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PROPERTIES OF HIGHLY PURIFIED LYSYL OXIDASE FROM EMBRYONIC CHICK CARTILAGE

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Summary

Lysyl oxidase the enzyme which oxidately deaminates lysine residues in collagen and elastin, was purified from embryonic chick cartilage by employing an affinity column of lathyritic rat skin collagen coupled to Sepharose, followed by separation on DEAE-cellulose. An enzyme preparation was obtained which was pure as shown by polyacrylamide gel electrophoresis. The specific activity was 1800-fold higher than that of the original extract. The pure enzyme utilized both collagen and elastin substrate. Furthermore, the ratios of enzyme activity with elastin substrate versus that with collagen substrate were the same at all stages of purity. Only one protein band was found after polyacrylamide gel electrophoresis of the pure lysyl oxidase in sodium dodecyl sulfate and mercaptoethanol. The molecular weight was estimated to be 28 000. It was found that the enzyme contained a large number of cysteine and tyrosine residues.

Evidence was obtained for molecular heterogeneity of lysyl oxidase. The enzyme eluted from DEAE-cellulose in at least four distinct regions. When the peaks were rechromatographed separately, they eluted at salt concentrations similar to those of the original chromatogram. However, the substrate specificity and the electrophoretic mobility on polyacrylamide gel were the same for all enzyme fractions.

Introduction

The tensile strength of connective tissue is largely determined by the number of intermolecular cross-links in collagen and elastin. In both proteins the cross-links occur between lysine residues of different peptide chains [1—5]. However, elastin and collagen are proteins with quite different properties and the amino acid sequences in which deaminated lysines (and hydroxylysines) occur

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in collagen [1,20—23] are unlike those in elastin [4,5]. The initial step in the cross-linking process is the oxidative deamination of peptide bound lysine to α-aminoadipic-δ-semialdehyde [1], a reaction catalyzed by the enzyme lysyl oxidase [6]. The question arose whether the deamination of both substrates could be attributed to a single enzyme or to the existence or more than one enzyme. Preliminary evidence [7—9] has been obtained using partially purified enzyme preparations which supports the hypothesis that lysine residues in both collagen and elastin can be deaminated by the same enzyme. Furthermore, the extraction medium employed in these studies [7—9] was shown to extract only a small portion of the total amount of lysyl oxidase present in the tissue. Therefore, it is possible that one molecular form of lysyl oxidase had been extracted selectively. The data reported in this paper indicate that highly purified lysyl oxidase from embryonic chick cartilage can utilize both elastin and collagen as substrate. In addition, evidence was obtained for the existence of different molecular forms of lysyl oxidase.

Materials and Methods

β-Aminopropionitrile hydrochloride was purchased from Calbiochem and β-aminopropionitrile fumarate from Aldrich Chemical Co. Inc. DL-[6-^{3}H] Lysine and L-[4,5-^{3}H] lysine were obtained from New England Nuclear. The Spex freezer/mill was purchased from Spex Industries Inc. (Metuchen, N.J.). Elastase (ESFF) was purchased from Worthington Biochemical Corp. and collagenase (Form III) from Advance Biofactures Corp. (Lynbrook, N.Y.). Dulbecco-Vogt's medium minus lysine and minus glutamine was prepared by Grand Island Biological Co. Penicillin G was obtained from E.R. Squibb and Sons, Inc. Lathyritic rat skin collagen was extracted with 0.5 M acetic acid and purified by the method of Gross [10]. The purified collagen was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the procedure of Cuatrecasas [11]. DEAE-cellulose (Whatman DE52) was prepared as recommended by the manufacturer and equilibrated with 6 M urea in 0.05 M Tris · HCl (pH adjusted to 7.5 at 4° C).

