sigma) were collected. LOX-V5 was purified using a nickel-nitrotriacetic acid-agarose resin (Qiagen) at 4°C, and the fractions immunoreactive to αV5 antibody were pooled, dialyzed against 2 M urea then 0.2 M sodium borate pH 8.2, and kept at –80°C until use. The protein concentrations were measured by a DC protein assay kit (Bio-Rad). To assess the purity of LOX-V5, aliquots of the sample were subjected to 4–12% SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue (CBB) R-250 or WB

analysis with α V5 antibody and two α LOX antibodies, α LOXi (Imgenex) and α LOXh [7–8, 10].

Lysyl oxidase Activity Assay

The LOX enzyme activity was measured by the method reported by Palamakumbura and Trackman [11] in the presence or absence of 500 μ M β -aminopropionitrile (BAPN) using F2000 spectrofluorometer (Hitachi).

LOX Protein Characterization by Mass Spectrometry

The major CBB stained protein bands at 30 and 35 kDa on the gel were cut, dehydrated and digested with sequencing grade trypsin (Promega) in 50mM ammonium bicarbonate (Fisher scientific) at 37°C overnight, using the automated in-gel digestion protocol described previously [12]. For endoproteinase Asp N (AspN, P3303, Sigma-Aldrich) digestion, the sample was dissolved in the same buffer and digested overnight at 35°C.

Mass Spectrometric Analysis and Database Searching

For protein identification from the gel bands, MALDI-MS and -MS/MS data were acquired using an Applied Biosystems Voyager 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Inc.), in the positive ion reflectron mode. For the ABI TOF/TOF analyses, a saturated solution of recrystallized α -cyano 4-hydroxycinnamic acid (Sigma-Aldrich) in 50:50 acetonitrile: 40 mM ammonium citrate/0.1% trifluoroacetic acid (TFA) was used as the matrix solution. A 0.3 μ L aliquot of each sample was spotted followed by 0.3 μ L of matrix solution, and allowed to dry at room temperature. MS and MS/MS spectra were acquired in automated mode in which the 8 most intense peaks with a signal to noise ratio greater than 25 were selected by the 4700 Data Explorer software for MS/MS analysis.

The peptide mass fingerprinting and sequence tag data from the TOF/TOF were evaluated using AB's GPS Explorer scores, which are derived from Mascot™ searches (www.matrixscience.com). Ion Scores were generated by submitting MS/MS spectra to the NCBI database *via* the Mascot search engine. Only proteins with Ion Scores greater than 20 were included in our analysis since those below 20 are not considered statistically significant by Mascot. The following search conditions were used: variable modifications: methionine oxidation, 2 missed cleavages, mass accuracy 100 ppm.

For the analysis of the enzymatic digests, samples were analyzed on a Bruker Ultraflex II, operated in the positive ion reflectron mode. Samples were spotted as above, using a matrix solvent consisting of recrystallized α -cyano 4-hydroxycinnamic acid in 50:50 ACN:water, 0.1% TFA.

Generation of Mutation and Deletion constructs of LOX

In order to obtain insights into the mechanism by which truncated LOX (tLOX) is generated, several LOX constructs were generated and expressed in 293 cells. The constructs generated were: 1. pcDNA3.1/LOX/V5-His (positive control), 2. pcDNA3.1/LOXD₁₆₃>E, D₁₆₄>G/V5-His (LOX with double mutations at the cleavage site by BMP-1/mTLD like proteases resulting in a non-processed proLOX) [13], and 3. pcDNA3.1/LOX Δ PP/V5-His (LOX with deletion of the propeptide domain) [14]. An empty vector (pcDNA3.1/V5-His) was used as a negative control. To generate the mutation/deletion constructs, the following additional primers were used. For pcDNA3.1/LOXD₁₆₃>E, D₁₆₄>G/V5-His: forward primer, 5'-CATGGTGGGCGAAGGCCCTACAATC-3' and 5'-GATTGTAGGGGCCTTCGCCACCATG-3', and for pcDNA3.1/LOX Δ PP/V5-His: forward primer, 5'-CTTCTCCGCTGCGACGACCCCTACAATCCCTAC-3' and reverse primer, 5'-GTAGGGATTGTAGGGGTCGTCGACGCGAGAAG-3'. The PCR product

was subcloned into the pcDNA3.1/V5-His-TOPO mammalian expression vector and sequenced at the UNC-CH DNA sequencing facility. 293 cells were transfected with 2.5 µg of each construct, or an empty vector. At 72 hours after transfection, the cultured medium was collected and subjected to IP-WB analysis with αV5 antibody [8].

