Purification and Properties of Four Species of Lysyl Oxidase from Bovine Aorta

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Lysyl oxidase of bovine aorta was resolved into four enzymically active species by elution from DEAE-cellulose with a salt gradient in 6M-urea, consistent with purification results obtained with enzyme of other tissues [Stassen (1976) *Biochim. Biophys. Acta* **438**, 49–60]. In the present study, each of the four peaks of activity was purified to apparent homogeneity by subsequent chromatography on gel-filtration media in 6M-urea. Each enzyme is eluted as a species with mol.wt. approx. 30000 under these conditions, although lysyl oxidase polymerizes to a series of multimers with molecular weights ranging up to 1 000 000 in the absence of urea. The apparent subunit molecular weight of each enzyme species determined by electrophoresis in sodium dodecyl sulphate and 8M-urea is approx. 32000–33000. The amino acid compositions of the purified forms of lysyl oxidase are similar to each other, although sufficient differences exist to conclude that each is a unique molecular species. Incorporation of α -toluenesulphonyl fluoride into the purification scheme does not alter the resolution of enzyme into four species, suggesting that proteolysis during isolation is not the basis of the heterogeneity. The similar sensitivities of each form of enzyme to chelating agents and to semicarbazide and isoniazid indicate that each requires the participation of a metal ion, presumably Cu^{2+} , and of a carbonyl compound for enzyme function. The present study describes a method for the purification of multiple species of lysyl oxidase and reveals that significant chemical differences exist between the different enzyme forms.

Lysyl oxidase plays a pivotal role in the maturation of collagen and elastin by oxidizing peptidyl-lysine in these connective-tissue proteins to peptidyl- α -amino-adipic δ -semialdehyde, the reactive precursor to a series of covalent inter- and intra-polypeptide cross-linkages (Pinnell & Martin, 1968). Collagen and elastin fibres become progressively more insoluble as spontaneous formation of cross-linkages proceeds from the aldehyde product of lysyl oxidase activity. This Cu^{2+} -dependent amine oxidase is strongly inhibited by β -aminopropionitrile, which is the apparent molecular basis of the lathyrogenic properties of this compound (Narayanan *et al.*, 1972). The enzyme has been purified partially or to apparent homogeneity from chick-embryo cartilage (Stassen, 1976; Siegel & Fu, 1976), chicken aorta (Harris *et al.*, 1974), bovine lung (Shieh & Yasunobu, 1976) and, as reported in a preliminary communication, from bovine aorta and ligamentum nuchae (Jordan *et al.*, 1977). There is evidence for a multiplicity of enzyme species in connective tissues, since at least two peaks (Shieh & Yasunobu, 1976; Vidal *et al.*, 1975), and in some cases four peaks (Stassen, 1976; Jordan *et al.*,

1977), of enzyme activity can be resolved on DEAE-cellulose. Thus far, physical-chemical comparisons of the multiple species of enzyme isolated from any one tissue have not been available, presumably owing to the lack of sufficient material of the appropriate degree of purity. However, unique immunological determinants have been indicated for two species of enzyme obtained from bovine aorta (Vidal *et al.*, 1975). It nevertheless appears to be important to establish more completely the physical-chemical properties and basis of the heterogeneity of this enzyme, since the existence of multiple species may be important to the physiological function (or functions) of lysyl oxidase.

In the present report, the purification of four species of enzyme from bovine aorta is described, as are factors that influence the stability and recovery of enzyme during the purification procedure. Amino acid analysis, molecular-weight determinations and inhibition patterns of the four species indicate that they are similar to, but not identical with, each other. Initial studies on the tendency of the enzyme to polymerize are described.

Experimental

Materials

DEAE-cellulose was purchased from Whatman, Clifton, NJ, U.S.A., and Sepharose CL-4B and Sephacryl S-200 were obtained from Pharmacia, Piscataway, NJ, U.S.A. Analytical-grade urea was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and was passed through a mixed-bed ion-exchange resin before use. Analytical-grade $(\text{NH}_4)_2\text{SO}_4$ was a product of Chemical Dynamics Corp., South Plainfield, NJ, U.S.A. Expendable gel-electrophoresis reagents and chemicals, including sodium dodecyl sulphate, were products of Bio-Rad, Richmond, CA, U.S.A. L-[4,5- ^3H]Lysine (43 Ci/ μmol) was obtained from Schwartz/Mann, Orangeburg, NY, U.S.A. The fumarate salt of β -aminopropionitrile was purchased from Aldrich. Bovine serum albumin, ovalbumin, bovine pancreas chymotrypsinogen, egg-white lysozyme, bovine liver catalase, rabbit muscle aldolase and bovine erythrocyte carbonic anhydrase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ferritin and thyroglobulin were obtained from Pharmacia, and bovine liver glutamate dehydrogenase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Concanavalin A coupled to agarose was obtained from Sigma. Dulbecco's Modified Eagle's Medium (Morton, 1970) lacking lysine was a product of Grand Island Biological Co., Grand Island, NY, U.S.A.