Elastin substrate was prepared from aortas of 16-day-old chick embryos [6, 7] by using β-aminopropionitrile to inhibit the activity of the endogenous lysyl oxidase. The aortas (18 per flask) were preincubated for 30 min at 37° C in lysine-free Dulbecco-Vogt's medium (10 ml per flask) containing β-aminopropionitrile hydrochloride (50 μg/ml), sodium ascorbate (50 μg/ml), penicillin G (2000 units/ml), and glutamine (2 mM). Subsequently, the medium was replaced by 15 ml of fresh medium plus 250 μCi DL-[6-^{3}H] lysine or L-[4,5-^{3}H] lysine * per flask and the aortas were incubated at 37° C for 24 h. The labeled aortas were rinsed with water and lyophilized. The dry aortas were powdered in the liquid nitrogen-cooled Spex freezer/mill for 3 min. The powder was sus-

* Either [6-^{3}H] lysine- or [4,5-$^3H_2$] lysine-labeled substrate can be used in the tritium-release assay for lysyl oxidase, because as soon as aldehyde forms at position 6, tritium at position 5 should become readily exchangeable [24]. This finding [7] was confirmed during this study.

pended in cold 0.1 M sodium phosphate (pH 7.8) containing 0.15 M NaCl (phosphate-buffered saline) by sonicating for 3 × 1 min at 50 W on a Biosonik IV instrument equipped with a needle probe 3 mm in diameter. The suspension was centrifuged at 18 000 × *g* for 10 min and the pellet was extracted twice with 0.5 M HCl and then twice with phosphate-buffered saline. Finally, the pellet was resuspended in phosphate-buffered saline (0.5 ml per aorta) and the substrate suspension was stored frozen.

Collagen substrate was prepared from calvaria of 16-day-old chick embryos [12]. β-Aminopropionitrile was used to inhibit the activity of the endogenous lysyl oxidase. The calvaria (20 bones per flask) were preincubated for 30 min and then incubated for 24 h with DL-[6-^{3}H]lysine or L-[4,5-^{3}H]lysine (see footnote) in the presence of β-aminopropionitrile as was described above for the preparation of the elastin substrate. The labeled calvaria were rinsed with water and lyophilized. The dry calvaria were powdered in the liquid nitrogen-cooled Spex freezer/mill for 3 min. The powder was extracted with 1.0 M NaCl in 0.05 M Tris · HCl (pH 7.5) for 18 h at 4°C. The extract was centrifuged at 28 000 × *g* for 10 min. The collagen was precipitated from the supernatant by the addition of solid NaCl to a concentration of 20%. The precipitated collagen was redissolved in phosphate-buffered saline (1 ml per 25 bones). The solution was extensively dialyzed against phosphate-buffered saline and finally stored frozen. The substrate preparation contained about 85% collagen, because 85% of the radioactivity could be solubilized with collagenase.

Extraction and purification of lysyl oxidase. Lysyl oxidase has been extracted and purified by a modification of methods previously reported by Narayanan et al. [8] and Siegel [9]. All procedures were carried out at 4°C. The cartilagenous ends of the tibias and femurs of 16-day-old chick embryos (100 g) were homogenized in 500 ml of 4.0 M urea in 0.05 M Tris · HCl (pH adjusted to 7.5 at 4°C) with a Sorvall omnimixer. The pellet obtained after centrifugation at 28 000 × *g* for 10 min was extracted with another 500 ml of 4 M urea in 0.05 M Tris · HCl (pH 7.5). The supernatants of the first and second extract were combined and centrifuged at 148 000 × *g* for 1 h. The 148 000 × *g* supernatant was filtered through glass wool. After the addition of one volume of phosphate-buffered saline, the crude extract was pumped onto an affinity column (2.6 × 25 cm) of lathyritic rat skin collagen coupled to Sepharose 4B [13] which had been equilibrated with phosphate-buffered saline. The column was then washed with phosphate-buffered saline followed by 1.0 M NaCl in 0.05 M Tris · HCl (pH 7.5), and 1 M urea in 0.0125 M Tris · HCl (pH adjusted to 7.5 at 4°C); finally the lysyl oxidase was eluted from the column with 6 M urea in 0.05 M Tris · HCl (pH 7.5). The 6 M urea eluate was applied to a DEAE-cellulose column (1.6 × 30 cm) which was equilibrated at 4°C with 6 M urea in 0.05 M Tris · HCl (pH 7.5). The column was developed at a flow rate of 55 ml/h with a 600 ml linear gradient from 0.0 to 0.3 M NaCl in 0.05 M Tris · HCl (pH 7.5) containing 6 M urea. Fractions of 14 ml were collected. When fractions containing lysyl oxidase were to be rechromatographed, they were dialyzed against 6 M urea in 0.05 M Tris · HCl (pH 7.5) to remove the NaCl, and then applied to another DEAE-cellulose column. Before the fractions were assayed for lysyl oxidase activity, the urea was removed by dialysis against phosphate-buffered saline.

