Biochimica et Biophysica Acta, 377 (1975) 229—238

BBA 67417

A PURIFICATION PROCEDURE FOR THE ISOLATION OF HOMOGENEOUS PREPARATIONS OF BOVINE AORTA AMINE OXIDASE AND A STUDY OF ITS LYSYL OXIDASE ACTIVITY

JAMES JII SHIEH, RANDOLPH TAMAYE and KERRY T. YASUNOBU*

Department of Biochemistry-Biophysics, University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.)

(Received September 5th, 1974)

Summary

It has been reported that bovine aorta amine oxidase oxidizes lysine residues in tropoelastin to allysine (Rucker, R.B. and O'Dell, B.L. (1971) Biochim. Biophys. Acta 235, 32—43). Pure bovine aorta amine oxidase was isolated by DEAE-cellulose, hydroxylapatite, Bio-Gel A-1.5 m and concanavalin A-Sepharose 4B chromatography. Enzymatic, chromatographic and immunochemical tests disclosed that pure bovine aorta amine oxidase was not a lysyl oxidase capable of oxidizing the lysine residues of tropoelastin to allysine. The bovine aorta amine oxidase preparation used by Rucker and O'Dell appears to have been contaminated with lysyl oxidase which is the enzyme that oxidizes some of the lysine residues in tropoelastin and tropocollagen to allysine.

Introduction

The biochemistry of the aorta is being actively investigated due to the current emphasis on cardiovascular diseases. A key structural protein present in the aorta is elastin and the great elasticity of the aorta is due to the presence of this structural protein [1,2]. However, the elastin is first biosynthesized in a precursor form, tropoelastin [3,4]. A copper-protein, lysyl oxidase [5], then oxidizes certain lysine residues in tropoelastin to allysine. The allysine then undergoes condensation to form cross-links such as desmosine [6,7] by some unknown mechanism.

Two different types of lysyl oxidase have been reported to be present in the aorta. One type was reported to be similar to the plasma amine oxidase [8] while the other type did not oxidize benzylamine, a standard substrate for amine

* To whom reprint requests should be addressed.

oxidase [9,10]. In the present investigation, pure preparations of bovine aorta oxidase were required for the investigation of the lysyl oxidase activity of the enzyme. A procedure was developed for the isolation of the pure enzyme and various experimental data were obtained to show the aorta amine oxidase was not a lysyl oxidase as reported.

Materials and Methods

Bovine aorta

Fresh bovine aorta was collected at the local abattoir, defatted and then frozen until needed. Benzylamine was purchased from the Sigma Chemical Co. Lysyl-vasopressin was purchased from Calbiochem. The remainder of peptides used in the investigation were obtained from Sigma Chem. Co. or were donated by Dr J. Stewart. Concanavalin A and Sepharose 4B were purchased from Pharmacia. Bovine plasma amine oxidase was purified as previously reported by this laboratory [11]. Highly purified chicken tropoelatin was a gift of Dr R. Rucker. [6-^{3}H]Lysine (42.74 Ci/mM) was purchased from New England Nuclear.

Partially purified bovine aorta lysyl oxidase was isolated by the procedure of Harris et al. [9]. The enzyme had been purified through the tropoelastin-Sepharose 4B affinity chromatography step.

Enzyme assay

The spectrophotometric assay of Tabor et al. [12], in which the oxidation of bezylamine is followed at 250 nm, was used to assay the enzyme. A unit of enzyme activity is the amount of enzyme which causes an absorbance change of 0.001 per min and specific activity is defined as the units per mg of enzyme. Lysyl oxidase activity was measured by the procedure of Pinnell and Martin [5]. [6-^{3}H]Lysine was incorporated into tropoelastin by the tissue culture procedure described by these workers. For the lysyl oxidase assay, the substrate in each tube contained about 100 000 cpm.

The relative rates of oxidation of various lysine derivatives by aorta amine oxidase was assayed as follows. The reaction mixtures contained 1.0 μmol of lysine derivative, 0.06 mg *o*-dianisidine, 0.1 mg peroxidase, 0.20 μmol of sodium phosphate buffer, pH 7.4 and 33 units of lysyl oxidase or plasma amine oxidase in a final volume of 3 ml. The rate of reaction was measured at 460 nm and 25 °C. This coupled amine oxidase-peroxidase assay was developed by McEwen et al. [13].