Isolation of Bovine Lysyl oxidase

Lysyl oxidase was isolated from bovine aorta by the method reported by Kagan and Cai [15] with some modifications. All preparations and purification were performed at 4°C in the presence of a cocktail of protease inhibitors (Sigma-Aldrich). Bovine aorta was cut into small pieces and pulverized in liquid nitrogen by a Spex Freezer Mill (Spex Certiprep) and washed by repeated centrifugation (X10,000g for 30min) with 0.4 M NaCl, 16 mM potassium phosphate pH 7.8, then with 16 mM potassium phosphate pH 7.8. The residues were extracted with 4 M urea, 16 mM potassium phosphate, pH 7.8 for 3 days, the supernatant was collected by centrifugation and this process was repeated twice. The supernatants pooled were then mixed with pre-equilibrated Bio-Gel HTF hydroxyapatite (Bio-Rad) for 30 min at 4°C. The supernatant was collected by centrifugation and concentrated by a ultrafiltration cell with a YM10 ultrafiltration membrane (Amicon). The concentrated extract was dialyzed exhaustively against 16 mM potassium phosphate, an equal volume of 1 M potassium phosphate, pH 7.8, was added and the precipitate was collected by centrifugation. The precipitate was dissolved in 6M urea, 16 mM potassium phosphate, pH 7.8 and separated on a Superdex 200 column (HiLoad 16/60, GE healthsciences) equipped with a Varian ProSatr HPLC system using the same buffer and the eluate was collected. An aliquot of each fraction was subjected to amine oxidase activity assay [11], the enzymatically active fractions were collected and pooled. The pooled samples were further dialyzed against 2 M urea, 16 mM potassium phosphate, pH 7.8 and separated on an anion exchange column (Protein-Pak DEAE 8HR, Waters) eluting with the same buffer with a gradient of 0 to 1M NaCl. An aliquot from every other fraction was subjected to amine oxidase activity assay and the relative fluorescence values were plotted. Two more aliquots from each fraction were also subjected to WB analysis with αLOXi and αLOXh antibodies respectively.

RESULTS

Identification, Characterization and Enzymatic Activity of LOX-V5 Protein

The purified mouse LOX-V5 protein was first characterized by SDS-PAGE and WB analyses. The results were consistent with our recent report [8]. When stained with CBB, two major protein bands at ~30 and 35 kDa, respectively, and a faint band at ~48 kDa were observed (Fig 1A, lane 1). By WB analyses with αV5 antibody (Fig. 1A, lane 3) and two αLOX antibodies (αLOXi and αLOXh) (Fig. 1A, lanes 4 and 5, respectively), the 30 kDa protein was immunopositive only to αV5 and αLOXi antibodies, but the 35 kDa band to all 3 antibodies (αV5, αLOXh and αLOXi antibodies). The ~48 kDa band was also immunopositive to all 3 antibodies. When the protein was incubated with normal rabbit serum, no immunoreactivity was detected (Fig. 1A, lane 2). The 48 kDa protein is likely full-length LOX as previously reported [4]. Then the protein was subjected to the amine oxidase assay (Fig 1B). The activity was readily detected and increased with dose, but was nullified by the addition of 500µM BAPN. These results demonstrate that the recombinant LOX-V5 generated is active as an amine oxidase.

The major mouse LOX-V5 bands migrated at 35 and 30 kDa were excised from the gel and subjected to MALDI-MS and MALDI-MS/MS analyses at the UNC-Duke Proteomics Center. Four tryptic peptides from the 35 kDa band (aa 225–231, 246–254, 263–277, and 372–391), and three from the 30 kDa band (aa 225–231, 263–277, 372–391) were identified