Assay of lysyl oxidase activity using labeled elastin. The enzyme activity was determined by the tritium release method described by Pinnell and Martin [6] and Siegel et al. [7]. The incubation mixture contained 0.5 ml of the labeled aorta suspension (one aorta; about $2 \cdot 10^6$ cpm), enzyme solution and phosphate-buffered saline up to a final volume of 2.0 ml. The incubation was carried out at 37°C for 2 h. The reaction was stopped by the addition of 0.2 ml 50% trichloroacetic acid, and after distillation 1.6 ml of the water was counted. The concentration of the elastin substrate was lower than the concentration required for substrate saturation. Therefore, for each experiment a standard curve was prepared which employed purified enzyme.

Assay of lysyl oxidase activity using labeled collagen. The enzyme activity was determined by the tritium release method of Siegel [9]. The labeled collagen substrate (0.2 ml containing about 0.1—0.2 mg collagen and approx. $2 \cdot 10^6$ cpm) was preincubated at 37°C for 1 h to promote fiber formation, a prerequisite for enzyme activity. Subsequently, warm phosphate-buffered saline and enzyme solution were added to a final volume of 2 ml. The incubation was continued for another 2 h. The reaction was stopped by the addition of 0.2 ml 50% trichloroacetic acid, and after distillation 1.6 ml of the water was counted. The concentration of the collagen substrate was lower than the concentration required for substrate saturation. Therefore, for each experiment a standard curve was prepared which employed purified enzyme.

Analytical gel electrophoresis was carried out as described by Davis [14] using 7.5% acrylamide gels, after the enzyme fractions were concentrated by ultrafiltration on PM 10 filters (Amicon Corporation). Prior to electrophoresis the urea was removed by dialysis against phosphate-buffered saline. After electrophoresis, the gels were either stained with Coomassie blue or they were frozen. The frozen gels were cut into 1 mm slices and the slices were extracted with phosphate-buffered saline at 0°C for 2 h. The extracts were assayed for lysyl oxidase activity.

Analytical gel electrophoresis on sodium dodecyl sulfate acrylamide gels. Sodium dodecyl sulfate gels (10%) were prepared according to the method of Laemmli [15]. The sample and the marker proteins were heated at 100°C for 2 min in 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. The molecular weights assumed for the marker proteins were: bovine serum albumin, 67 000; ovalbumin, 45 000; chymotrypsinogen, 25 000; hemoglobin, 16 000; and ribonuclease, 13 700. The electrophoresis was carried out on a vertical slab gel electrophoresis cell from BioRad Laboratories. The gels were stained with Coomassie blue.

Amino acid analysis. Hydrolysis was carried out under N_2 in 6 M HCl at 110°C for 24 h and the amino acid composition was determined by Dr. S. Stein using fluorescamine which has recently been applied to the automated determination of amino acids [16].

Protein concentration was determined by the method of Lowry et al. [17] with bovine serum albumin as the standard. The amount of protein was also determined by calculation from the values obtained by amino acid analysis.

Hydroxyproline was determined after hydrolysis of the protein sample in 6 M HCl at 100°C for 24 h according to the procedure of Peterkofsky and Prockop [18].