Protein determination

Protein concentrations of solutions of the aorta amine oxidase were determined spectrophotometrically, the $E^{1\%}_{1\,cm}$ value of 20.8 nm was assumed. Otherwise, the protein concentration was determined by the biuret procedure of Gornall et al. [14].

Molecular weight estimation

The molecular weight of the enzyme was estimated by molecular seiving on a 1.2 cm × 116 cm column of Biogel A-1.5 m by the procedure described

by Whitaker [15]. Standard proteins used to calibrate the column were bovine heart cytochrome *c*, bovine serum albumin, alcohol dehydrogenase, catalase and blue dextran.

Preparation of affinity gels

Concanavalin A-Sepharose 4B and tropoelastin-Sepharose 4B affinity supports were prepared by coupling 80 mg of concanavalin A with 30 ml of Sepharose 4B and 10 mg of highly purified chicken tropoelastin with 10 ml of Sepharose 4B at pH 9.0 as described by Cuatrecasas and Anfinsen [16].

Disc gel electrophoresis

About 100 μg of enzyme was tested by polyacrylamide gel electrophoresis as described by David [17]. Electrophoresis was carried out in a solution of glycine and diethylbarbiturate at pH 7.0 with a 7.5% total polyacrylamide gel. On completion of the electrophoresis run, the gel was strained with a solution of Coomassie Blue and destained in a methanol/acetic acid solution. The enzyme, 8 mg per ml in 0.1 M sodium phosphate buffer, pH 7.4, was also analyzed in the Beckman-Spinco Model E analytical ultracentrifuge at 20 °C and 59 870 rev/min.

Results

Purification procedure

All operations were carried out at 4 °C.

(a) Preparation of the homogenate. About 6000 g of cleaned and rinsed aorta was passed through a meat grinder. The minced aorta was then added to two volumes of 0.03 M sodium phosphate buffer solution, pH 7.4. The suspension was then homogenized in a Waring blender for 5 intervals, each of one min duration. The suspension was then centrifuged for 12 min at 16 000 × *g*. The precipitate was discarded and 9550 ml of supernatant was obtained. About 23 779 units of enzyme with specific activity of 0.32 was found in the supernatant solution.

(b) Fractionation with $(NH_4)_2SO_4$. The supernatant solution from the previous step was fractionated with ammonium sulfate and the protein precipitating between 40 and 60% saturation was collected. (A saturated solution was taken as 760 g of $(NH_4)_2SO_4$ per l). The precipitate was dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, and was found to contain 13 350 units of enzyme with a specific activity of 1.06.

(c) Chromatography on DEAE-cellulose. The enzyme was placed on a column of DEAE-cellulose (3 l bed volume) previously equilibrated with 0.01 M sodium phosphate buffer, pH 7.4. The column was washed with about 3.5 l of the same buffer followed by about 3.5 l of 0.03 M sodium phosphate buffer, pH 7.4. The enzyme which eluted from the column with 0.07 M sodium phosphate buffer, pH 7.4 was collected. The elution pattern of the enzyme is shown in Fig. 1. The enzyme extract was then brought to 40 and 60% saturation with respect to the ammonium sulfate concentration. The resulting precipitate was dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, and was found to contain 9835 units with a specific activity of 15.4.

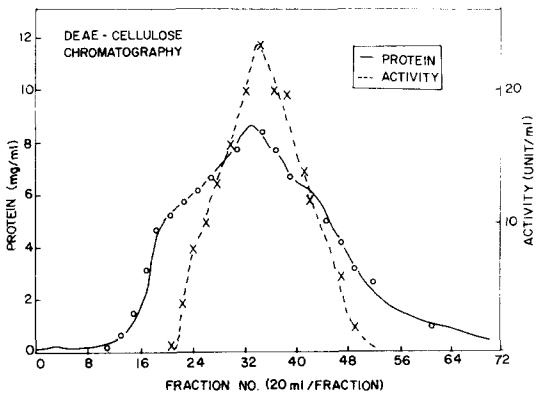

Fig. 1. DEAE-cellulose chromatography of bovine aorta amine oxidase. The enzyme, 12.71 g containing 13 350 units, was added to a column of 3 l bed-volume after it had been purified by ammonium sulfate fractionation. Fractions eluted by 0.07 M sodium phosphate buffer, pH 7.4 were pooled.

