Purification and Characterization of Lysyl Oxidase from Fetal Bovine Aorta in the presence of protease inhibitors
-Evidence against polymorphism-

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CONTENTS

Ⅰ. SUMMARY

Lysyl Oxidase from fetal bovine aorta was purified to homogenity using urea extraction, Sephacryl S200HR chromatography, Hydropore AX ion-exchange high performance liquid column chromatography, Cibacron blue affinity chromatography, and Sephacryl S-300 HR chromatography in the presence of protease inhibitors. The purified enzyme was active toward lathyritic collagen as well as elastin and was sensitive to aminonitriles such as BAPN. Upon Sephacryl S-300 HR chromatography, the enzyme was eluted as a peak with a K_{av} value of 0.45 (65% of V_t) and it eluted from high performance liquid ion-exchange column (Hydropore AX) at single position (ionic strength, $I = 0.1 \sim 0.15$). Once purified, it showed one band upon SDS-PAGE. It migrated to a band the mobility of which corresponded to a Mr of 33,500 upon reduction while it migrated to a 24,500 Mr position under the non-reducing condition. In constrast to other reports, it is concluded that fetal bovine aorta contains only one type of lysy oxidase.

Ⅱ. INTRODUCTION

Lysyl oxidase is an extracellular, copper containing amine oxidase and is responsible for the initiation of inter- and intramolecular cross-linking of collagens and elastin by catalyzing oxidative deamination of the ε-amino group of lysine or hydroxylysine residue of the proteins yielding peptidyl (δ-hydroxy,) α-aminoadipic acid δ-semialdehyde (Fig. 1). These aldehydes, through non-enzymatic spontaneous condensations, subsequently can form various covalent inter- and intra-chain crosslinks (Fig. 2~4) which stabilize and insolubilize these proteins[1-3]. Since it was shown by Narayanan et al. that lysyl oxidase is stable in buffers containing urea[4], the enzyme has been successfully purified from various animal tissues[4-16]. Also, the enzyme was cloned, its cDNA sequenced and its chromosomal localization was studied[17-20]. It has been reported that the enzyme has copper at its active site and require molecular oxygen as a co-substrate[21-24]. Preparations of lysyl oxidase from various tissues including human tissues were reported heterogeneous, with

multiple peaks of activity in DEAE-cellulose chromatography[14-16], Mr being about 30,000 by gel filtration in the presence of 6M urea and by SDS-PAGE. No distinct differences in terms of Mr, amino acid composition, specific activity, peptide mapping, etc. were found between different fraction of DEAE chromatography. The existence of the multiple lysyl oxidase forms is probably not explained by cabamylation of amino groups on the enzyme due to the cyanate derived from urea since none of the enzyme species contain any detectable amount of homocitrulline, the cabamylated derivative of lysine[14-16].

The nature of the organic prosthetic group involved in the lysyl oxidase reaction has been a controversial issue for many years, the issue being whether it was pyridoxal phosphate (PLP) or some kind of quinone[25-30] (Fig. 5). It now seems that this long-standing problem has been solved, since the cofactor in bovine serum asime oxidase and porcine kidney diamine oxidase appears to be covalently bound PQQ (pyrroloquinoline quinone), a compound already shown to be the cofactor in a number of different bacteraial oxidoreductases. Derivatization of the prothetic group of the lysyl oxidase with DNPH and subsequent isolation of C-5 hydrazone adduct