as mouse LOX peptides (accession protein# NP_034858.1), thus, confirming both proteins as mouse LOX. The gel extracts from 30 and 35 kDa mouse LOX-V5 bands were further characterized to identify a proteolytic cleavage site by MALDI-MS on a Bruker Ultraflex II mass spectrometer. The relevant peptides identified are summarized in Table 1 and Fig 1C. The tryptic fragments, T1 and 2 (the first and second tryptic peptides from the N-terminus of mature LOX-V5), were identified in gel extracts from the 35 kDa band, but not from the 30 kDa band (Table 1, Fig 1C). A tryptic peptide, T3, including the N-terminal asparagine (N)-arginine (R)₁₉₂ sequence was also found in the 35 kDa LOX-V5. The C-terminal of R₁₉₂ was not cleaved by trypsin due to the presence of proline (P) located C-terminal to this residue [16]. The T3 was also identified from the 30 kDa band but the N-terminal NR₁₉₂ was missing, thus, designated as T3 (partial). However, in some cases, the peptide bond between R and P could be cleaved by trypsin [17]. Thus, to determine whether or not the carboxy terminus of Arg₁₉₂ is the actual proteolytic cleavage site, the 30 and 35 kDa mouse LOX-V5 protein bands excised from the gel were manually digested with AspN. The resulting MALDI-MS spectra from AspN digestion from 1600 to ~2600 m/z are shown on the right panel in Fig. 1C. The peak at m/z 2625.30 in the AspN digest of 35kDa LOX-V5 (upper spectra) corresponds to the calculated m/z for ERPRGSRNRPGYGTGYFYGLP (AspN6, Table 1) (see also peptide illustration below the spectra in Fig 1C) where the cleavage occurred at the N-terminus of glutamic acid (E) [18]. This peak was not found in the AspN digest of the 30kDa LOX-V5 (lower spectra). Instead, there was a new peak at m/z 1689.84 (lower spectra), which corresponds to the calculated molecular weight for NRPGYGTGYFYGLP (designated as AspN6 partial in Table 1 and Fig 1C), the N-terminus of which is a non-AspN cleavage site. These results strongly indicate that this is the N-terminus of the 30 kDa mouse LOX with a novel proteolytic cleavage site, the C terminus of Arg₁₉₂. The AspN1–5 fragments were all identified in the 35 kDa band but not in the 30 kDa band (Table 1) (also, see peptide illustration in Fig 1C).

Transfection and mRNA Expression of Deletion Mutation LOX

In order to determine if the presence of propeptide or BMP-1 processing is involved in the generation of tLOX, we generated full-length LOX (Fig 2, left panel, construct 1), full-length LOX with point mutations induced at the BMP-1 cleavage site (LOXΔBMP-1) (Fig. 2, construct 2), and mature LOX (signal peptide and mature LOX: residue 1–16 and 163–411, mLOX) (Fig. 2, construct 3). The protein expression was assessed by IP-WB analysis (Fig 2, right panel). In Ev (negative control), an IgG heavy chain indicated by an asterisk was observed at ~50 kDa. When full-length LOX was expressed, both 35 and 30 kDa mature LOX proteins were identified (lane 1). The full-length LOX (proLOX) band was observed at ~48 kDa partially overlapping with an IgG heavy chain. The full length LOX construct devoid of the BMP-1 cleavage site (LOXΔBMP-1, construct 2) did not produce the 35 kDa LOX but the 30 kDa LOX was detected (lane 2) indicating that this novel processing is not dependent on the processing by BMP-1. However, when the propeptide was absent, the 35 kDa LOX but not the 30 kDa LOX was detected, (lane 3) indicating the critical importance of the propeptide for the novel processing.

The Presence of the Novel Processing of LOX in Bovine Aorta

In order to investigate if the novel processing occurs *in vivo*, LOX was isolated from bovine aorta. The enzymatically active fractions pooled from the initial molecular sieve chromatography were subjected to DEAE column chromatography and the fractions collected were analyzed for amine oxidase activity (solid bars in Fig 3) and immunoreactivities with αLOXi and αLOXh antibodies (two panels below the chromatograph, Fig 3). Two enzymatically active peaks were identified, one passed through the column (peak I) and the other eluted with a NaCl concentration of >0.4M (peak II) (Fig. 3). With WB analysis, both ~25 and ~30 kDa immunoreactive bands were detected with

α LOXi antibody (upper panel) that corresponded to the enzymatically active peaks I and II, respectively. However, with α LOXh antibody, only ~30 kDa band, but not the 25 kDa band, was detected (lower panel). These two immunoreactive, enzymatically active bovine LOX proteins (I and II) well correspond to the two molecular species of LOX-V5 proteins expressed by 293 cells in terms of the molecular size (30 and 35 kDa with a ~5 kDa V5 tag) as well as the pattern of immunoreactivity.