(d) Chromatography on hydroxylapatite. The enzyme from the previous step was applied to a 2.5 cm × 15 cm hydroxylapatite column [18] equilibrated with 0.01 M sodium phosphate buffer. The elution was followed by 0.01, 0.3 and 0.07 M sodium phosphate buffer, pH 7.4. The elution pattern is shown in Fig. 2. The fractions eluted by 0.03 and 0.07 M buffer which contained most of the activity was fractionated with ammonium sulfate and the protein precipitating between 40 and 60% ammonium sulfate saturation was collected. The precipitate was then dialyzed against 0.07 M sodium phosphate buffer, pH 7.4, and it contained 5760 units with a specific activity of 32.1.

(e) Chromatography on Bio-Gel. The sample was then chromatographed on a 1.5 cm × 90 cm Bio-gel A 1.5-column previously equilibrated with 0.07 M sodium phosphate buffer, pH 7.4. The eluate obtained with 0.07 M buffer was collected and precipitated with 70% ammonium sulfate (Fig. 3). The precipitate after dialysis against 0.1 M sodium phosphate buffer, pH 7.4, contained 4554 units and the specific activity of the enzyme was 109.

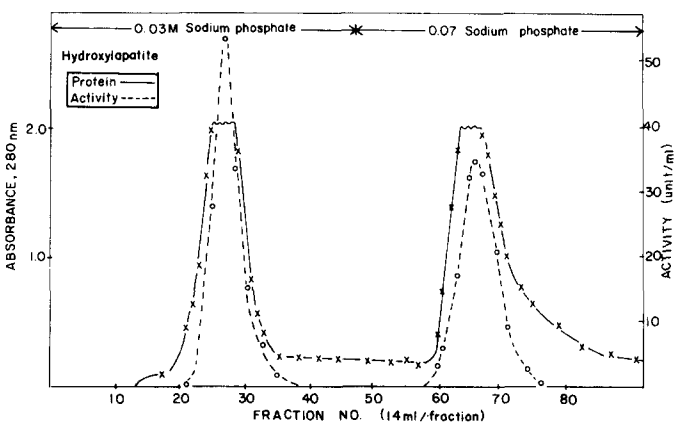

Fig. 2. Hydroxylapatite chromatography of purified aorta amine oxidase. The enzyme, 637 mg containing 9835 units, was applied to a column (4 cm × 25 cm) and developed stepwise with 0.01, 0.03, 0.07 and 0.15 M sodium phosphate buffer, pH 7.4. Fractions eluted by 0.03 and 0.07 M buffer were pooled.

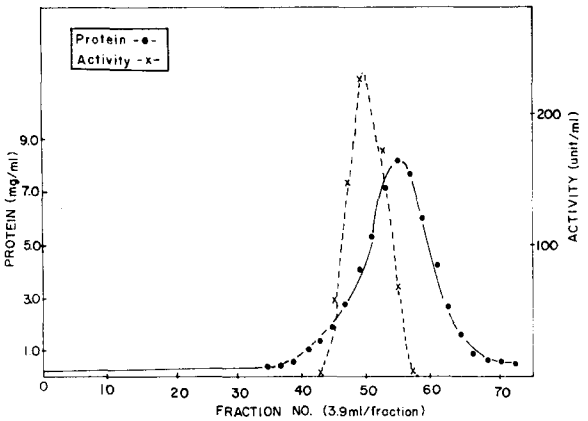

Fig. 3. Bio-gel chromatography of purified aorta amine oxidase. The enzyme (179 mg, 5760 units) was applied to a column (1.5 cm × 90 cm) and eluted with 0.07 M sodium phosphate buffer, pH 7.4.