Preparation of substrate and assay

Substrate was prepared by the method of Pinnell & Martin (1968) with some modifications. Aortae were removed from 16-day chick embryos and preincubated at 37°C for 30 min in Dulbecco's Modified Eagle's Medium lacking lysine and supplemented with 5 mg of β -aminopropionitrile fumarate, 5 mg of sodium ascorbate, 6 mg of penicillin and 10 mg of streptomycin/100 ml. The preincubation medium was then removed and replaced with the same medium further supplemented with 2.4 mCi of L-[4,5- ^3H]lysine/100 ml. Aortae were incubated at a ratio of 60 aortae/25 ml of medium at 37°C for 24 h. After incubation, the aortae were rinsed with water, freeze-dried and stored at -20°C until used. These aortae were prepared for use as substrate in assays by extracting twice with 0.15 M-NaCl, once with 1 M-HCl to inactivate endogenous enzyme (Kagan *et al.*, 1974), once with 0.1 M-sodium borate/0.15 M-NaCl, pH 8.0, and twice with 0.15 M-NaCl. Portions of the final aortic substrate suspension equivalent to 125 000 c.p.m. of ^3H were added to each assay tube. Assays included substrate (0.2–0.1 ml), enzyme and 0.1 M-sodium borate/0.15 M-NaCl, pH 8.0 (0.5 ml). The final assay volume was 0.75 ml. Reaction mixtures were incubated at 37°C with shaking for 2 h.

Reactions were stopped by cooling on ice, and $^3\text{H}_2\text{O}$ formed during the assay was distilled in a micro-distillation apparatus and cooled at -50°C. Portions (0.5 ml) of the distillates were added to 5 ml of Beckman Ready-Solv EP scintillation fluid in polyethylene scintillation vials and radioactivity was assayed in a Packard liquid-scintillation spectrometer. One unit of enzyme activity is defined as 1 c.p.m./ml of assay reaction mixture released in 2 h at 37°C. Enzyme-catalysed release of ^3H was linear with time and with enzyme concentration at activity levels which released not more than 1200 c.p.m. of ^3H in 2 h of assay. All activity data are corrected for the non-enzymic release of ^3H measured in enzyme-free reaction mixtures. All activities reported are fully inhibited by β -aminopropionitrile fumarate at 50 μg /ml of reaction mixture.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Dilute samples of enzyme were prepared for protein analyses by dialysis against water and freeze-drying. In some cases, protein samples in urea solutions were analysed by the Lowry *et al.* (1951) procedure, with bovine serum albumin in urea as a standard. The presence of urea did not significantly affect the colour yield in the Lowry *et al.* (1951) assay.

Preparation of collagen affinity columns

Citrate-soluble collagen was purified from the skins of 2.5–3 kg albino male rabbits by the procedure described for the purification of calf skin collagen by Gallop & Seifter (1963). The collagen was finally dialysed against water and stored as a freeze-dried product. Collagen was coupled to Sepharose CL-4B which had been previously activated by treatment with CNBr (Cuatrecasas, 1970). The degree of substitution of collagen on the Sepharose beads was established by the analysis of acid hydrolysates of the coupled Sepharose for hydroxyproline (Stegemann, 1958).

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in urea was performed by a modification of the method of Swank & Munkres (1971). Samples in 6 M-urea/0.016 M-potassium phosphate, pH 7.7, were concentrated by dialysis against 10% (v/v) poly(ethylene glycol) (mol.wt. 20 000) in 6 M-urea/0.016 M-phosphate, followed by dialysis into electrophoresis sample buffer containing 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol, 0.01 M-phosphoric acid, 10% (v/v) glycerol, 0.0025% Bromophenol Blue and 8 M-urea, adjusted to pH 6.8 with Tris base. The concentrated dialysed samples were heat-denatured at 100°C for 10 min and applied to tube gels composed of 7.5% acrylamide, 0.1%

sodium dodecyl sulphate, 8M-urea and 0.1M-Tris/phosphate buffer, pH6.8, with an acrylamide/methylenebisacrylamide ratio of 37:1 (w/w). The gels were electrophoresed at constant current at 4mA/gel for 4h and stained for 2h in Coomassie Brilliant Blue R-250 as described by Weber & Osborn (1969). Gels were destained in 10% methanol/7% acetic acid at 37°C. Gels were also stained for carbohydrate with the periodic acid/Schiff stain (Glossmann & Neville, 1971).