Results

Extraction and purification of lysyl oxidase. More than 90% of the lysyl oxidase extractable from embryonic chick cartilage with 4 M urea in 0.05 M Tris · HCl (pH 7.5) was present in the first two extracts. There was no loss of enzyme activity from the 28 000 × *g* supernatant upon centrifugation at 148 000 × *g*. Under the conditions used, all the enzyme activity was removed from the 148 000 × *g* supernatant (diluted with one volume phosphate-buffered saline) by passing the extract through an affinity column of collagen coupled to Sepharose (Fig. 1). The column was washed with 1 M NaCl and then with 1 M urea to remove the salt. A small amount of protein was eluted with NaCl, but no lysyl oxidase activity was detected in the NaCl or in the urea eluates. Lysyl oxidase was eluted with 6 M urea in 0.05 M Tris · HCl (pH 7.5). The enzyme in this affinity eluate had been purified 200-fold and 94% of the activity of the crude extract was recovered (Table I). Based upon the determination of hydroxyproline, no collagen could by detected in a concentrated affinity eluate of lysyl oxidase. The affinity column could be reused after the urea was removed by washing with phosphate-buffered saline.

The affinity eluate of lysyl oxidase was chromatographed on DEAE-cellulose in 0.05 M Tris · HCl (pH 7.5) containing 6 M urea (Fig. 2A). Lysyl oxidase ac-

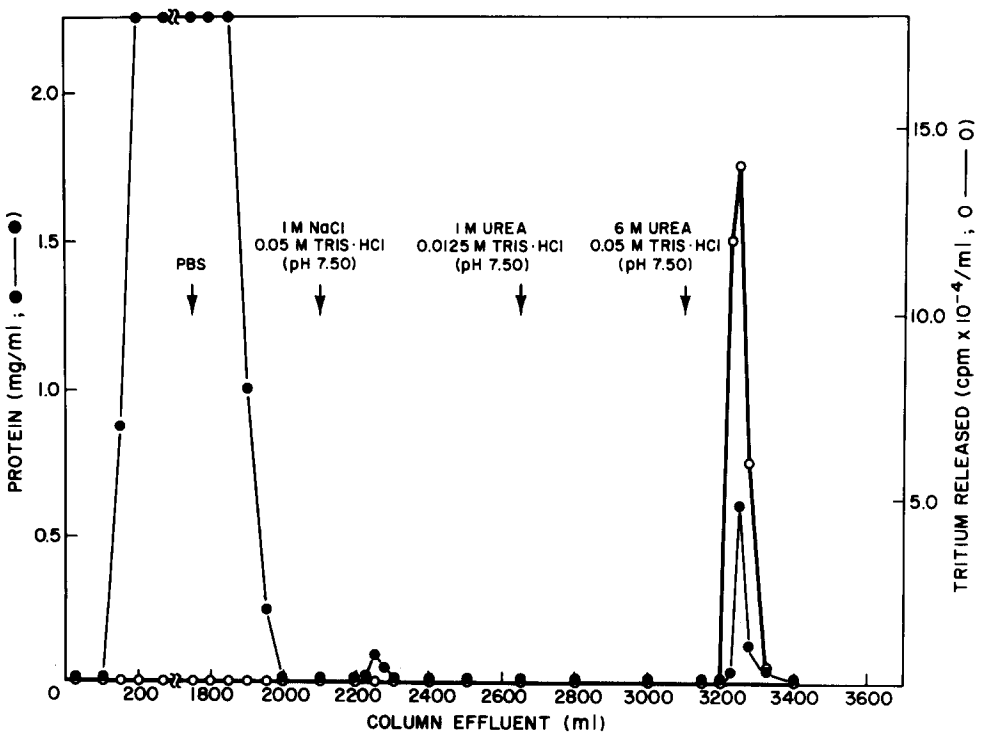

Fig. 1. Purification of lysyl oxidase by affinity chromatography. The amount of protein and the lysyl oxidase activity were determined on aliquots which had been dialyzed against phosphate-buffered saline. The lysyl oxidase activity was determined with L-[4,5-$^3H_2$]lysine-labeled elastin as substrate. The other experimental details are described in Materials and Methods.

TABLE I

PURIFICATION OF LYSYL OXIDASE FROM EMBRYONIC CHICK CARTILAGE

The urea was removed by dialysis against phosphate-buffered saline. Both elastin and collagen were labeled with L-[4,5-^{3}H]lysine.