(f) Chromatography on concanavalin A-Sepharose 4B. The resulting enzyme from previous step was applied to a 1.2 cm × 25 cm column of concanavalin A-Sepharose 4B which had been equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. Following the removal of unbound protein, the column was eluted with the same buffer containing 0.1 M α-methyl-D-glucoside (Fig. 4). The eluate was pooled and precipitated with 70% ammonium sulfate. The precipitate was dissolved in 0.1 M sodium phosphate, pH 7.4, and was then dialyzed against the same buffer. About 1335 units of enzyme with a specific activity of 267 was isolated. However, preparations with a specific activity of 318 have been isolated.

A summary of the purification data of lysyl oxidase is shown in Table I. The enzyme was purified about 800-fold and the yield was about 5.6%.

Purity checks

The purified enzyme exhibited only one protein band after disc gel elec-

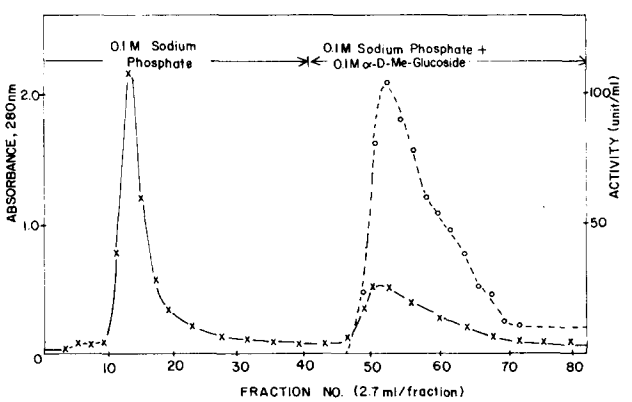

Fig. 4. Concanavalin A-Sepharose 4B affinity chromatography of purified bovine aorta amine oxidase. The enzyme (42 mg, 4554 units) was applied to a column (1.2 cm × 25 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. After the non-glycoproteins had passed through the column, buffer which contained 0.1 M α-methyl-D-glucoside was used. The fractions which contained enzyme were pooled.

TABLE I

PURIFICATION OF AMINE OXIDASE FROM BOVINE AORTA

The amine oxidase was extracted from 6 kg of bovine aorta.

Procedure	Volume (ml)	Total units	Total protein (mg)	Specific activity	Yield (%)	Purification
Extract	9550.0	23 779	74 868.0	0.318	100.0	1.0
Ammonium Sulfate fractionation	2540.0	13 350	12 708.0	1.06	56.1	3.3
DEAE-cellulose chromatography	41.5	9835	637.0	15.4	41.4	48.5
Hydroxylapatite chromatography	8.0	5760	179.0	32.14	24.2	101.1
Bio-gel chromatography	2.3	4554	42.0	108.5	19.2	341.1
Concanavalin A Sepharose chromatography	1.0	1335	5.0	267.1	5.6	840.0

trophoresis. In order to confirm that the protein band was due to the enzyme itself, the coupled amine oxidase-peroxidase assay of McEwen [13] was used. The results of the disc electrophoresis runs are shown in Fig. 5 and showed that the purified enzyme was in a monodisperse state. Analysis of the purified protein in the analytical ultracentrifuge showed the protein was monodisperse.

Oxidation of lysine peptides

Rucker and O'Dell [8] reported that bovine aorta amine oxidase oxidized the lysine residue in lysyl-vasopressin. In agreement with these investigators, the amine oxidase-peroxidase-*o*-dianisidine coupled assay indicated that the homogeneous preparations of the enzyme oxidized the hormone at about a third of the rate of benzylamine at pH 7.2. However, none of the other lysine derivatives in which the lysine was substituted in various positions or was present in different parts of the peptide were oxidized. These derivatives include Gly-Lys-HCl, Lys-*p*-nitroanilide, *N*-acetyllysine methyl ester, lysylglutamyl acetate and Lys-Gly-sulfate. Other lysine peptides which were not oxidized by amine oxidases were: Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg,