Amino acid analysis

Salts and urea were removed from protein samples by dialysis against deionized distilled water. Dialysed samples were freeze-dried in 16mm×125mm thick-walled glass ignition tubes. The dried protein residue was hydrolysed for 22h at 110°C in 1 ml of 5.85M-HCl after freezing, thawing, flushing twice with N₂ and sealing the tubes *in vacuo*. Oxidation of protein samples with performic acid was performed by the method of Hirs (1967) for the conversion of cysteine residues into cysteic acid residues before the acid hydrolysis of proteins. Hydrolysates were dried under a stream of N₂ gas and redissolved in 0.01M-HCl. Analyses were performed on these solutions on a Beckman model 119C amino acid analyser.

Purification of lysyl oxidase

The methods of Harris *et al.* (1974), Siegel & Fu (1976) and Stassen (1976) were adopted and modified in the procedure used in these studies for the isolation of lysyl oxidase from bovine aorta. Thoracic aortae of 2–6-week-old calves were cleaned of adhering tissue before extraction. Extraction and all subsequent procedures were carried out at 4°C. Aortae were obtained from a local slaughterhouse and were stored frozen for 1 to several weeks before extraction. The duration of storage did not affect the quantity of extractable enzyme or the overall results of the purification procedure. Cleaned aortae were finely ground by passage through a chilled electrically powered meat grinder. The ground aortae were homogenized by blending for 90s in 0.12M-NaCl/0.016M-potassium phosphate, pH7.7, at a ratio of 2.5ml of buffer/g of ground tissue. The homogenate was centrifuged at 15000g for 30min, and the pellet was extracted once more with the same buffer. The two buffered salt extracts contained negligible activity and were discarded. The resulting pellet obtained from the salt extractions was further extracted with 0.016M-potassium phosphate, and then with 4M-urea in this phosphate buffer. The urea-soluble material was decanted and kept and the pellet was extracted twice more with 4M-urea/0.016M-phosphate. The three urea extracts were pooled and diluted with an equal volume of 0.016M-potassium phosphate, pH7.7, to adjust the urea concentration to 2M. This solution was directly applied to a collagen-

Sephacrose CL-4B affinity column (13cm×5cm) and sequentially eluted at pH7.7 with 600ml of each of the following: 2M-urea/0.016M-phosphate; 0.016M-phosphate; 1M-NaCl/0.016M-phosphate; 0.016M-phosphate; 6M-urea/0.016M-phosphate. The enzyme activity was eluted in 6M-urea/0.016M-phosphate, pH7.7, and this solution was applied to a column (2.5cm×84cm) of DEAE-cellulose previously equilibrated in 0.016M-phosphate/6M-urea, pH7.7. The column was eluted with a linear gradient of NaCl (0–0.4M) in 6M-urea/0.016M-potassium phosphate, pH7.7; 12ml fractions were collected. The total volume of the gradient was 1500ml. Fractions containing enzyme activity were pooled as separate peaks. Enzymes were precipitated by the addition of solid (NH₄)₂SO₄ to 70% saturation. The precipitate was sedimented at 100000g for 30min. The pellets were suspended in 6M-urea/0.016M-phosphate buffer, and dialysed for 24h against 2 litres of this buffer. The dialysed solutions were clarified by centrifugation at 20000g for 30min and applied to a column (120cm×3cm) of Sephacryl S-200 previously equilibrated with 6M-urea/0.016M-phosphate, pH7.7. The column was eluted with this buffer.

Results

Effect of urea on enzyme activity

Since urea is a common component of the buffers used in the purification procedure, the effect of urea on the activity of lysyl oxidase was examined (Fig. 1).

Fig. 1. Effect of urea on the assay of lysyl oxidase activity. Urea was present in the assays (0.75 ml final volume) at the indicated concentrations. Assay mixtures were constituted and incubated as described in the Experimental section.

Table 1. Purification of lysyl oxidase on Sepharose CL-4B coupled with native or heat-denatured collagen

	$10^{-4} \times$ Total activity (units)	Total protein (mg)	$10^{-4} \times$ Specific activity (units/mg)	Activity recovered (%)	Purification (fold)	$10^{-3} \times$ Enzyme recovered (units/mg of collagen bound to Sepharose)
4M-Urea extract*	7.50	69	0.109	(100)	—	
6M-Urea eluates of affinity columns						
Native collagen	3.29	3.28	1.00	43.9	9.2	2.28
Denatured collagen	5.87	3.05	1.92	78.3	17.6	6.73
Ratio $\frac{\text{Denatured}}{\text{Native}}$	1.78	0.94	1.92	1.78	1.91	2.95

* The first 4M-urea extract of 4g of aortic tissue previously extracted with 0.012M-NaCl/0.016M-potassium phosphate, pH 7.7, was used in this experiment.

