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Xanthine Oxidase and Aldehyde Oxidase: A Simple Procedure for the Simultaneous Purification from Rat Liver[1]

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Aldehyde oxidase (AO) and xanthine oxidase (XO) are cytosolic enzymes that have been involved in some pathological conditions and play an important role in the biotransformation of drugs and xenobiotics. The increasing interest in these enzymes demands for a simple and rapid procedure for their purification. This paper describes for the first time a method that allows simultaneous purification of both enzymes from the same batch of rat livers. It involves few steps, is reproducible and offers high enzyme yields with high specific activities. The rat liver homogenate was fractionated by heat denaturation and by ammonium sulphate precipitation to give a crude extract containing both enzymes. This extract was chromatographed on an Hydroxyapatite column that completely separated AO from XO. Further purification of XO by anion exchange chromatography on a Q-Sepharose Fast Flow column resulted in a highly purified (1200-fold) preparation, with a specific activity of 3.64 U/mg and with a 20% yield. AO was purified about 1000-fold at a yield of 15%, with a specific activity of 3.48 U/mg, by affinity chromatography on Benzamidine-Sepharose 6B. The purified enzymes gave single bands of approximately 300 kDa on a polyacrylamide gel gradient electrophoresis and displayed the characteristic absorption spectra of highly purified enzymes. © 2002 Elsevier Science (USA)

Key Words: **aldehyde oxidase; xanthine oxidase; xanthine dehydrogenase; purification; rat liver.**

Aldehyde oxidoreductase and xanthine oxidoreductase (XOR)[3] are cytosolic enzymes that belong to the family of molybdenum-containing hydroxylases (1, 2). These enzymes are homodimers, containing one molybdopterin, one FAD and two different iron-sulfur centers per each 145-kDa subunit (1, 2). The XOR monomer can be divided into three domains of approximately 20, 40, and 85 kDa (from N- to C-terminal), containing the iron-sulfur, FAD, and molybdopterin centers, respectively (2–4). These three domains are relatively resistant to proteolysis, while the two linker segments are easily hydrolyzed by proteases (3, 4).

Aldehyde oxidoreductase (EC 1.2.3.1) exists exclusively as an oxidase, aldehyde oxidase (AO),[4] but XOR can undergo interconversion between a NAD^+-dependent dehydrogenase form (EC 1.1.1.204, xanthine dehydrogenase (XD)) and an oxidase form (EC 1.1.3.22, xanthine oxidase (XO))[5] (2). The distinction between XD and XO is based on the electron acceptor used by each form. XO transfers the reducing equivalents only to O_2, whereas XD preferentially transfers them to NAD^+, although it can also use O_2 (2). *In vivo,* mam-

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[3] Abbreviations used: A, absorbance; AO, aldehyde oxidase; DMAC, *p*-dimethylaminocinnamaldehyde; D/O, dehydrogenase to oxidase ratio; DTT, dithiothreitol; HTP, hydroxyapatite; PAGGE, polyacrylamide gradient gel electrophoresis; SDS, sodium dodecyl sulphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

[4] The Nomenclature Committee of the IUBMB (http://www.chem.qmw.ac.uk/iubmb/enzyme/) recommends the name aldehyde oxidase for aldehyde oxidoreductase (last update 15 August, 2001).

[5] The Nomenclature Committee of the IUBMB (http://www.chem.qmw.ac.uk/iubmb/enzyme/) recommends the names xanthine oxidase and xanthine dehydrogenase for the two interconvertible XOR forms (last update 15 August, 2001).