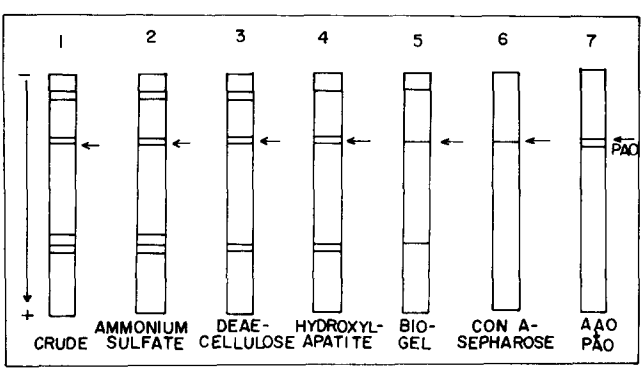

Fig. 5. Disc-gel electrophoresis of aorta amine oxidase. The horizontal arrow indicates the position of the aorta amine oxidase. The direction of migration was from cathode to anode. Enzyme activity was determined by the assay system described under Results. In the disc 7, a mixture of aorta amine oxidase (AAO) and plasma amine oxidase (PAO) was examined.

TABLE II

LYSYL OXIDASE ACTIVITY OF BOVINE AORTA AMINE OXIDASE

The assay procedure described by Pinnell and Martin [5] was used. The total volume in each case was 2.0 ml and contained enzyme, substrate (100 000 cpm) and 1 mM lysine in 0.1 M potassium buffer containing 0.16 M NaCl, pH 7.4. The temperature was 37°C and the reaction time was 8 h.

Enzyme	Tritium release (cpm)
1. Control*	157
2. Aorta amine oxidase**	150
3. Aorta amine oxidase***	660
4. Aorta lysyl oxidase†	575

* The reaction mixture included the indicated components except enzyme.
** 0.4 mg of highly purified enzyme with specific activity 286 was used for the assay.
*** 1.0 mg of partially purified enzyme with specific activity 43 was used.
† 1.25 mg of bovine aorta lysyl oxidase which had been purified through the tropoelastin-Sepharose 4B was used.

Glu-Lys-Trp-Ala-Pro, Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Gly, pyrrolidone carboxyl-Trp-Pro-Arg-Phe-Lys-Trp-Ala-Pro. Thus the ability of the enzyme to oxidize lysine peptides in general appears to be very limited.

Action of tritiated tropoelastin

The results with the purified aorta enzyme are summarized in Table II. No tritium was released when the aorta enzyme was incubated with the radioactive substrate. However, a partially purified preparation of the enzyme and a partially purified aorta lysyl oxidase preparation were active in the assay.

Chromatography on tropoelastin-Sepharose 4B

Harris et al. [9,10] reported a purification procedure for bovine aorta lysyl oxidase. In the second step of purification, an affinity column, tropoelastin-Sepharose 4B was used. The enzyme was retained by this column and 0.1 M potassium phosphate containing 6 M urea and 0.2 M NaCl, pH 8.4 was required to elute the enzyme from the column. When the bovine aorta amine oxidase was applied to the Sepharose 4B column, the enzyme was not retained by the column (Fig. 6). However, a partially purified lysyl oxidase preparation was applied to the column. It was retained which demonstrated that the column was effective in absorbing lysyl oxidase.

Effect of tropoelastin on the benzylamine oxidase activity

Mixed substrate experiments were performed. When 4 mg of highly purified tropoelastin was added to 100 units of the aorta amine oxidase with and without 5 min preincubation, no inhibition of benzylamine oxidation was observed which indicated that tropoelastin was not a substrate of the enzyme.

Immunochemical investigations

The Ouchterlony double immunodiffusion test indicated that the aorta amine oxidase (50 μg) cross reacted with the rabbit anti-bovine plasma amine

