# A PARTIAL CHARACTERIZATION OF LYSYL OXIDASE FROM LIGAMENTUM NUCHAE

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ABSTRACT

Lysyl oxidase from bovine ligamentum nuchae has been purified by two different methods. The first method utilized urea extraction, ion exchange chromatography, and affinity chromatography. The second method consisted of urea extraction followed by two successive runs on a Sephadex G-100 column. The different extracts were compared with respect to specific activity, copper values, and chelation effects. Significantly different results were obtained which tend to suggest the possibility of two different forms of the lysyl oxidase enzyme.

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## INTRODUCTION

The lysyl oxidase enzyme is responsible for the oxidation of specific lysyl residues of elastin and collagen.(1) The enzyme is thought to catalyze the first step in elastin and collagen formation with subsequent steps occuring spontaneously by Schiff base reactions or aldol condensations.(2) Also evidence has been shown that copper is an integral part of the enzyme.(3) Lysyl oxidase activity is present in vivo wherever elastin and collagen synthesis is taking place.

Connective tissue in cartilage, skin, and blood vessels have the highest amounts of lysyl oxidase activity. These tissues have been used extensively as an enzyme source for isolation and purification of lysyl oxidase. Ligamentum nuchae from cattle have one of the highest concentrations of elastin known. The ligament, being mostly extra-cellular tissue, is approximately seventy-five per cent elastin. Therefore, this would be an excellent choice for enzyme isolation.

Substantial purification methods have been reported in chick aorta using urea buffers in combination with ion exchange and affinity chromatography.(4) With the use of these purification methods, lysyl oxidase can be purified from bovine ligamentum nuchae and characterized.

## MATERIALS AND METHODS

The first step in enzyme isolation was urea extraction from tissue. Fifty grams of ligament were cleaned of fascia and homogenized in 150 ml of 0.15M NaCl-0.1M $KH_2PO_4$, pH 7.7 at 4C. All subsequent steps were carried out at 4C. The mixture was centrifuged at 17,500g for 20 min. The pellot was resuspended in an additional 150 ml of 0.15M NaCl-0.1M $KH_2PO_4$, pH 7.7 and centrifuged at 17,500g for 20 min. The pellot was allowed to soak overnight in 150 ml of 6M Urea-0.015M NaCl-0.01M $KH_2PO_4$, pH 8.3. The mixture was centrifuged at 17,500g for 30 min and the supernatent was collected. The remaining pellot was suspended in an additional 150 ml of 6M Urea-0.015M NaCl-0.01M $KH_2PO_4$, pH 8.3 and recentrifuged at 17,500g for 30 min. The supernatents were combined and filtered through glass wool to remove lipid components. The supernatents were dialyzed against 2 liters of Buffer A, 0.12M NaCl-0.015M $KH_2PO_4$, pH 7.5 with three changes of buffer over a two-day period. This dialyzed supernatent was designated as the urea extract. The urea extract was centrifuged at 17,500g for 30 min. to remove precipitated collagen.

The first method of purification was by ion-exchange and affinity chromatography. Thirty-six grams of DE-11 cellulose were washed in 1N NaOH and 1N HCl ending up with acid as the final wash. The cellulose was then washed several timed with distilled, de-ionized water. The column(2.5cm high X 7cm wide) was equilibrated with Buffer A. The dialyzed urea extract was applied to the column. This, followed by 100 mls of Buffer A. Next, 100 mls of 0.52M NaCl-0.015M $KH_2PO_4$, pH 7.2 was added to the column. Buffer A was then put on the column until $A_{279}$

fell below 0.01. Finally, 100 mls of 6MUrea-0.015M NaCl-0.01M $KH_2PO_4$, pH 8.3 was applied to the column to elute lysyl oxidase. This urea fraction was collected and dialyzed against 2 liters of Buffer A with three changes over a two-day period. This was designated as the DEAE extract.