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malian XOR exists predominantly as xanthine dehydrogenase, but can be readily converted to xanthine oxidase, either reversibly, through oxidation of the cysteine residues 535 and 992 (5) or, irreversibly, by proteolysis at the interconnecting segment between the 40- and 85-kDa domains (3, 5). AO and XOR show a broad and complementary specificity for reducing substrates, i.e., compounds not readily oxidised by one of the two enzymes are good substrates for the other (6). Both enzymes oxidise a wide variety of substituted pyrimidines, purines, pteridines, and related compounds. This has suggested their involvement in xenobiotic metabolism, oxidizing the more-polar nitrogen-containing heterocycles. XOR and AO, together with the cytochrome P450-containing system, which most efficiently oxidizes lipophilic aromatic compounds, appear to be responsible for the detoxification of aromatic compounds in animals (7). In addition, XOR and AO have been implicated on the toxic activation of azo dyes (used as colorants in food, drink, and cosmetics) (8) and on the reductive activation of anti-neoplasic agents (9, 10) and anti-hypertensive drugs (11), just to mention a few examples of the toxicological and pharmacological importance of the AO and XOR enzymes.

Furthermore, the ability of AO and XOR to catalyse the reduction of molecular oxygen, generating $O_2^{\bullet -}$ and H_2O_2, suggests their involvement in some reactive oxygen species-mediated diseases (12–14), such as ischemia-reperfusion injury (15), and ethanol hepatotoxicity (16, 17).

The proposed roles of both AO and XOR in a range of physiological and pathological states have resulted in a considerable and increasing interest in these enzymes. The present work describes a simple method for the simultaneous purification of XOR, in its XO form, and AO from the same batch of rat livers. This procedure is quite simple and reproducible, involves few steps, and recovers significant quantities of highly purified XOR and AO.

MATERIALS AND METHODS

Materials. Adult male Sprague–Dawley rats (3–4 months old) were obtained from Instituto de Investigação Científica Bento da Rocha Cabral (Lisboa, Portugal). Chromatographic media were from Pharmacia Biotech (Uppsala, Sweden) with the exception of Hydroxyapatite (HTP) that was from Bio-Rad (CA). Molecular weight markers were also from Pharmacia Biotech (Uppsala, Sweden). All the reagents were of the highest quality available and were used as supplied. Xanthine, *p*-dimethylaminocinnamaldehyde, NAD^+, *p*-aminobenzamidine, and dithiothreitol (DTT) were from Sigma Chemical Co. (Madrid, Spain). All the other reagents were from Merck (Darmstadt, Germany).

Enzyme assays. Activity assays and spectra were recorded on a PC-linked UV2-100 Unicam spectrophotometer. AO activity was assayed by following the oxidation of 25 μM *p*-dimethylaminocinnamaldehyde at 398 nm (ϵ = 30,500 M^{-1} cm^{-}) in 50 mM phosphate buffer pH 7.8, at 25°C, in air-equilibrated solution. XO activity was measured using 20 μM xanthine in 50 mM phosphate buffer pH 7.8, at 25°C, in air-equilibrated solution, monitoring the production of urate at 295 nm (ϵ = 9500 M^{-1} cm^{-1}). XD activity was measured using the same assay mixture as described for XO plus 85 μM NAD^+ and monitoring NADH production at 340 nm (ϵ = 6220 M^{-1} cm^{-1}). One unit (U) of catalytic activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol/min of substrate, under our experimental conditions. The dehydrogenase/oxidase (D/O) ratio of XOR, as defined by Waud and Rajagopalan (18), was determined as the ratio of aerobic formation of urate, measured at 295 nm, in the presence of NAD^+ to that in the absence of NAD^+.

Protein determination. The protein concentration was estimated by the biuret method (19), using bovine serum albumin as a standard.

Purification of AO and XO. All chromatographic procedures were performed using C-columns, a peristaltic pump P-1, a monitor UV-1 (λ = 280 nm), a fraction collector FRAC-100, a recorder, and a gradient mixer GM-1 from Pharmacia Biotech (Uppsala, Sweden). The eluent was monitored at 280 nm. Enzyme samples were concentrated using an Amicon ultrafiltration cell with YM-100 membranes. The enzyme samples were desalted and the buffers exchanged by gel filtration chromatography on a small Sephadex G-25 column equilibrated with the desired buffer solution. All buffer solutions used contained 1 mM EDTA.