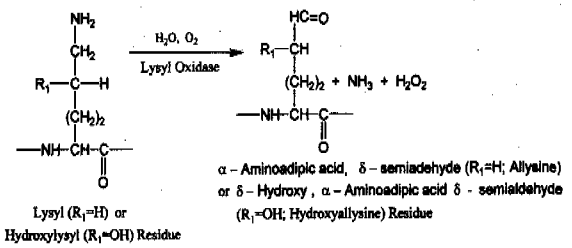

Fig. 1. Reaction driven by lysyl oxidase

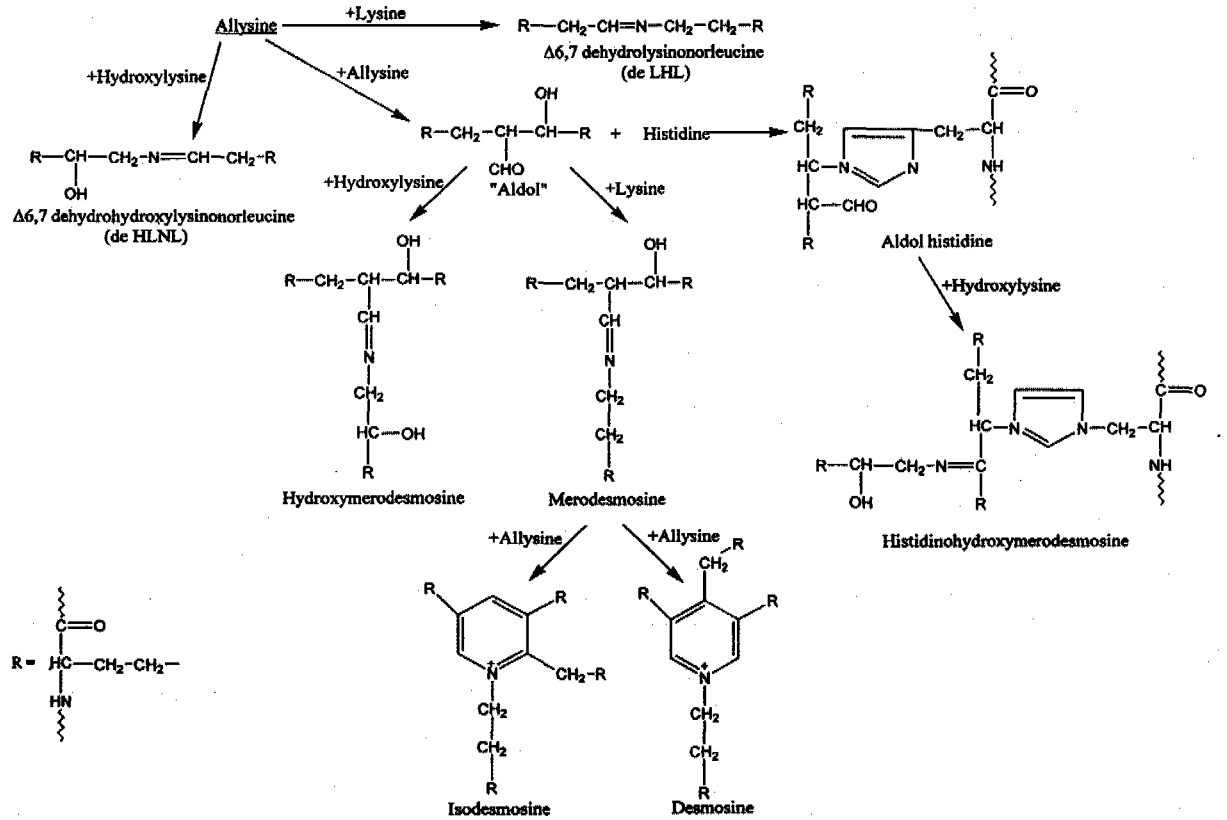

Fig. 2. Chemical structure of allysine derived crosslinks

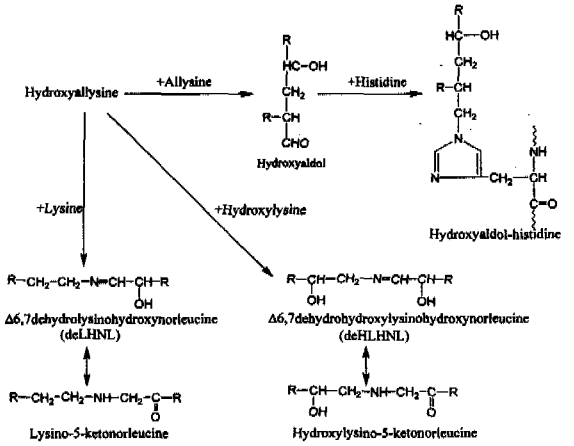

Fig. 3. Chemical structure of hydroxyallysine derived crosslinks.