Sixty mls of DEAE extract were applied to a Sepharose 4-B affinity gel prepared by method of Harris.(4) The gel was washed with 60 mls of Buffer A and then followed by 0.52M NaCl-0.015M $KH_2PO_4$, pH 7.2. Buffer A was again applied until $A_{279}$ fell below 0.01. The lysyl oxidase was separated from the gel by addition of 6M Urea-0.12M NaCl-0.015M $KH_2PO_4$, pH 8.3. The urea fraction was collected and dialyzed against 2 liters of Buffer A with three changes over a two-day period. This was designated as the affinity extract.

The second method of purification was by Sephadex chromatography. Thirty grams of Sephadex G-100 was swelled in distilled, de-ionized water overnight. The gel was washed three times with 0.1N HCl followed by washing with distilled, de-ionized water. The gel was poured as a slurry into a 2.5cm X 40cm column with the column buffer, 6M Urea-0.12M NaCl-0.05M TRIS, pH 7.8. Meanwhile, the urea extract was lypholized and resuspended in 5 ml of column buffer. This was applied to the column and fractions were collected in acid-washed tubes. The peak lysyl oxidase activity fractions were combined and lypholyzed. The material was resuspended in 2.5 ml of column buffer and put through the column a second time. Peak lysyl oxidase activity fractions were once again combined. These were dialyzed against 2 liters of Buffer A with three changes over a two-day period.

The assay for lysyl oxidase consists of measuring the amount of tritiated water released from a substrate labelled with 6-$H^3$lysine. An insoluable substrate isolated from chick aorta and prepared by the method of Harris(4) was used for activity determinations. The assay consisted of adding to a culture tube; 0.1ml substrate, 0.2ml enzyme sample, and 0.7ml Buffer A. One drop of toluene was added to inhibit bacterial growth. The tubes were incubated at 40C for 4 hrs. The samples were then vaccuum-distilled to collect tritiated water formed. 0.2ml of recovered water were added to 5ml of Aquasol and counted on a liquid scintillation counter. Enzyme activity units were CPM of released $H_2^3O$ per ml of enzyme sample. Also, protein values for enzyme samples were determined by the method of Lowry.(5)

The chelation experiments were done on both the affinity extract and the second Sephadex extract. All chelating agents used; dithiothreitol, dithizone, bathocuproine, and sodium diethyldithiocarbamate, were made to a concentration of $10^{-4}$ M in Buffer A. Three ml of enzyme sample were dialyzed against 1 liter of chelating agent in Buffer A. The samples were then dialyzed against 1 liter of Buffer A with three changes over a two-day period. Copper values were determined by atomic absorbance spectrophotometry.

## RESULTS

The two purification methods have roughly the same final specific activity, with the ion exchange-affinity procedure having a slightly higher value. The specific activity in the Sephadex method actually decreased after the first run through the column. This indicated a loss

of enzyme activity. The specific activity in the ion exchange-affinity method increased throughout purification. (See Table I)

Table I. Purification Results

| Sample | Protein* | Activity** | %Cu | Spec. Act. |
|---|---|---|---|---|
| (1st method) | | | | |
| Urea extract | 400 | 3751 | - | 9 |
| DEAE extract | 150 | 1810 | 0.04 | 12 |
| Aff. extract | 28 | 4800 | 0.43 | 171 |
| (2nd method) | | | | |
| Urea extract | 597 | 5350 | - | 6 |
| 1st Seph. run | 447 | 1570 | - | 4 |
| 2nd Seph. run | 22 | 3400 | 0.07 | 154 |