DISCUSSION

It has been well documented that pro LOX is processed by PCP/BMP-1 [5–6] and other extracellular proteases including tolloid-like-1 and 2 proteases (mTLL-1 and -2) [19] between Gly₁₆₂ and Asp₁₆₃ (numbered according to the mouse sequence). In our recent report, we noted that two molecular species of mature LOX may exist [8]. In the current study, we have identified a truncated form of mature LOX (tLOX) from a cell culture system as a ~30kDa LOX-V5 recombinant protein and as a ~25kDa LOX protein from bovine aorta. tLOX is a novel proteolytic product of proLOX between Arg₁₉₂ and Asn₁₉₃ and appears to retain amine oxidase activity. Interestingly, tLOX was immunoreactive with α LOXi but not α LOXh. This can be explained by the specific epitopes for the respective antibodies. The peptide sequence used to generate α LOXi antibody is residues 305–320 (DLLDANTQRRVAEGHK) (RES # 305–320 in human LOX) located near the C terminus of LOX while that for α LOXh antibody is residues 176–197 (KYSDDNPYYNYDTYERPRPGG) which is near the PCP/BMP-1 cleavage site, thus near the N-terminus, of human LOX [7, 10]. The difference between LOX and tLOX is the presence or absence of the residues 163–192 (DDPYNPYKYS/TDDNPYYNYDTYERPRGSR) (RES # 179–198 in human LOX) where the epitope for the α LOXh antibody resides. This is most likely the reason for the lack of immunoreactivity of tLOX with α LOXh antibody. The biological significance of the presence or absence of this peptide is not clear at present. However, the relative abundance of acidic amino acid residues in this peptide could be important for the substrate specificity, protein binding, stability and folding. The lack of this acidic peptide in tLOX could be the reason why it failed to bind to anionic exchange resin while LOX did (Fig 3). Interestingly, the DDPYNPYKYS(T)DDNPY sequence in this peptide is similar to the DDPYDPYPYEPYEPY sequence in the N-terminal region of fibromodulin, a collagen-binding small leucine-rich proteoglycan. Some of the Tyr residues in this sequence of fibromodulin have been reported to be sulfated [20]. Though we failed to identify the tyrosine sulfation in LOX by MALDI-MS analysis, this could be due to the lability of this modification under the conditions used [20–23]. Further studies are warranted to investigate this potential modification in LOX and its significance.

It has been reported that, beside PCP/BMP-1, proLOX can be also processed by MMP-2 at the specific site in the propeptide region. In the current study, the MMP-2 processed LOX resulting from the cleavage between Asn and Leu [6] was not observed. This might be due to the difference in the cell type and vectors for the generation of recombinant LOX. In the system reported in this study, the LOX-V5 protein secreted is processed and enzymatically active [8]. This is also different from the recombinant LOX protein generated in S4 cells in which it is secreted as a proLOX and cannot be further processed [4, 6].

This study is the first to show this novel processing that results in producing an enzymatically active 25 kDa LOX both in a cell culture system and bovine aorta. This novel processing requires an unknown serine protease, which might bind to LOX propeptide in order to process proLOX between R₁₉₂ and D₁₉₃ (mouse sequence) since the deletion of the LOX propeptide prevents this novel processing.

Acknowledgments

The authors thank Drs. Trackman and Pamalakumbura for their valuable suggestions for LOX purification and activity assay, and Drs. Csiszar and Fong for providing the α LOXh antibody. This work was supported by NIH-NIDCR R21 DE019569.