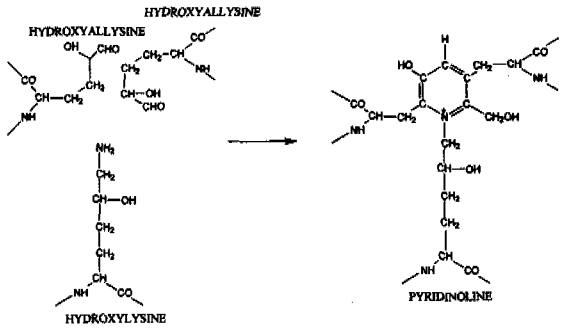

Fig. 4. Chemical structure of trifunctional crosslink, pyridinoline

was reported[30]. More recent study, employing Edman sequencing, mass spectrometry, and Raman resonance spectroscopy indicated that lysyl oxidase enzyme contains LTQ (lysine tyrosyl quinone) but neither PQQ nor TPQ (topa quinone) while bovine serum albumin amine oxidase contain TPQ[31, 32].

III. MATERIALS AND METHODS

L-[4,5-^{3}H]lysine was obtained from NEN. Chicken embryos were purchased from SPAFAS while cell culture media were from GIBCO. Fetal bovine aorta were from Pel-Freeze Biologicals. Chemicals for SDS-PAGE were purchased from BioRad while chromatographic materials are from Pharmacia and Sigma. Other chemicals purchased from various companies were at least ACS grade.

1. Preparation of substrate

Calvaria were taken out from 17-day-old chicken embryos and their surrounding periosteum were removed with a fine forcep and were cultured in DMEM lacking lysine and glutamine, supplemented with 10% fetal bovine serum, 50μg/ml ascorbic acid, 50μg/ml glycine, 40μg/ml proline, 50μg/ml BAPN and 20 μCi/ml L-[4,5-^{3}H]lysine for 36 hrs. The organ-cultured calvaria was rinsed with PBSA two times, frozen with liquid nitrogen and were pulverized with a mortar and pestle. Newly synthesized collagen was extracted from the powdered calvaria with 50mM Tris, 0.5M NaCl, pH, 7.4 in the presence of PI (protease inhibitors; 5mM EDTA, 5mM NEM, 1mM PMSF, 5mM Benzamidine) and was precipitated with a final 20% (w/v) NaCl. After standing overnight, the precipitate was recovered by centrifugation at 35,000g for 1 hr. The pellet was re-dissolved with 50mM Tris, 0.15M NaCl, pH, 7.4, dialyzed against the same buffer and was centrifuged at 35,000g. The supernatant was used for further purification of the collagen. Ethanol (-20 °C) was added dropwise to the supernatant to a final 18% (v/v) and the precipitate was centrifuged at 35,000g for 1 hr. The pellet was re-dissolved with 50mM Na_2HPO_4, 0.15M NaCl, pH, 7.4, dialyzed against the same buffer and was clarified at 100,000g. The purity of the L-[4,5-^{3}H] lysine-labeled collagen was monitored by amino acid analysis, collagenase digestion and SDS-PAGE followed by fluorography.

2. Enzyme assay

Lysyl oxidase activity was assayed against solube collagen substrate prepared from chicken embryo calvaria as described by others[33-35] (Fig. 6). Briefly, for the enzyme assay, 500,000-650,000 cpm of the collagen substrate in 50mM Na_2HPO_4, 0.15M NaCl, pH, 7.4 was pre-incubated for 1 hr at 37 °C to promote fibril formation of the collagen. Enzyme was