Fig. 2. Polyacrylamide-gel electrophoresis in 8M-urea and sodium dodecyl sulphate

Gel 1, from top to bottom, protein standards and their molecular weights are: bovine serum albumin, 67000; aldolase, 40000; carbonic anhydrase, 29000; lysozyme, 14000. Gel 2, crude 4M-urea extract of aorta. Gel 3, 6M-urea effluent of native-collagen affinity column. Gel 4, 6M-urea effluent of denatured-collagen affinity column. Lysyl oxidase would appear as a species slightly larger than carbonic anhydrase under these conditions. A faint band is seen in this position in gel 4. Proteins migrated toward the anode (+, bottom of gels, as pictured).

This inhibition plot was used to correct for the inhibition of lysyl oxidase activity caused by the presence of urea in assays of samples of enzyme taken from the various urea-containing buffers used in the purification procedure, thus obviating the necessity

for dialysis of enzyme into urea-free buffer before assays. The correction factors were found to pertain to partially purified and pure lysyl oxidase.

Affinity chromatography

An initial study was made of the relative effectiveness of native or denatured collagen as the ligand of choice for the affinity-column step of the purification procedure. Rabbit skin collagen intended as an affinity ligand in the native form was dissolved in 0.05% acetic acid at 2mg/ml. The collagen was maintained at 0–4°C before and during the coupling of collagen to the activated beads of Sepharose CL-4B. It was established that collagen was in its native conformation by comparing its c.d. spectrum recorded in a Cary model 61 CD spectrophotometer with that previously reported for native collagen (Piez & Sherman, 1970). Another sample of collagen was intentionally denatured by dissolving it in 0.05% acetic acid, adjusting the pH to 7 with 1M-NaOH, and heating at 90°C for 10min. The c.d. spectrum of this sample verified that the collagen had been fully denatured. Both solutions of collagen were quickly adjusted to pH 9.5 and added to separate equal amounts of activated Sepharose beads suspended in 0.1M-NaHCO₃, pH 9.5. Coupling was continued for 24h at 4°C. Each product was washed free of unbound collagen and poured into columns. An initial extract in 4M-urea/0.016M-potassium phosphate, pH 7.7, of the salt-extracted bovine aorta pellet was prepared as described in the Experimental section and diluted to a final urea concentration of 2M. Equal volumes of the diluted extract were passed into each affinity column and each column was eluted with a series of washing and eluting buffers described in the Experimental section. Enzyme was released from both collagen affinity columns by only the 6M-urea/0.016M-potassium phosphate elution buffer. The yields and specific activities of enzyme determined in this fraction are shown in Table 1.

The column coupled with denatured collagen yielded more enzyme activity per mg of Sepharose-

coupled collagen than did the column coupled with native collagen. Furthermore, the specific activity of the enzyme eluted from the denatured collagen ligand was higher than that eluted from the native collagen ligand. The crude extract and the two 6M-urea effluents were compared by polyacrylamide-gel electrophoresis (Fig. 2). Although both affinity-column effluents contain protein bands in common, there is less stainable protein in the sample eluted from the denatured collagen affinity column, particularly in the region of 40000–50000 mol.wt. These results led to the use of an affinity column coupled with heat-denatured rabbit skin collagen for the purification procedure.

The complete elution profile of an affinity column coupled with denatured collagen and loaded with the pool of three consecutive 4M-urea extracts of 200 g of bovine aorta is shown in Fig. 3. There is a considerable amount of material that does not bind to the column in 2M-urea. An additional class of proteins binds to the column, but is eluted with 1M-NaCl/0.016M-potassium phosphate, pH 7.7, and lysyl oxidase activity is eluted in the 6M-urea/0.016M-potassium phosphate effluent.

Chromatography on DEAE-cellulose

The enzyme eluted from the affinity column in 6M-urea was directly applied to a column of DEAE-

cellulose which had been equilibrated in 6M-urea/0.016M-phosphate, pH 7.7. The column was eluted with a linear gradient of NaCl in 6M-urea/0.016M-potassium phosphate. Four distinct peaks of enzyme activity were eluted under these conditions (Fig. 4a).