Fraction	Lysyl oxidase activity				Ratio elastin/collagen
	Specific activity (cpm × 10^{-3}/mg protein)		Elastin substrate		
	Elastin substrate	Collagen substrate	Recovery (%)	Purification factor	
Crude extract (140 000 × *g*)	2.5	0.9	100	1	2.67
Affinity extract	507.5	205.6	94	203	2.47
Peak b	1979.0	717.0	7	792	2.76
Peak c	2941.0	1392.0	24	1176	2.11
Peak d	4533.0	1633.0	11	1813	2.78

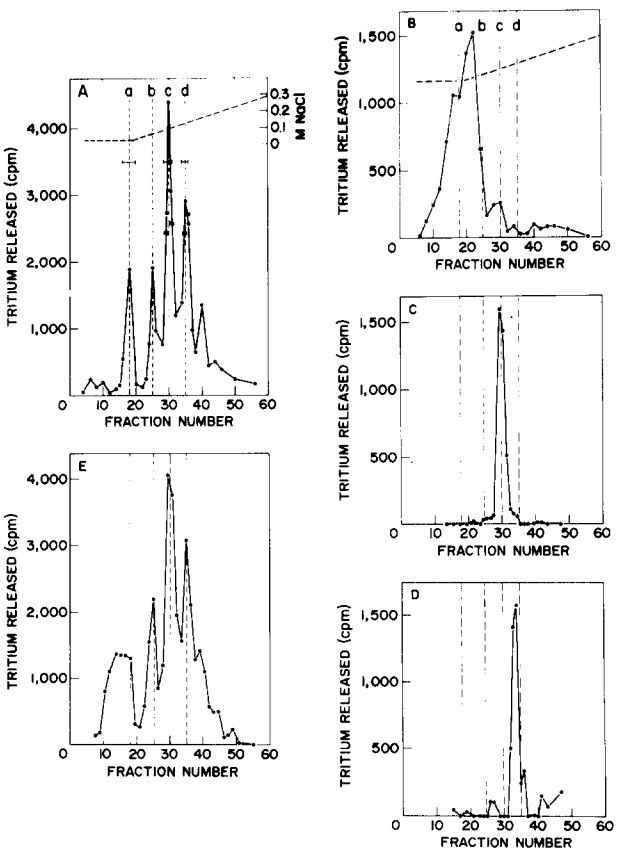

Fig. 2. Ion-exchange chromatography of lysyl oxidase extracts on DEAE-cellulose. A, lysyl oxidase eluate from the affinity column shown in Fig. 1; B, C and D show the elution profile after re-chromatography of the peaks a, c and d of frame A, respectively; E, the 148 000 × *g* supernatant of a cartilage extract in buffered 6 M urea. The lysyl oxidase activity was determined with L-[4,5-$^3H_2$]lysine-labeled elastin as substrate. The other experimental details are described in Materials and Methods.

tivity eluted in at least four distinct regions, marked peaks a, b, c and d. Peak d contained only one protein band after electrophoresis on polyacrylamide gel (Fig. 3). On an unstained gel the lysyl oxidase activity was found at a relative electrophoretic mobility identical to that of the protein band (Fig. 4). The