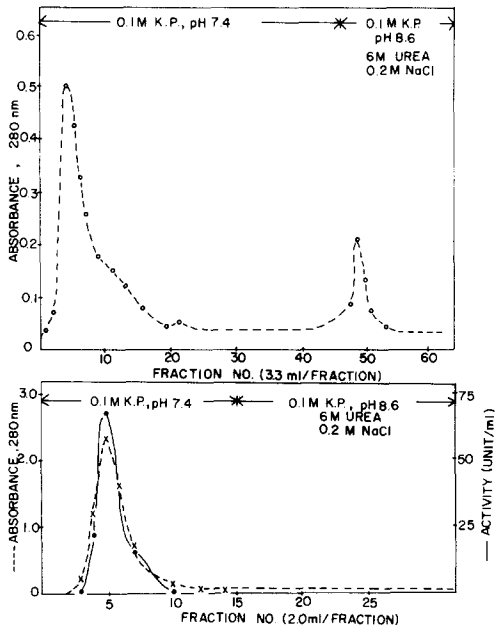

Fig. 6. (A) Chromatography of aorta amine oxidase and partially purified lysyl oxidase on chicken tropoelastin-Sepharose 4B columns. In (A) about 7 mg of bovine aorta lysyl oxidase in 0.1 M phosphate buffer, pH 7.0 was applied to the column. 0.1 M potassium phosphate (K.P.) buffer containing 6 M urea and 0.2 M NaCl used for the elution of the enzyme is shown in the figure. (B) 15 mg of purified aorta amine oxidase in 0.1 M phosphate buffer, pH 7.4 was applied to the column (1.1 cm × 7 cm) and the indicated amounts of the two buffers were passed through the column.

oxidase (0.5 ml) but not with partially purified lysyl oxidase (50 μg). However, a spur appeared between adjacent outer wells which contained the bovine aorta and plasma enzymes with the spur pointing towards the aorta enzyme. Thus, the aorta enzyme is deficient in some antigenic sites present in the plasma enzyme but both enzymes have some common antigenic sites. Advantages of this cross-reactivity of the aorta enzyme to the anti-plasma amine oxidase was utilized to show the purity of the highly purified bovine aorta amine oxidase. The antisera obtained by the injection of crude aorta extract in the rabbit showed a single precipitin band with the purified aorta amine oxidase in the double immunodiffusion test.

Approximate molecular weight

Molecular seiving on Bio-Gel A 1.5 mm was used to estimate the molecular weight of the purified bovine aorta lysyl oxidase. It is estimated that the molecular weight of the enzyme is about 180 000.

Discussion

Our laboratory has embarked on a research program to investigate the lysyl oxidase present in different tissues. Lysyl oxidase was originally discovered by Pinnell and Martin [5] in chicken bone extracts. Since that time, Rucker and O'Dell reported that bovine aorta amine oxidase also possessed

lysyl oxidase activity [8]. Layman et al. also reported that human skin extracts contain lysyl oxidase [19]. Nagatsu et al. did not check the dental pulp amine oxidase for lysyl oxidase activity but suggested that it probably was a lysyl oxidase [20]. Harris et al. have obtained chicken aorta lysyl oxidase in a highly purified form [9,10].

In order to investigate the lysyl oxidase activity of the bovine aorta amine oxidase, a pure preparation of the enzyme was needed. This objective was accomplished in the present study and the procedure for obtaining the homogeneous enzyme is described. The enzyme was purified about 800-fold as compared to the previously reported purification procedure of Rucker and O'Dell who obtained about 180-fold purification of the enzyme. Two forms of the enzyme were detected in the hydroxylapatite chromatography step. Since disc electrophoresis, ultracentrifugation and immunochemical tests showed that the enzyme was pure and our objective was to check the lysyl oxidase activity of the total amine oxidase present in the aorta, the two forms were not separately investigated in the present investigation.

The purified enzyme was used to check the lysyl oxidase activity. The following experiments were conducted: (1) The bovine aorta amine oxidase was able to oxidize lysyl-vasopressin but not a number of other lysine peptides. (2) The enzyme did not release tritium from tropoelastin (tritium labelled in 6C atom of the lysine residues) obtained from the aorta tissue culture experiments. (Partially purified aorta lysyl oxidase was active in releasing tritium). (3) The enzyme did not show affinity for a chicken tropoelastin-Sepharose 4B affinity column. (4) Antibody to the bovine plasma amine oxidase cross-reacted with the bovine aorta amine oxidase but not with partially purified aorta lysyl oxidase. These experiments led to the conclusion that bovine aorta amine oxidase does not possess lysyl oxidase activity as reported by Rucker and O'Dell [8]. It appears that their 150-fold purified enzyme contained some lysyl oxidase of the type described by Harris et al. [9,10]. We have also observed lysyl oxidase activity in bovine aorta amine oxidase preparations which were not pure.