Livers were homogenised in 3 vol of 50 mM phosphate buffer, pH 7.8. The homogenate was rapidly heated to 55°C on a water bath, maintained at this temperature for 10 min, and then cooled quickly to below 10°C in an ice bath. During both the heating and cooling procedures the homogenate was stirred. The heat-treated homogenate was centrifuged at 10,000*g* for 3 h and the precipitate discarded. Enough solid ammonium sulfate was added to the supernatant in order to bring it to 60% saturation. The solution was stirred for 30 min, the precipitate was collected by centrifugation and dissolved in 100 mM phosphate buffer, pH 7.8. The solution was clarified by centrifugation, equilibrated in the same buffer and chromatographed on a HTP column (2.6 × 33 cm). Elution with 100 mM phosphate buffer, pH 7.8, was carried out until the large protein peak had been clearly eluted from the column. AO is eluted during this first elution step. A 100–400 mM phosphate linear gradient was then applied and the XO eluted. All the fractions collected were assayed for AO and XO activity.

XO-containing fractions were combined and further purified by ion exchange chromatography on a Q-Sepharose Fast Flow column (2.6 × 15 cm) equilibrated in 100 mM Tris–HCl buffer, pH 7.8. XO was eluted with a 0–300 mM NaCl linear gradient.

AO-containing fractions were equilibrated in 15 mM pyrophosphate buffer, pH 9.0, and applied to a Benzamidine-Sepharose 6B column (1.6 × 10 cm). AO was eluted with 10 mM *p*-aminobenzamidine (in pyrophosphate buffer). Owing to the strong absorption of *p*-aminobenzamidine at 280 nm, the automatic eluent monitoring was stopped and the elution was followed recording the visible spectrum of the fractions. The fractions showing the characteristic AO visible spectrum were pooled and the *p*-aminobenzamidine was removed by gel filtration on a Sephadex G-25 column equilibrated in 100 mM phosphate buffer, pH 7.8.

Preparation of XD. XD was obtained through reversible reduction of oxidized XO sulphydryl groups. Purified XO was incubated with 5 mM DTT, for 1–2 h at 30°C, and then passed through a small G-25 column equilibrated in 100 mM Tris–HCl buffer, pH 7.8.

Electrophoresis. Samples were electrophoresed on both native and denaturing conditions along with molecular weight markers. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (20) with 5% acrylamide stacking gel and 7.5% resolving gel using Hoffer Pharmacia Biotech equipment. Samples for SDS–PAGE were treated with sample buffer containing 1% SDS and 5% β-mercaptoethanol at 95°C for 5 min prior to electrophoresis. Electrophoresis was performed at a

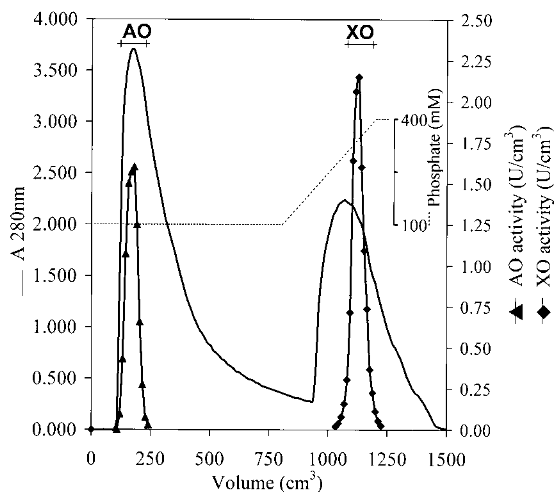

FIG. 1. Elution profiles of total protein and of AO and XO activities from an HTP column. The ammonium sulphate extract was applied on an HTP column and the elution was performed with the indicated phosphate buffer concentration (···). The AO (▲) and XO (◆) activities were determined as described under Materials and Methods. The protein concentration was monitored spectrophotometrically at 280 nm (—).

constant voltage of 55 V. The standard molecular weight markers for SDS–PAGE were as follows: myosin, 212 kDa; α2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa. The electrophoresed samples were stained for protein with 0.25% Coomassie brilliant blue R-250.