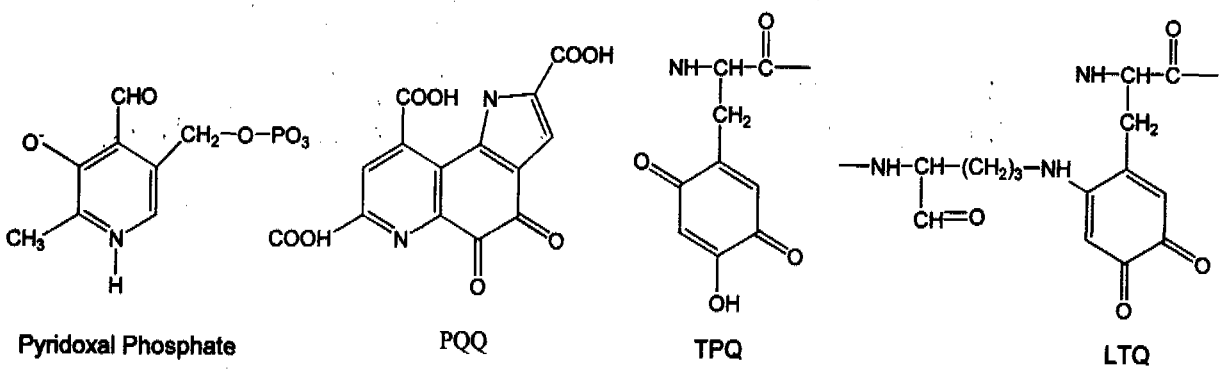

Fig. 5. Structure of presumptive cofactors in amine oxidases
PQQ: pyrroloquinoline quinone TPQ: topa quinone LTQ: lysine tyrosine quinone

then introduced to the reaction tube and the mixture was incubated for 2 more hours at 37 °C. At the end of the assay, tritiated water formed during the reaction was collected by vacuum sulbimation using Thumberg tubes after passing through Dowex 50 (H^+ form) column (bed volume 1.0 ml) and its quantity was measured by liquid scintillation counting of aliquots.

3. Preparation of enzyme

a. Extraction of the Enzyme

900g of fetal bovine aorta were minced and were homogenized with 3 volumes of 20 mM Na_2HPO_4, 0.12M NaCl, pH, 7.4. After centrifugation at 17,000g for 10 min, the pellet was homogenized under the same condition and recentrifuged. Both extracts, which have no enzyme activity, were discarded. Lysyl oxidase was then extracted with 3 volumes of 6M urea, 20mM Na_2HPO_4, pH, 7.4 and centrifuged at 17,000g for 10 min and the extraction was repeated one more time.

b. DEAE-Cellulose Batch Chromatography

Urea extract (ca. 13,000 mg in 5,000 ml) was pooled and directly applied onto a column of CM-celluose (10x10cm) which had been equilibrated with 6M urea, 20mM Na_2HPO_4 buffer, pH, 7.4. The flow-through fraction from the column was directly

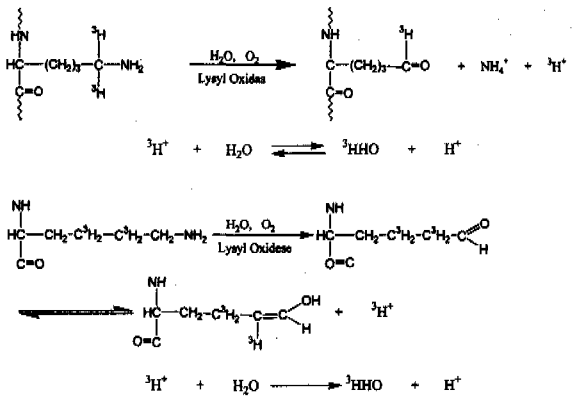

Fig. 6. Tritium release from L-[6-^{3}H]lysine- or L-[4,5-^{3}H]lysine-labeled collagen or elastin by lysyl oxidase

applied onto a column of DEAE-cellulose (10x20cm) that had been equilibrated with 6M urea, 20mM Na_2HPO_4, pH, 7.4 and the column was washed with 1 column volume of equilibration buffer. The enzyme was then eluted with 1.5 column volume of 6M urea, 20mM Na_2HPO_4, 0.35M NaCl, pH, 7.4.