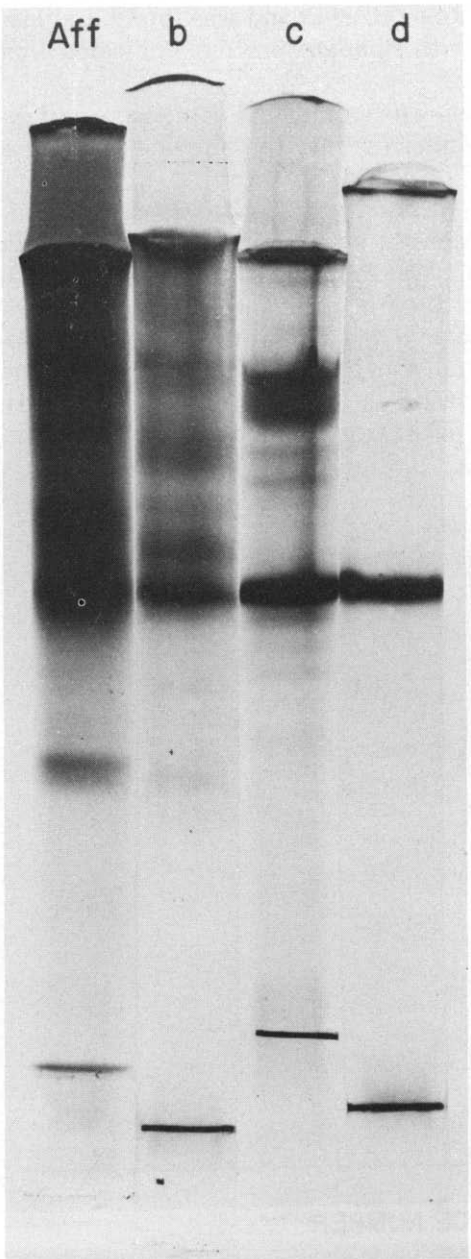

Fig. 3. Polyacrylamide gel electrophoresis of lysyl oxidase extracts. Aff: lysyl oxidase eluate from the affinity column shown in Fig. 1 (60 μg of protein); b, c and d represent the different peaks as shown in Fig. 2A (20 μg of protein each).

specific activity of the electrophoretically pure enzyme was more than 1800-fold higher than the specific activity of the crude extract; the pure enzyme accounted for 11% of the original enzyme activity (Table I).

Molecular weight determination by electrophoresis on sodium dodecyl sulfate-acrylamide gels. The pure enzyme (peak d) was treated with sodium dodecyl sulfate and mercaptoethanol and then examined by acrylamide electrophoresis in sodium dodecyl sulfate. Only one protein band was found. Calibration of the sodium dodecyl sulfate gels with standard proteins indicated that the molecular weight was about 28 000.

Amino acid composition of lysyl oxidase. The amino acid composition of lysyl oxidase is shown in Table II. It is of interest that the enzyme contains a large amount of cysteine and tyrosine.

Molecular heterogeneity and substrate specificity of lysyl oxidase. The different peaks of lysyl oxidase activity found with elastin as substrate after DEAE-cellulose chromatography (Fig. 2A) could represent lysyl oxidases with different substrate specificities. Therefore, the fractions were also assayed with collagen as substrate. It was found that the activities determined with both substrates were superimposable. This is illustrated in Table I which shows that the ratio of the lysyl oxidase activity with elastin as substrate versus the activity with collagen is the same for the different peaks from the ion-exchange column,