References

1. Siegel RC. Collagen cross-linking. Synthesis of collagen cross-links in vitro with highly purified lysyl oxidase. *J Biol Chem.* 1976; 251:5786–5792. [PubMed: 9402]
2. Kagan, HM. Characterization and regulation of lysyl oxidase. Orlando, Fla: Academic Press; 1986.
3. Yamauchi M, Shiiba M. Lysine hydroxylation and crosslinking of collagen. *Methods Mol Biol.* 2002; 194:277–290. [PubMed: 12029842]
4. Trackman PC, Bedell-Hogan D, Tang J, Kagan HM. Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor. *J Biol Chem.* 1992; 267:8666–8671. [PubMed: 1349020]
5. Cronshaw AD, Fothergill-Gilmore LA, Hulmes DJ. The proteolytic processing site of the precursor of lysyl oxidase. *Biochem J.* 1995; 306 (Pt 1):279–284. [PubMed: 7864821]
6. Panchenko MV, Stetler-Stevenson WG, Trubetskoy OV, Gacheru SN, Kagan HM. Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C-proteinase. *J Biol Chem.* 1996; 271:7113–7119. [PubMed: 8636146]
7. Fogelgren B, Polgar N, Szauter KM, Ujfaludi Z, Laczko R, Fong KS, Csiszar K. Cellular fibronectin binds to lysyl oxidase with high affinity and is critical for its proteolytic activation. *J Biol Chem.* 2005; 280:24690–24697. [PubMed: 15843371]
8. Atsawasuwan P, Mochida Y, Katafuchi M, Kaku M, Fong KS, Csiszar K, Yamauchi M. Lysyl oxidase binds transforming growth factor-beta and regulates its signaling via amine oxidase activity. *J Biol Chem.* 2008; 283:34229–34240. [PubMed: 18835815]
9. Mochida Y, Duarte WR, Tanzawa H, Paschalis EP, Yamauchi M. Decorin modulates matrix mineralization in vitro. *Biochem Biophys Res Commun.* 2003; 305:6–9. [PubMed: 12732188]
10. Li PA, He Q, Cao T, Yong G, Szauter KM, Fong KS, Karlsson J, Keep MF, Csiszar K. Up-regulation and altered distribution of lysyl oxidase in the central nervous system of mutant SOD1 transgenic mouse model of amyotrophic lateral sclerosis. *Brain Res Mol Brain Res.* 2004; 120:115–122. [PubMed: 14741400]
11. Palamakumbura AH, Trackman PC. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem.* 2002; 300:245–251. [PubMed: 11779117]
12. Parker, CEW.; MR; Loiselle, DR.; Dicheva, NN.; Scarlett, CO.; Borchers, CH. Identification of Components of Protein Complexes. Vol. 301. Patterson, New Jersey: Humana Press; 2005.
13. Seve S, Decitre M, Gleyzal C, Farjanel J, Sergeant A, Ricard-Blum S, Sommer P. Expression analysis of recombinant lysyl oxidase (LOX) in myofibroblastlike cells. *Connect Tissue Res.* 2002; 43:613–619. [PubMed: 12685868]
14. Kagan HM, Reddy VB, Panchenko MV, Nagan N, Boak AM, Gacheru SN, Thomas KM. Expression of lysyl oxidase from cDNA constructs in mammalian cells: the propeptide region is not essential to the folding and secretion of the functional enzyme. *J Cell Biochem.* 1995; 59:329–338. [PubMed: 8567751]
15. Kagan HM, Cai P. Isolation of active site peptides of lysyl oxidase. *Methods Enzymol.* 1995; 258:122–132. [PubMed: 8524144]
16. Keil, B., editor. Specificity of Proteolysis. Springer-verlag; Berlin And Heidelberg: Gmbh & Co. K; Berlin, Germany: 1992.
17. Rodriguez J, Gupta N, Smith RD, Pevzner PA. Does trypsin cut before proline? *J Proteome Res.* 2008; 7:300–305. [PubMed: 18067249]
18. Ingrosso D, Fowler AV, Bleibaum J, Clarke S. Specificity of endoproteinase Asp-N (*Pseudomonas fragi*): cleavage at glutamyl residues in two proteins. *Biochem Biophys Res Commun.* 1989; 162:1528–1534. [PubMed: 2669754]
19. Uzel MI, Scott IC, Babakhanlou-Chase H, Palamakumbura AH, Pappano WN, Hong HH, Greenspan DS, Trackman PC. Multiple bone morphogenetic protein 1-related mammalian

- metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures. *J Biol Chem.* 2001; 276:22537–22543. [PubMed: 11313359]
20. Onnerfjord P, Heathfield TF, Heinegard D. Identification of tyrosine sulfation in extracellular leucine-rich repeat proteins using mass spectrometry. *J Biol Chem.* 2004; 279:26–33. [PubMed: 14551184]
 21. Yagami T, Kitagawa K, Futaki S. Liquid secondary-ion mass spectrometry of peptides containing multiple tyrosine-O-sulfates. *Rapid Commun Mass Spectrom.* 1995; 9:1335–1341. [PubMed: 8534883]
 22. Talbo, GaMM. *Techniques in Protein Chemistry V.* San Diego: Academic Press; 1994.
 23. Monigatti F, Hekking B, Steen H. Protein sulfation analysis--A primer. *Biochim Biophys Acta.* 2006; 1764:1904–1913. [PubMed: 16952486]

Fig. 1.