It is possible that the multiplicity of enzyme species resolved on DEAE-cellulose reflects the proteolytic conversion of a parent larger molecule into the multiple species either before or during the purification procedure. As one control for such possibilities, the proteinase inhibitor α -toluenesulphonyl fluoride phenylmethanesulphonyl fluoride) was incorporated into the purification procedure after trials had established that addition of this compound to assays at 0.001 M had no effect on lysyl oxidase activity of either crude or purified fractions. The inhibitor was added to a final concentration of 0.001 M to each salt extract buffer and to each 4M-urea extract buffer just before homogenization of the aortic tissue in each case. Otherwise the purification followed that detailed for the purification of enzyme described in this report. An aortic extract prepared in this fashion and passed through a denatured-collagen affinity column still yielded four peaks of enzyme activity on chromatography on DEAE-cellulose, as shown in Fig. 4(b). The final yields and specific activities of enzyme purified through the entire procedure were essentially the same regardless of

Fig. 3. *Chromatography of 4M-urea extract on a denatured-collagen-Sepharose CL-4B affinity column*
The first major A_{280} peak represents protein eluted in 2M-urea/0.016M-phosphate, pH 7.7. The second peak (fractions 152–164) was eluted with 1M-NaCl/0.016M-phosphate, pH 7.7. The third peak, containing both A_{280} and enzyme activity (fractions 218–240), was eluted with 6M-urea/0.016M-phosphate, pH 7.7. Fraction volume was 15 ml. —, A_{280} ; □, enzyme activity.

Fig. 4. Chromatography of 6M-urea effluent on affinity column on DEAE-cellulose. Extraction was performed in the absence (a) or presence (b) of 0.001 M- α -toluenesulphonyl fluoride. ----, Enzyme activity; —, A_{280} ; •, salt gradient. The preparation in the absence of α -toluenesulphonyl fluoride began with 250g of tissue. The volume of each fraction was 5 ml.

Fig. 5. Chromatography of lysyl oxidase on Sepharose 4B in 0.12M-NaCl/0.016M-potassium phosphate, pH 7.7. Approximate molecular weights shown were determined by calibrating the column with bovine serum albumin (67000), catalase (250000), ferritin (440000) and glutamate dehydrogenase (1015000).

Fig. 6. Chromatography of DEAE-cellulose peaks I-IV on Sephacryl S-200 in 6M-urea/0.016M-potassium, pH7.7. Abbreviations used to identify elution positions of standards are: v, void volume; a, bovine serum albumin; o, ovalbumin; c, carbonic anhydrase; cy, cytochrome c. Fraction size was 4ml. Roman numerals refer to peaks of activity resolved on DEAE-cellulose, as labelled in Fig. 4. ----, Enzyme activity; —, A_{280} .

the presence or absence of the proteinase inhibitor during the extraction procedure. Therefore α -toluenesulphonyl fluoride was omitted from the purification scheme for routine preparation of enzyme.

Gel exclusion chromatography of lysyl oxidase

The absorption profile of the eluate of the DEAE-cellulose column (Fig. 4a) suggests that the enzyme peaks are significantly contaminated with other proteins at this stage, a likelihood verified by polyacrylamide-gel electrophoresis. In consideration of gel exclusion chromatography as a subsequent purification step, the behaviour of enzyme on gel exclusion media was tested in the presence and absence of 6M-urea. Enzyme was eluted at the void volume of a calibrated column of Sephadex G-100 in 0.12M-NaCl/0.016M-potassium phosphate, pH7.7, consist-

ent with a mol.wt. ≥ 100000 . In contrast, enzyme was eluted from the same column of G-100 with an approx. mol.wt. of 30000 when the column was equilibrated and eluted with 6M-urea/0.012M-NaCl/0.016M-potassium phosphate, pH7.7. The large aggregate of enzyme formed in the absence of urea can be resolved further if enzyme is eluted from a column of Sepharose 4B in 0.12M-NaCl/0.016M-potassium phosphate, pH7.7 (Fig. 5). This chromatographic profile indicates that enzyme polymerizes in the absence of urea into a series of multimeric states which are detectable by their catalytic activity.

Gel exclusion chromatography in 6M-urea was applied to the further purification of the enzyme. Each peak of enzyme activity eluted from DEAE-cellulose was separately concentrated and passed through a column of Sephacryl S-200 in 6M-urea/0.016M-potassium phosphate, pH7.7. As shown in

Table 2. *Purification of four species of lysyl oxidase from bovine aorta*

Purification began with 200g of bovine aorta. The specific activity of the 4M-urea extract (7530 units/mg) exceeds that of the experiment described in Table 1 (1090 units/mg), since three consecutive urea extracts of the salt-extracted aorta pellet were pooled in the present experiment and only the first 4M-urea extract was used in the study of Table 1. The secondary and tertiary urea extracts of aorta have considerably higher specific activities than the primary urea extract.