c. Sephacryl S-200 HR Chromatography

The enzyme fractions (ca. 960 mg in 2300 ml) eluted from the DEAE-Cellulose column was pooled, concentrated and was clarified at 50,000g for 1 hr. 100 ml (960 mg) of the sample thus prepared was loaded onto a Sephacryl S200 HR column (5 x 100cm)

that had been equilibrated with 6M urea, 20mM Na_2HPO_4, 0.05M NaCl, pH, 7.4 and the enzyme was eluted with the same buffer.

d. Hydropore AX-300 HPLC

The enzyme fractions eluted from Sephacryl S300 HR column was pooled, concentrated and an aliquot of the sample was loaded onto a Hydropore AX-300 column (1.0 x 25 cm) that had been equilibrated with 6M urea, 20mM Na_2HPO_4, pH, 7.0. The enzyme was eluted using stepwise salt gradient.

e. Cibacron Blue 3GA-agarose affinity column chromatography

Fractions (total 25 mg) that contain enzyme activity from Hydropore AX-300 HPLC column was pooled, dialyzed vs. 20 mM Na_2HPO_4, pH, 7.4 and were loaded onto a Cibacron Blue 3GA-agarose column (Type 3000 CL, 2.5cm x 8cm) that had been equilibrated with 20 mM Na_2HPO_4, pH, 7.4. After washing 2 column volume of equilibration buffer, the column was treated with sequentially with 1.5 column volume of 20 mM Na_2HPO_4, 1M NaCl, pH, 7.4, with 1 column volume of equilibration buffer, with 2 column volume of 2M urea, 20 mM Na_2HPO_4, pH, 7.4 then finally with two column volume of 6M urea, 20 mM NaH_2PO_4, pH 7.4.

f. Sephacryl S-300 HR chromatography

Fractions that was eluted from the Cibacron Blue-agarose affinity column with 6M urea, 20 mM Na_2HPO_4, pH 7.4 were pooled, concentrated and were loaded onto a Sephacryl S-300 column that had been equilibrated with 6M urea, 20mM Na_2HPO_4, 0.05M NaCl, pH, 7.4. The enzyme was eluted with the same buffer.

g. Protein Determination

Protein concentration was determined by the method of Lowry et al.[36] using bovine serum albumin as a standard. When concentration of collagen was estimated, acid soluble bovine skin collagen was used as a standard.

h. SDS-PAGE

SDS-PAGE was carried out using Laemmli buffer system as described[37].

Ⅳ. RESULTS AND DISCUSSION

1. Purification of Substrate

L-[4,5-^{3}H]lysine labeled newly synthesized lathyritic neutral salt soluble collagen was purified from chicken embryo calvaria by standard method, namely extraction with high concentration of NaCl, buffered at neutral pH, salt precipitation and ethanol precipitation. The collagen thus prepared was subjected to SDS-PAGE to monitor purity and integrity of it. The Coomassie blue staining pattern showing two bands (α1, and α2 subunit band) matched two bands on fluorogram (lane 2 of Fig. 7).

More than 99% of the radioactivity on the gel was accounted for by Type I collagen components, which did not appear if prior incubation in bacterial collagenase was carried out (lane 1 of Fig. 7). Amino

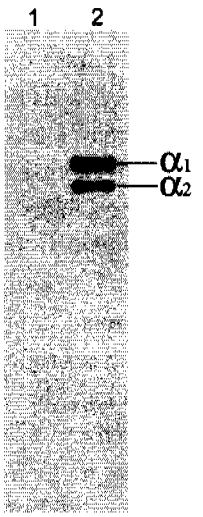

Fig. 7. Fluorogram of purified L-[4,5-^{3}H]lysine labeled collagen
lane 1: bacterial collagenase digested L-[4,5-^{3}H]lysine labeled lathyritic collagen
lane 2: undigestfed L-[4,5-^{3}H]lysine labeled lathyritic collagen

acid composition of the sample showed it to be Type I collagen. Specific activity of the L-[4,5-^{3}H]lysine labeled collagen was 3,000,000 cpm/mg and 500,000-650,000 cpm of collagen was used for lysyl oxidase assay.