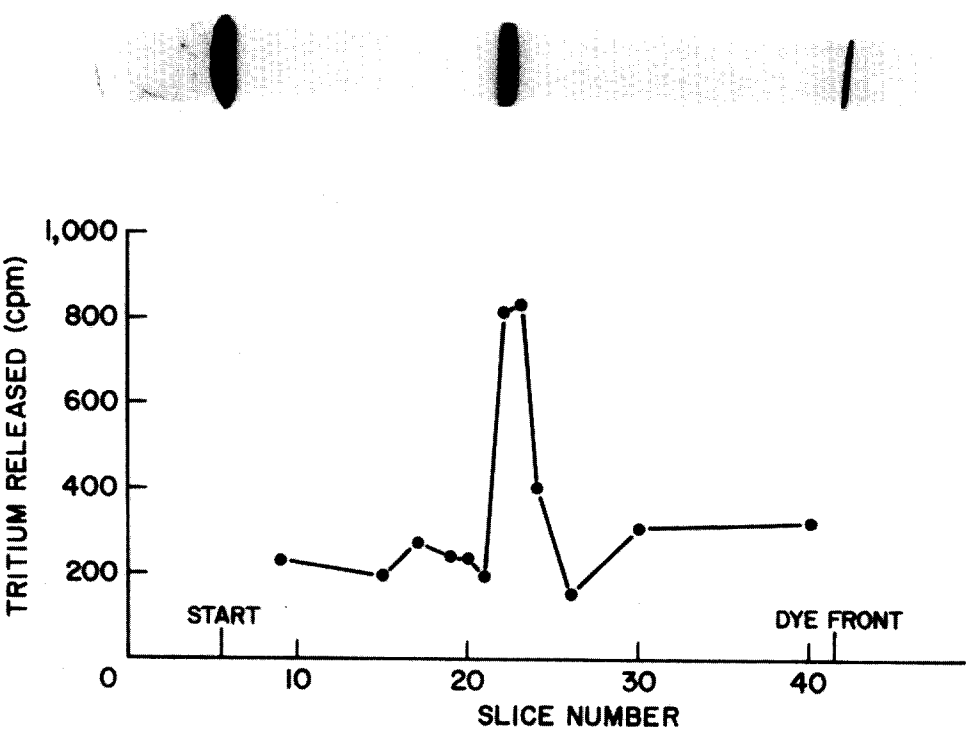

Fig. 4. Location of lysyl oxidase activity on a polyacrylamide gel after electrophoresis. Samples of peak d (Fig. 2A) were applied to duplicate gels. One gel was stained with Coomassie blue, while the other one was cut into 1 mm slices. Phosphate-buffered saline extracts of these slices were assayed for lysyl oxidase activity using L-[4,5-$^{3}H_2$]lysine-labeled elastin as substrate.

TABLE II

AMINO ACID COMPOSITION OF LYSYL OXIDASE FROM EMBRYONIC CHICK CARTILAGE

Values are the mean of duplicate analyses carried out with fluorescamine on hydrolysates in HCl of peak d. The individual values did not differ from the mean by more than 5%.

Amino acid	Amino acid residues/1000
Lys	31
His	29
Arg	59
Asx	136
Thr	53
Ser	82
Glx	106
Pro	58
Gly	97
Ala	66
Cys	30
Val	39
Met	15
Ile	40
Leu	67
Tyr	65
Phe	27

the affinity eluate and the crude extract. The data presented in Table III confirm the findings of Narayanan et al. [19] that the substrate activity of the suspension of labeled aortas is indeed mainly due to elastin and not to collagen. Only 12% of the substrate activity was lost after extensive treatment with collagenase, while all the substrate activity was abolished by digesting the substrate with elastase. The collagen substrate contained about 85% pure collagen, because 85% of the radioactivity could be solubilized with collagenase. In addition, when the different lysyl oxidase fractions were examined by acrylamide gel electrophoresis (Fig. 3) and also by sodium dodecyl sulfate-acrylamide gel electrophoresis, each sample, including that from the affinity eluate, contained the same protein band which had been identified as lysyl oxidase (Fig. 4).

TABLE III

EFFECT OF COLLAGENASE AND ELASTASE DIGESTION ON THE SUBSTRATE ACTIVITY OF LABELED AORTAS FROM EMBRYONIC CHICKS

The L-[4,5-$^3H_2$]lysine-labeled elastin substrate was treated with collagenase at 37°C for 2 h (300 units per 5 aortas [25]), or with elastase at 37°C for 1 h (10 units per 5 aortas [26]). After digestion, the suspensions were washed with phosphate-buffered saline and incubated with a standard amount of purified lysyl oxidase.

Treatment	Amount of tritium-labeled protein solubilized (%)	Amount of tritium released after incubation with lysyl oxidase (%)
Without collagenase	4.3	100
With collagenase	7.8	88
Without elastase	9.1	100
With elastase	87.3	0

The different molecular forms of lysyl oxidase were found not only when affinity eluates were chromatographed on DEAE-cellulose, but also after DEAE-cellulose chromatography of crude tissue extracts (Fig. 2E). Furthermore, when peaks a, c and d (Fig. 2A) were re-chromatographed individually (Figs. 2B, 2C and 2D, respectively), they eluted at salt concentrations similar to those of the original chromatogram (Fig. 2A).

Kinetic investigation of the lysyl oxidase reaction with elastin and collagen as substrates. The release of tritium from the elastin substrate by purified lysyl oxidase (peak c; Fig. 2A) was linear with time up to 2 h. The double reciprocal plot of the velocity of the lysyl oxidase reaction as a function of the substrate concentration was linear and the apparent K_m was found to be equal to the amount of elastin in 1.3 aortas per incubation mixture (final volume of 2 ml). This finding indicates that the assay of lysyl oxidase activity with elastin as substrate as reported by other workers [6,8,9] was not being carried out at a saturating substrate concentration (only the elastin of one aorta was used per 1.5 ml incubation mixture).