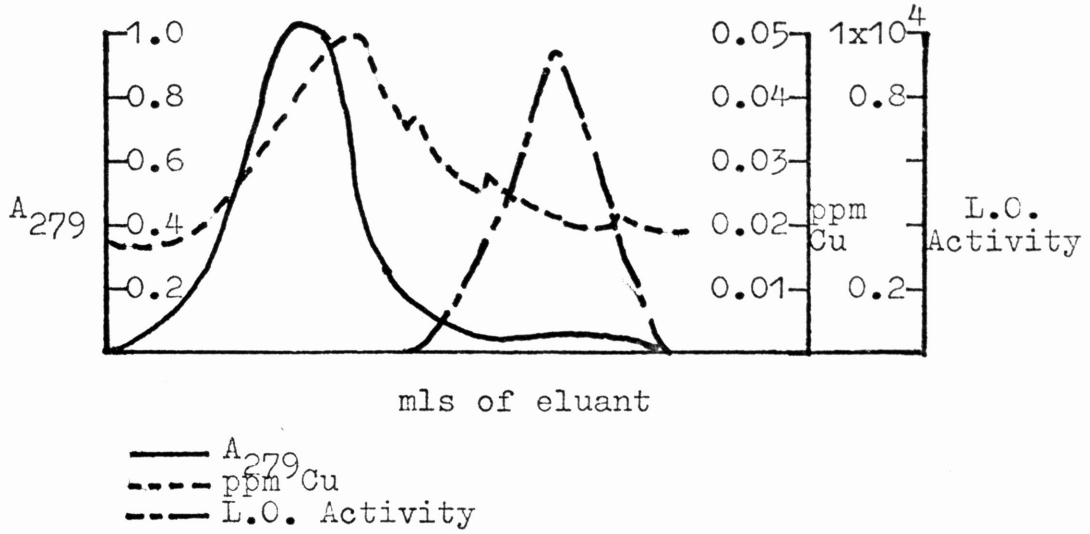
* protein measured in micrograms per ml
** activity measured in CPM of tritiated $H_2^3O$ released per ml of sample

The final extracts of both purification methods were completely inhibited by $10^{-4}$M BAPN.

In the ion exchange-affinity method, the per cent copper value was observed to increase as purity increased. This was not observed in the Sephadex method. However, in the Sephadex method, a graph comparing protein profile, copper profile, and lysyl oxidase activity profile shows the highest copper value to be associated with the protein peak and not the lysyl oxidase activity peak.(See Fig. 1) In fact, the copper value through the lysyl oxidase activity peak falls almost to the background level.

The most startling results have been produced by the chelation experiments.(See Table II) When the affinity extract was exposed to $10^{-4}$M dithiothreitol in Buffer A, the sample lost all enzyme activity and the copper value was decreased by one-half. But when the second pooled Sephadex fractions were exposed to the same chelator,

Figure 1. Sephadex Elution Profile




100% of lysyl oxidase activity was recovered. Also the per cent copper level of the pooled fractions was unaffected and remained at its previous low level. When other chelators were tested on the pooled Sephadex fractions it was found that none of them significantly decreased the per cent copper value. Moreover, a substantial amount of lysyl oxidase activity was recovered.

Table II. Chelations Results

| Chelating Agent | %Cu before | %Cu after | %Recovered Act. |
|---|---|---|---|
| Dithiothreitol* | 0.46 | 0.23 | 0 |
| Dithiothreitol | 0.07 | 0.07 | 100 |
| Bathocuproine | 0.07 | 0.07 | 66 |
| Diethyldithiocarbamate | 0.07 | 0.06 | 77 |
| Dithizone | 0.07 | 0.07 | 75 |

*enzyme source of this test was affinity extract, while all others were from 2nd pooled Sephadex fractions.

## DISCUSSION

Since the final extracts from the two methods of purification release tritiated water in the lysyl oxidase assay and are inhibited by BAPN, the requirement for lysyl oxidase activity is satisfied. However, the per cent copper values of the two final extracts are quite different. This would suggest the possibility of two forms of the lysyl oxidase enzyme. The possibility becomes more realistic when completely different results appear in the dithiothreitol treatment of the samples. Dithiothreitol completely destroys activity in the affinity extract, but has no effect on the pooled Sephadex extract. The preceding results strongly suggest two different enzyme forms.

A theory has been proposed by E. D. Harris that not only do two different forms of the enzyme exist, but they may actually be two different enzymes responsible for different steps in collagen and elastin formation. The two enzymes, or forms of enzymes, are probably present in the affinity extract. But in the final Sephadex extract, only the copper-independent form is present. The other form seems to have lost activity. This is very probable since only one-tenth of the lysyl oxidase activity applied to the Sephadex column was recovered. The copper-dependent lysyl oxidase seems to have been inactivated during the purification. The overall implications of these findings are that the spontaneous reactions occuring after the oxidation of lysine in elastin and collagen synthesis may actually have an enzyme responsible for the "spontaneous" step.

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