Purification step	$10^{-6} \times$ Total activity (units)	Total protein (mg)	$10^{-5} \times$ Specific activity (units/mg)	Yield (%)	Purification (fold)
4M-Urea extract	11.7	1554	0.075	100	1
Affinity chromatography	8.75	160	0.547	74.7	7.3
DEAE-cellulose chromatography					
Peak I	3.02	30.4	0.994	25.8	13.2
Peak II	1.59	35.8	0.443	13.5	5.9
Peak III	1.03	16.4	0.627	8.8	8.3
Peak IV	0.41	7.4	0.557	3.5	7.4
(Sum of four peaks)	(6.05)	(90.0)		(51.6)	
Sephacryl S-200 chromatography					
Peak I	0.49	0.54	11.0	4.2	146
Peak II	0.30	0.39	6.9	2.3	92
Peak III	0.48	0.67	12.2	4.1	162
Peak IV	0.26	0.37	6.0	2.2	80
(Sum of four peaks)	(1.54)	(1.97)		(12.8)

Fig. 7. Polyacrylamide-gel electrophoresis of four purified species of lysyl oxidase in 8M-urea and sodium dodecyl sulphate

Standards stained in the first gel (left) from top to bottom are bovine serum albumin, aldolase, carbonic anhydrase and lysozyme. Roman numerals refer to resolved enzyme species. Proteins migrated from the top of the gels to the bottom, as pictured, toward the anode (+).

Fig. 6, enzyme in DEAE-cellulose peaks I, II, III or IV was eluted at a position approximating to that of carbonic anhydrase (29000 mol.wt.) in this buffer. Further, the chromatographic profiles suggest that purity is achieved for each of the enzyme species by this procedure.

Summary of the purification procedure

A summary of the purification procedure is presented in Table 2. The purification described involved a denatured-collagen affinity column and was carried out in the absence of α -toluenesulphonyl fluoride. Crude and partially purified enzyme fractions lost 30–50% of their activity over periods of 2–3 weeks of storage at 4°C. This rate of loss of activity was markedly decreased if the enzyme was protected from light both during storage and during the purification procedure. The reproducibility and absolute values of the final specific activities and yields of product were improved by purifying the enzyme with as much protection from light as possible.

Polyacrylamide-gel electrophoresis of the isolated enzymes

Polyacrylamide-gel electrophoresis of the isolated enzyme species purified through the Sephacryl procedure was performed in sodium dodecyl sulphate and 8M-urea, as described in the Experimental section. As shown (Fig. 7), each peak of enzyme yields an apparently homogeneous somewhat diffuse band

under these conditions. The molecular weights of the enzyme species were determined by comparison of their electrophoretic mobilities in sodium dodecyl sulphate and 8M-urea to those of standard proteins. The standards used and their molecular weights are as follows: albumin, 67000; catalase, 57600; ovalbumin, 43000; aldolase, 40000; carbonic anhydrase, 29000; chymotrypsinogen, 25500; lysozyme, 14000. The linear plot relating the logarithm of the molecular weights of the standards to the electrophoretic migration distance yielded molecular weights for the enzyme from peaks I, II, III and IV of 32100 ± 1000 , 33100 ± 1000 , 32600 ± 900 and 31700 ± 400 respectively. These values represent averages of duplicate electrophoretic analyses and approximate to the values expected on the basis of the elution positions of each species on the calibrated Sephacryl column (Fig. 6). Polyacrylamide-gel electrophoresis of enzyme isolated with α -toluenesulphonyl fluoride present in the extracting buffers as described yielded molecular weights which are not readily distinguishable from those obtained in the absence of proteinase inhibitor. Thus peak II has mol.wts. of 33100 ± 900 and 32100 ± 800 when isolated in the presence or absence of α -toluenesulphonyl fluoride respectively. Similarly, peak III enzyme has mol.wts. of 32000 ± 900 and 33200 ± 150 when isolated in the presence or absence of the proteinase inhibitor respectively.

Analyses for carbohydrate content

The four purified species of lysyl oxidase were

tested for carbohydrate content by applying the periodic acid/Schiff stain to polyacrylamide gels of each enzyme form after electrophoresis in 8M-urea and sodium dodecyl sulphate and removal of sodium dodecyl sulphate from the gels (Glossmann & Neville, 1971). None of the four species gave a positive reaction in this procedure, in comparison with the positive reaction obtained with ovalbumin at equivalent concentrations of protein. Similarly, concanavalin A coupled to agarose, which serves as an affinity adsorbent for glycoproteins containing α -D-glucopyranosyl or α -D-mannopyranosyl residues (Lis & Sharon, 1973), did not bind any of the enzyme in a crude 4M-urea extract of aorta after dialysis of the extract into 0.016M-potassium phosphate buffer, pH 7.7, containing 0.1 mM concentrations of CaCl_2 , MgCl_2 and MnCl_2 . The column (3 ml volume, containing 47 mg of bound concanavalin A) was equilibrated and eluted with this urea-free buffer. More than 90% of the applied enzyme activity was found in the initial eluate of the column, which is consistent with a lack of binding of enzyme to the affinity ligand. Further, no additional enzyme activity was freed from the column by elution with 1M-NaCl or with 6M-urea.