After the collagen substrate was preincubated for 1 h at 37°C, the release of tritium by purified lysyl oxidase (peak c; Fig. 2A) was linear with time up to 6 h. The double reciprocal plot of the velocity of the lysyl oxidase reaction as a function of the substrate concentration was linear and the apparent K_m was $2.4 \cdot 10^{-6}$ M (calculated using a value of 300 000 as the molecular weight of collagen). The apparent K_m value is in good agreement with that reported earlier [9].

Discussion

Lysyl oxidase oxidatively deaminates specific lysine residues in elastin, and lysine and hydroxylysine residues in the non-helical amino and carboxy termini of collagen. However, elastin and collagen are quite different proteins and the amino acid sequences in which the deaminated lysines (and hydroxylysines) occur in collagen [1,20—23] are unlike those in elastin [4,5]. Therefore, it was of interest to determine whether both proteins were substrates for the same enzyme or whether there was more than one form of lysyl oxidase present in the enzyme preparations. This study was made possible by the recent findings that lysyl oxidase is remarkably stable in concentrated solutions of urea and that the enzyme can be purified by ion-exchange and affinity chromatography using this solvent [8]. Furthermore, Siegel [9] demonstrated that collagen can only be a substrate for lysyl oxidase after fiber formation has occurred and he described the conditions under which collagen can be used as substrate in a rapid and quantitative assay of lysyl oxidase activity.

Electrophoretically pure lysyl oxidase, which was obtained after affinity and ion-exchange chromatography of a urea extract from embryonic chick cartilage, was shown to oxidatively deaminate lysine in both a collagen and an elastin substrate. Therefore, it appears that the same enzyme can use both elastin and collagen as substrate. It was also found that the ratio of the lysyl oxidase activity determined with elastin as substrate versus the activity with collagen as substrate was the same for the enzyme preparation at all stages of purity. This indicates that the crude urea extract from embryonic chick cartilage contains

only one type of lysyl oxidase. These findings, however, do not exclude the possibility that other tissues contain lysyl oxidases with different substrate specificities.

Lysyl oxidase appeared to be remarkably stable in urea solutions. This property indicates special structural features that allow easy renaturation of the enzyme molecule. Disulfide bonds could contribute to the stability of the molecule. Lysyl oxidase contains a sufficient number of half cysteines to allow for the existence of several disulfide bonds between half cysteines of the same protein chain or between cysteines of different subunits.

The finding that lysyl oxidase was eluted from the DEAE-cellulose column in at least four distinct regions in a highly reproducible way indicated the existence of different molecular forms of lysyl oxidase. It should be noted that the multiple peaks were obtained from embryonic chick cartilage after ion-exchange chromatography when either a crude urea extract or an urea extract purified by affinity chromatography was applied. In contrast, other authors reported only one peak of lysyl oxidase activity after DEAE-cellulose chromatography [8,9]. The fact that these investigators did not resolve more peaks could be due to their use of a much steeper salt gradient than that described in this paper. Another explanation is that only one molecular form of lysyl oxidase was extracted from the tissue by phosphate-buffered saline, the solvent employed by these workers, while the urea used in this report also extracted less soluble forms of the enzyme. It seems unlikely that the lysyl oxidase peaks obtained by ion-exchange chromatography represent different enzyme-substrate complexes, because collagen could not be detected in the affinity eluate of lysyl oxidase. The urea extract may contain different aggregates consisting of enzyme molecules or enzyme subunits. This hypothesis is supported by the finding that on polyacrylamide gel electrophoresis the lysyl oxidase activity of all enzyme fractions was found in the same protein band. In addition, after electrophoresis on sodium dodecyl sulfate-acrylamide gel, a protein band with a molecular weight of 28 000 was observed in all enzyme fractions. Since the possibility exists that the different molecular forms are generated by the extraction procedure itself, the physiological significance should be determined.

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