Characterization of a novel processing of proLOX. A. SDS-PAGE and Western blot analysis. Purified LOX-V5 protein was subjected to SDS-PAGE analysis stained with Coomassie Brilliant Blue (CBB) (lane 1) and WB analysis with normal rabbit serum (NRS) (lane 2), anti(α)(V5) antibody (lane 3), αLOXi antibody (Imgenex) (lane 4) and αLOXh antibody (lane 5). Two major CBB stained bands were observed at ~30 and ~35 kDa bands. Both proteins are immunoreactive with αV5 (lane 3) and αLOXi (lane 4) antibodies but only the ~35kDa band was immunoreactive with αLOXh antibody (lane 5). No immunoreactivity was found with NRS (lane 2). B. Amine oxidase activity of LOX-V5 protein. Note that the amine oxidase activity of LOX-V5 protein is retained and increased in

a dose-dependent manner, and the activity was nullified by 500 μ M of BAPN. C. Upper panel: MALDI-MS spectra from the tryptic (T) and AspN digestion of the 35 kDa (mature) and 30 kDa (truncated) LOX-V5. T1, T2 and T3 were identified in the 35kDa LOX-V5, while in the 30 kDa LOX-V5 only a shorter form of T3, denoted as T3 (partial), was identified. In the AspN digests, a shorter form of AspN6, AspN6 (partial), was found only in the 30kDa LOX-V5. The peptides identified by mass spectrometry (T1–3, partial T3, AspN1–6, partial AspN6) are illustrated in the lower panel. The residue numbers are based on the mouse proLOX. For the detailed information about each peptide, see Table 1.

\$watermark-text

\$watermark-text

\$watermark-text

Fig. 2.

Generation of LOX constructs and their expression pattern. Three LOX-V5 constructs were generated and shown on left. 1. LOX-V5, 2: LOX(D₁₆₃D₁₆₄>EG)-V5, 3: ΔPPLOX-V5 (lacking propeptide). The protein expression of each construct was analyzed by IP-WB with αV5 antibody and shown on right. An asterisk at ~50 kDa indicates IgG heavy chain. Ev: Control cells transfected with an empty vector (negative control). Lane 1: LOX-V5, lane 2: LOX(D₁₆₃D₁₆₄>EG)-V5, lane 3: ΔPPLOX-V5. When LOX-V5 was expressed, a full-length (~48kDa just below the IgG heavy chain), a mature LOX-V5 (35 kDa) and a truncated LOX-V5 (30 kDa) were observed (lane 1). With LOXΔPCP expression, full-length (50kDa) and a truncated LOX-V5 (30 kDa) but not mature LOX-V5 (35 kDa) were observed (35 kDa) (lane 2). With ΔPPLOX, only mature LOX (35 kDa) was observed (lane 3)

Fig. 3. DEAE chromatograph of bovine aorta derived LOX. Upper panel: the solid line represents the amine oxidase activity in the fractions and the dotted line the percentage of 1M NaCl. Lower panel: Western blot analyses of the fractions with anti(α)LOXi (upper section) and α LOXh antibody (lower section). The unbound fractions (peak I) contained tLOX (25 kDa) which was immunoreactive only with α LOXi but not with α LOXh while the bound fractions (peak II) contained mature bovine LOX (30 kDa) which was immunoreactive with both α LOXi and α LOXh.

TABLE 1

Enzymatic digests of 35 and 30 kDa mouse LOX-V5 with trypsin (T1–3) and AspN. (AspN1–6) identified by mass spectrometry. Calculated (M+H)⁺ of each peptide and the residue number of mouse LOX are also shown. Note that partial (pt) T3 and AspN6 were identified only in the 30kDa LOX-V5

Enzyme digest	Peptide sequence	(M+H) ⁺	Residues	35k	30k
T1	DDPYNPYK	1011.44	163–170	+	
T2	YSDDNPYYNYDTYERPRGSR	2791.20	171–192	+	
T3	NRPGYGTGYFQYGLPDLVPDPYYIQASTYVQK	3670.79	193–224	+	
T3 (pt)	PGYGTGYFQYGLPDLVPDPYYIQASTYVQK	3398.71	195–224		+
AspN1	D	134.05	163	+	
AspN2	DPYNPYKYS	1146.51	164–172	+	
AspN3	D	134.05	173	+	
AspN4	DNPPYNNY	1111.44	174–181	+	
AspN5	DTY	398.16	182–184	+	
AspN6	ERPRGSRNRPGYGTGYFQYGLP	2625.31	185–207	+	
AspN6 (pt)	NRPGYGTGYFQYGLP	1689.84	193–207		+