Amino acid analysis

The amino acid compositions of the four enzymes of bovine aorta are shown in Table 3. The amino acid composition of the species of chick-embryo

Table 3. *Amino acid analyses of purified lysyl oxidase species*

Values presented for each bovine enzyme species are the averages of analyses of duplicate samples of protein acid-hydrolysed for 22h at 110°C. Duplicates were within 6% of each other. Tryptophan was not determined. Cysteine was analysed as cysteic acid in separate samples oxidized with performic acid as described in the Experimental section.

Content (residues/1000 residues)

Residue	Peak I	Peak II	Peak III	Peak IV	Chick-embryo cartilage enzyme*
Aspartic acid	123.1	125	121.5	94.3	136
Threonine	56.7	55	55.7	47.5	53
Serine	103.7	86.3	103.7	121.6	82
Glutamic acid	112.7	135.7	135.8	124.1	106
Proline	59.8	51.1	49.8	50.6	58
Glycine	120.3	86.8	107.7	176.8	97
Alanine	71.1	80.7	82.8	85.4	66
Valine	39	42.3	34.7	31.7	39
Cysteine	26.9	24	18.3	14.3	30
Methionine	15.3	16.1	14.6	16.9	15
Isoleucine	30	27.2	30.6	28.5	40
Leucine	64.3	86.1	78.3	64.7	67
Tyrosine	25	31.4	21.4	17.8	65
Phenylalanine	27.2	30.3	25.9	24.8	27
Lysine	30.8	35.8	35.6	28.5	31
Histidine	38.6	25.4	27.1	22.7	29
Arginine	55.5	60.5	56.3	49.3	59

* Data of Stassen (1976).

Table 4. *Inhibition of lysyl oxidase species by chelating and carbonyl reagents*

Values in the Table are the averages of duplicate or triplicate assays with maximal variation from the average values of $\pm 8\%$. The average radioactivity (c.p.m.) released in 2h of assay above enzyme-free controls in the absence of added inhibitors was 664, 533, 565 and 290 for peaks I, II, III and IV respectively. Each inhibitor was present in the assay at 0.001 M.

Inhibitor	Inhibition (%)			
	Peak I	Peak II	Peak III	Peak IV
$\alpha\alpha'$ -Bipyridine	21	19	21	30
Sodium diethyl-dithiocarbamate	55	66	61	73
NaCN	82	87	91	89
Semicarbazide	98	98	99	96
Isoniazid	63	69	69	74

cartilage lysyl oxidase previously reported by Stassen (1976) is included for comparison. The compositions of the enzymes of bovine aorta are very similar to each other, although sufficient differences exist which support the conclusion that they are distinct molecular species. Each has a considerable excess of dicarboxylic amino acids over basic amino acids, indicating that these proteins are acidic, which is consistent with their affinity for DEAE-cellulose. The amide content of these proteins was not determined. In addition to the acidic amino acids, each is relatively enriched in serine and glycine. The composition of the chick-embryo cartilage enzyme resembles the compositions of the bovine enzymes in several respects. However, the chick enzyme has a significantly higher content of tyrosine than the bovine enzymes.

Effect of inhibitors on enzyme activity

Lysyl oxidase has been shown to be a Cu^{2+} -dependent amine oxidase (Siegel *et al.*, 1970; Harris *et al.*, 1974). Nutritional studies (Chou *et al.*, 1970; Rucker & O'Dell, 1970) and inhibition patterns (Kagan *et al.*, 1974) suggest that the enzyme also contains a catalytically functional cofactor with carbonyl reactivity. Recent evidence indicates that a form of vitamin B-6 is incorporated into cartilage lysyl oxidase in chick embryos (Murray & Levene, 1977), suggesting that a form of pyridoxal may be the source of carbonyl reactivity. The four species of aortic enzyme isolated in the present study were tested with a series of chelating agents and with compounds reactive with carbonyl groups (Table 4). As shown, each species is similarly inhibited by $\alpha\alpha'$ -bipyridine, NaCN and diethyldithiocarbamate, all potentially reactive with Cu^{2+} . Similarly, semicarbazide and isoniazid inhibit each enzyme to

similar extents, suggesting carbonyl reactivity in each species.

Discussion

The present report describes a method for the purification to apparent homogeneity of four species of lysyl oxidase from bovine aorta. The use of gel exclusion chromatography in urea after the elution of the four peaks of activity from DEAE-cellulose appears to be a convenient and effective final step for achieving purity of each peak. The use of denatured collagen as an affinity ligand and protection of enzyme against exposure to light also are important factors in the purification method described in this study.