2. Purification of Lysyl Oxidase

Lysyl oxidase activity is essential for cross-linking of collagen and elastin[1]. The enzyme has been extracted from various tissues such as cartilage, aorta, lung, ligamentum nuchae and placenta[4-16]. One of the characteristics of the enzyme is that various preparations of the enzyme is inhibited by aminonitrile and they catalyze the oxidative deamination of peptidyl-ε-NH_2 groups in collagen and elastin. Many workers have reported multiple peaks of enzyme activity during purification especially in DEAE-Cellulose chromatography step[14-16] though relationship between such peaks in a particular enzyme preparation have not been diciphered. One possibility is that lysyl oxidase was randomly cabamylated by cyanate ion present in concentrated urea solution, though there is no report that the enzyme preparations did contain any detectable amount of homocitrulline, the cabamylated derivatives of lysine[14, 16]. The second possibility is that the differences may exist at the level of some post-translational modification of the enzyme protein[16]. Another possibility is that the enzyme might undergo degradation by proteolysis during long purification step[16]. It also can not be ruled out that each peak represents the translation product of a group of lysyl oxidase mRNAs that result from different RNA splicing. In order to study the polymorphism reported by others, it is decided that the enzyme be purified under more stringent, defined condition. Overall strategy for the purification of the enzyme was very similar to the reports by others[14-16]. For the purification of the enzyme in this study, several precautions were taken. Firstly, the enzyme preparation were maintained in the presence of protease inhibitors (5mM NEM, 1mM PMSF, 5mM Benzamidine, 1ug/ml pepstatin, 10μg/ml leupeptin) until the last step of purification to avoid any possible degradation of the enzyme. Secondly, the enzyme was maintained in 6M urea throughout the purification steps since maintaining the enzyme in urea, lower than 6M might induce aggregation of the enzyme[14], inducing unpredictable behaviour in column chromatography procedure. Also, HPLC step was employed since it gives a better resolution than conventional column chromatography. The prior extraction of aorta with phosphate buffered saline efficiently removed the extractable proteins. The most efficient purification step was Sephacryl S-200 HR chromatography step that eliminates the bulk of the non-enzyme protein. The enzyme was eluted from the column at the descending part of the first broad protein peak (Fig. 8). Since the enzyme activity peak was not symmetrical, frontal part (ascending part) of the enzyme activity peak

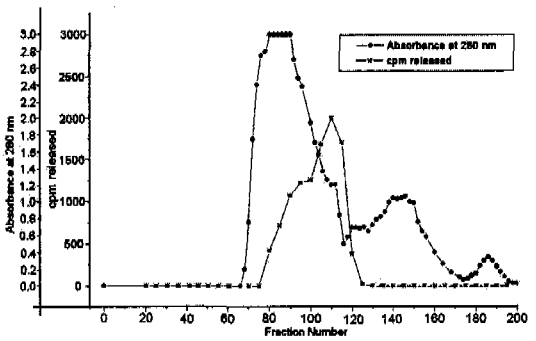

Fig. 8. Sephacryl S-200 HR Chromatography of lysyl oxidase
Lysyl oxidase, batch-eluted from DEAE-Cellulose column with 6M urea, 20 mM Na_2HPO_4, 0.35M NaCl, pH, 7.4 was pooled, concentrated with a Minitan apparatus (Millipore). 100 ml of the concentrated enzyme (ca. 960 mg) was loaded onto a Sephacryl S200HR column (5 cm x 135 cm) that was equilibrated with 6M urea, 20 mM Na_2HPO_4, 50 mM NaCl, pH, 7.4 and was eluted with the same buffer. Flow rate was 2.63 ml/min or 8.037 cm/hr. Fraction volume was 12.625 ml.