A few of the properties of lysyl oxidase and the amine oxidase are summarized below. As noted by Rucker and O'Dell [8], the bovine plasma amine oxidase and bovine aorta amine oxidases have very similar properties. Both enzyme oxidize benzylamine, and have molecular weight of about 170 000—180 000. However, the two enzymes differ in that the aorta enzyme was capable of oxidizing lysyl-vasopressin while the plasma enzyme was not. Also, they differ in the mode and sensitivity to inhibition by β-aminopropionitrile [8]. Lysyl oxidase on the other hand cannot oxidize benzylamine [9,10] but does oxidize certain lysine residues in the structural proteins, tropoelastin [21] and procollagen [22]. The aorta lysyl oxidase has been reported to have a molecular weight of about 60 000 [10] and the bone lysyl oxidase to have a molecular weight of about 170 000 [23]. Thus, there may be two types of lysyl oxidases with different molecular weights.

In summary, from the various observations made with pure preparations of bovine aorta amine oxidase, it was concluded that this enzyme was not a lysyl oxidase nor a plasma amine oxidase.

Acknowledgements

The authors are greatly indebted to the National Institutes of Health GM 21085 and Contract Grant NHLI 743-NOI-HR-42944, the National Science Foundation Grant GB 18739, the American Heart Association 71-769 and the Hawaii Heart Association.

References

1 Ross, R. and Bornstein, P. (1969) J. Cell Biol. 40, 366—381
2 Ross, R. (1973) J. Histochem. Cytochem. 21, 199—208
3 Sandberg, L.B., Weismann, N. and Smith, D.W. (1969) Biochemistry 8, 2940—2945
4 Sandberg, L.B., Weismann, N. and Gray, W.R. (1971) Biochemistry 10, 52—56
5 Pinnell, S.R. and Martin, G.R. (1968) Proc. Natl. Acad. Sci. U.S. 61, 708—716
6 Thomas, J., Elsden, D.F. and Partridge, S.M. (1963) Nature 200, 651—652
7 Starcher, B.C., Partridge, S.M. and Elsden, D.F. (1967) Biochemistry 6, 2425—2432
8 Rucker, R.B. and O'Dell, B.L. (1971) Biochim. Biophys. Acta 235, 32—43
9 Harris, E.D., Gonnerman, W.A. and O'Dell, B.L. (1973) Fed. Proc. 32, 2132
10 Harris, E.D., Gonnerman, W.A., Savage, J.E. and O'Dell, B.L. (1974) Biochim. Biophys. Acta 341, 332—344
11 Yamada, H. and Yasunobu, K.T. (1962) J. Biol. Chem. 237, 1511—1516
12 Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954) J. Biol. Chem. 208, 645—661
13 McEwen, Jr, C.M., Cullen, R.T. and Sober, A.J. (1966) J. Biol. Chem. 241, 4544—4556
14 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
15 Whitaker, J.R. (1963) Anal. Chem. 35, 1950—1953
16 Cuatrecasas, P. and Anfinsen, C.B. (1971) in Methods in Enzymology, Vol. XXII, pp. 345—378, Academic Press, New York
17 David, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404
18 Levin, O. (1962) in Methods in Enzymology, Vol. V, pp. 27—32, Academic Press, New York
19 Layman, D.L., Narayanan, A.S. and Martin, G.R. (1972) Arch. Biochem. Biophys. 149, 97—101
20 Nagatsu, T., Nakano, G., Muzutani, K. and Harada, M. (1972) in Advances in Biochemical Psycopharmacology, Vol. V, 25—36
21 Narayanan, A.S., Page, R.C. and Martin, G.R. (1974) Biochim. Biophys. Acta 351, 126—132
22 Siegel, R.C. and Martin, G.R. (1970) Biochemistry 245, 1653—1658
23 Siegel, R.C., Pinnell, S.R. and Martin, G.R. (1970) Biochemistry 9, 4486—4492