Multiple forms of lysyl oxidase have now been found in bovine aorta, lung (Shieh & Yasunobu, 1976), ligament (Jordan *et al.*, 1977) and chick-embryo cartilage (Stassen, 1976), and thus this seems to be characteristic of this enzyme activity in connective tissues. The present study reveals that the multiple peaks of activity have very similar subunit molecular weights. Further, there are strong similarities in the amino acid compositions of the isolated enzymes, although their amino acid compositions are not identical. The chemical similarity of the four peaks of enzyme suggests the possibility that proteolysis of a common parent molecule could be the basis of their formation. If this is true, the degree of proteolysis is not revealed by significant differences in molecular weight, nor is it prevented by incorporation into the preparation of α -toluenesulphonyl fluoride, an inhibitor of proteolytic enzymes with functional serine residues. It was also noted in these experiments that 4M-urea extracts which had been aged for several days at 4°C or processed immediately after extraction yielded identical multiplicity of peaks on DEAE-cellulose. Thus, if proteolysis is the basis of heterogeneity, it is more likely that it occurs *in vivo*, possibly as an important processing event in the biosynthesis and/or secretion of lysyl oxidase. It is also possible that other post-translational events influence the results obtained, although the present studies tend to exclude different degrees of glycosylation as bases for the differences noted.

It is possible that amino groups of the enzyme become carbamoylated during the preparation of the enzyme, since the purification is carried out in urea solutions. Nevertheless, precautions against this were taken by using freshly deionized urea throughout this procedure. It also seems unlikely that random carbamoylation of a parent species of enzyme would result in the consistency of patterns obtained on DEAE-cellulose from preparation to preparation.

Therefore the present results indicate that the difference between the multiple species is reflected both in their elution from DEAE-cellulose and in

their amino acid compositions, even though they generally seem related to each other. The molecular weights of the four species are within 1400–1500 of each other, and, if they do differ in size, the differences are subtle. Further studies on the primary structure of each isolated species should clarify the relationship between these enzymes, and additional studies on their biosyntheses are required to establish whether the multiple species each have independent genomic origin or derive from a common molecule by post-translational processing.

Stassen (1976) has reported that the four species of enzyme of chick-embryo cartilage each oxidize both elastin and collagen substrates. However, it is possible that lysines in different environments within each of these connective-tissue proteins may require enzyme species of different specificities. Furthermore it is conceivable that multimers of lysyl oxidase composed of various mixtures of the individual subunit species might exist *in vivo* which could have unique substrate specificities.

The differential effect of native and denatured collagen as affinity ligands not only has significance for the purification procedure, but also indicates that lysyl oxidase binds quite strongly to both states of collagen. This is of interest in light of the finding of Siegel (1974) that the chick cartilage enzyme has a marked preference for the native insoluble fibrillar state of collagen as a substrate and only poorly oxidizes denatured α -chains. The gels of the 6M-urea effluents of the affinity columns illustrate that there are proteins in aorta which have differential binding affinities for the two forms of collagen, thus contributing to the result that the specific activity of the enzyme eluate is higher on elution from the affinity column containing the denatured collagen ligand. However, the principal difference between the two columns is seen in the greater yield of enzyme activity in the eluate of the column coupled with denatured collagen. This result has obvious pragmatic importance to the efficiency of the purification, although the basis of this effect requires investigation. These results could be explained by differential binding affinities of enzyme for the two states of collagen or by the simultaneous elution from the native collagen of lysyl oxidase together with an enzyme inhibitor.

The tendency of the enzyme to polymerize to a series of multimeric states, many of which are quite large, would appear to be inconsistent with the presumptive extracellular function of this enzyme. The study described in the present paper was carried out with a partially purified mixture of the four species of the enzyme. However, preliminary studies indicate that the isolated species of enzyme undergo similar degrees of polymerization under analogous conditions (K. Sullivan & H. Kagan, unpublished work). Thus, although it is possible that the poly-

merization of the partially purified enzyme may reflect interaction with collagen molecules, the tendency of purified enzyme to polymerize indicates that this is an intrinsic property of lysyl oxidase in the absence of urea. It seems therefore that a biological mechanism may exist which maintains enzyme in a limited state of aggregation for optimal solubility, interaction with substrate, and catalytic production of α -amino adipic semialdehyde in elastin and collagen of the extracellular milieu.

In summary, multiple species of lysyl oxidase can be purified by the procedure detailed in the present paper. The multiple species of enzyme are quite similar, although sufficient differences do exist to raise further questions about the potential physiological significance of apparently heterogeneous enzyme species of this enzyme. The possibility that the multiple species may function as homopolymers or heteropolymers is of interest.

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