(fraction 80-100 of Fig. 8) was re-chromatographed and the enzyme came out as a single symmetrical peak (data not shown), elution position of which moved to the position of original major enzyme peak, suggesting that even in concentrated urea solution, at the high concentration of protein mixture (9.6 mg/ml), some aggregation occurred or more likely, the column was overloaded (3.77% of the column volume vs. recommended 1~2% of the column volume by the vendor). As a part of purification step, and to ascertain polymorphism reported by others, anion-exchange HPLC method was employed. When the enzyme was eluted from Hydropore AX-300 HPLC column, the protein came out from the column at a single position (Ionic strength I = 0.1~0.15)

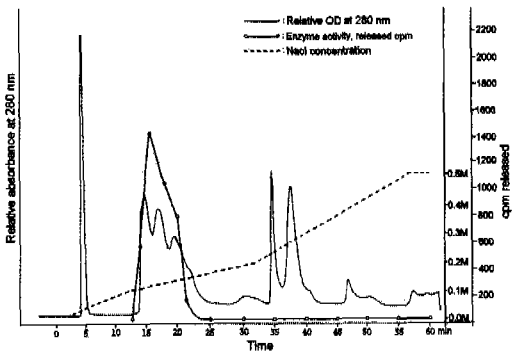

Fig. 9. HPLC of fetal bovine aortic lysyl oxidase. Fractions from Sephacryl S-200 HR chromatography was concentrated and loaded onto a Hydropore AX-300 HPLC ion-exchange column (1.0 cm x 25 cm). Flow rate was 4.0 ml/min. The enzyme was eluted using gradient method (see below).
Equilibration Buffer (Buffer A): 6M urea, 20 mM Na_2HPO_4, pH, 7.0
Limiting Buffer (Buffer B): 6M urea, 20 mM Na_2HPO_4,, 0.5 M NaCl, pH, 7.0
0-2min: Buffer A, 100 %
2-12 min: linear gradient to Buffer A, 80 % and Buffer B 20%
12-32 min: linear gradient to Buffer A, 60% and Buffer B, 40 %
32-57 min: linear gradient to Buffer B 100 %

(Fig. 9). Employment of a shallow gradient by increasing total volume of eluant from 12.2 column volume to 24.4 column volume did neither resolve individual protein peak nor separate the enzyme activity peak into its presumptive, putative components. The result suggests that the enzyme was not polymorphic.

The enzyme preparation thus purified was further purified by employing Cibacron blue affinity chromatography. Some of the contaminating proteins were removed by high salt (1.0M NaCl, 20mM Na_2PO_4, pH,7.4) and 2M urea buffer sequentially. The enzyme was finally eluted from the column with 6M urea, 20mM Na_2HPO_4, pH, 7.4 (data not shown). Final purification was performed on a Sephacryl S300 HR column. All of the rest contaminants were well separated from the enzyme peak (fraction 80~90 of Fig. 10). The enzyme activity peak matched protein peak and was symmetrical. In order to

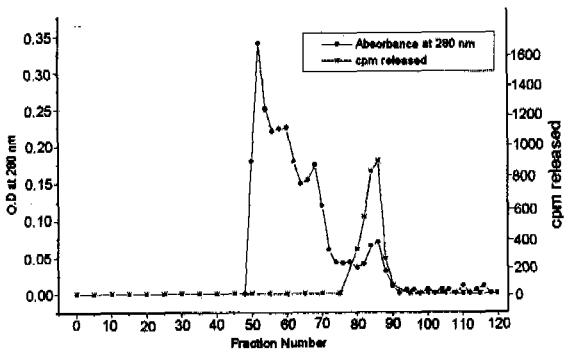

Fig. 10. Sephacryl S-300 HR chromatography of lysyl oxidase
Lysyl oxidase enzyme fraction eluted from Cibacron blue affinity column was pooled and concentrated by ultrafiltration using PM-10 membrane (Amicon). ca. 8 mg of the enzyme was loaded onto a Sephacryl S-300 HR column (1.5 cm x 95 cm) that was equilibrated with 6M urea, 20 mM Na_2HPO_4, 50 mM NaCl, pH, 7.4 buffer. The enzyme was eluted with the same buffer. Fraction volume: 1.27 ml; fraction time: 2.5 min; flow rate: 0.5 ml/min

ascertain the purity of the enzyme thus prepared, it was subjected to SDS-PAGE using buffer system described by Laemmli[37]. Upon staining with Coomassie brilliant blue, it migrated to a position, the mobility of which corresponded to a Mr of 33,500, just above the standard marker, carbonic anhydrase (Mr=29,000) if it is reduced with 50 mM dithiothreitol (lane 1 of Fig. 11). However, if it is not reduced, it migrated to a position of Mr of 24,500 (lane 2 of Fig. 11) just below the standard marker, carbonic anhydrase. Its electrophoretic behavior can be explained by the fact that hydrodynamic radius of the unreduced form of a protein was usually smaller than that of the reduced form because of intramolecular disulfide bond formation. Given the result from ion-exchange HPLC, it is concluded that bovine aorta contain only one type of lysyl oxidase. Polymorphism reported by others probably are due to minor proteolysis occurred during the purification steps.

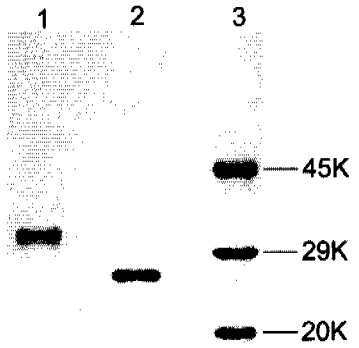

Fig. 11. SDS-PAGE of purified lysyl oxidase
lane 1: lysyl oxidase, reduced with 50 mM dithiothreitol
lane 2: lysyl oxidase, unreduced
lane 3: Mr. standard, ovalbumin (45K), carbonic anhydrase (29K), and soybean trypsin inhibitor (20K)

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The following abbreviations are used:
BAPN: β-aminopropionitrile
DMEM: Dulbecco's modified Eagle's minimum media
DPNH: dinitrophenylhydrazine
EDTA: ethylene diamine tetracetic acid
NEM: N-ethyl maleimide

PAGE: polyacrylamide gel electrophoresis
PBSA: phosphate buffered saline without calcium and magnesium
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulfate

국문초록

소(牛) 胎兒 大動脈으로부터 단백 분해효소 억제제 존재 하에 Lysyl Oxidase의 純粹分離
-多形性에 對한 反論-

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한 송

소(牛) 태아 대동맥으로부터 Urea 추출, Sephacryl S200 HR 크로마토그라피, Hydropore AX 이온교환 크로마토그라피, Cibacron blue-agarose 친화 크로마토그라피, 그리고 Sephacryl S300 HR 크로마토그라피를 이용하여 lysyl oxidase를 순수분리를 하였고 분리기간 중 항상 단백분해 억제제를 첨가하였다. 순수 분리된 효소는 가교결합이 없는 교원단백질과 엘라스틴에 활성을 보였고, BAPN 같은 아미노나이트릴에 의하여 억제되였다. Sephacryl S300 HR 크로마토그라피로 분리 될 경우, 이 효소는 0.45의 K_{av} (V_t의 65%)값을 보였고, 이온교환 고속액체 크로마토그라피의 경우에서는 이온 강도가 0.1-0.15 사이에서 하나의 피크로 용리되었다. 순수 분리된 이 효소는 SDS 폴리아크릴아마이드 전기영동에서는 하나의 밴드로 이동하였는데, 환원이 될 경우에는 분자량이 33,500, 비환원이 될 경우에는 분자량이 24,500의 위치로 이동하였다. 이온교환 고속액체 크로마토그라피의 결과를 참조하여, 다른 보고서와는 달리 소(牛) 胎兒 大動脈에는 여러 종류가 아닌 한 종류의 lysyl oxidase가 존재한다고 결론을 내렸다.