Native polyacrylamide gradient gel electrophoresis (PAGGE) was performed with a 5% acrylamide stacking gel and 7.5–20% gradient resolving gel using Hoffer Pharmacia Biotech equipment. Electrophoresis was performed at a constant voltage of 125 V for 36 h. The standard molecular weigh markers for the native-PAGGE were as follows: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; albumin, 67 kDa. The electrophoresed samples were stained for protein with 0.25% Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

Purification of AO and XO. Crude extracts of rat liver, prepared by homogenization, heat treatment and ammonium sulfate precipitation, contain both AO and XO. Trial experiments revealed that high AO and XO purification degrees are achieved at different saturation percentages (AO at 40% and XO at 60%). The degree of AO purification was, however, sacrificed for higher recovery of both enzymes, implementing a precipitation with 60% ammonium sulfate as described in the methods section.

The 60% ammonium sulphate pellet, containing both AO and XO, was applied to a HTP column. The elution profiles of protein and of XO and AO activities are shown in Fig. 1. AO did not adsorb to the HTP column and was eluted during the "wash-procedure" with 100 mM phosphate buffer. XO, on the contrary, was adsorbed on the HTP column and was eluted only after the linear gradient had been applied. All the XO activity was present in the large peak eluting at approximately 300 mM phosphate. It should be noted that after this purification step AO is completely separated from XO.

Highly purified XO was obtained after ion exchange chromatography on Q-Sepharose Fast Flow, being XO eluted as a single peak at approximately 100 mM with a NaCl linear gradient. Further purification of AO was achieved by affinity chromatography on Benzamidine-Sepharose 6B, as suggested by Stell *et al.* (21).

Typical results for the purification of both XO and AO are illustrated in Tables I and II. Following the described purification procedure, XO and AO were purified about 1200- and 1000-fold, respectively, with yields between 25 and 15% for XO and between 10 and 15% for AO. The final specific activity of purified XO and AO were 3.64 and 3.48 U/mg, respectively, which are favorably comparable to the highly purified enzymes described in the literature (21–23).

Absorption spectra. Additional criteria of purity of the enzymes are their absorption spectra. The visible absorption spectra of purified AO and XO (Fig. 2) were qualitatively the same as those reported by other authors for highly purified enzymes (18, 21, 24, 25). The visible absorption spectra of AO and XO reflect essentially the presence of flavin and iron in a ratio of 1/4, being the ratio of absorbances at 450 and 550 nm between 2.8 and 3.1. The absorbance ratio A_{280}/A_{450}, indicative of the ratio of protein to flavin plus iron, were between 5.3 and 5.8 and were comparable to the

TABLE I

Purification of Rat Liver XO

Purification step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Homogenate	49.0×10^3	153	3.12	1	100
Heat treatment	12.1×10^3	83.7	6.92	2	55
60% $(NH_4)_2SO_4$	1.99×10^3	57.0	28.6	9	37
HTP	70.7	38.3	542	174	25
Q-Sepharose F. F.	7.97	29.0	3.64×10^3	1167	19

TABLE II

Purification of Rat Liver AO

Purification step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Homogenate	49.0×10^3	180	3.67	1	100
Heat treatment	12.1×10^3	136	11.2	3	76
60% $(NH_4)_2SO_4$	1.99×10^3	71.8	36.1	10	40
HTP	614	63.9	104	28	36
Benzamidine–Sepharose	6.51	22.7	3.48×10^3	945	13

values obtained with the best preparations of XO (25, 26) and AO (21, 24).

Electrophoretic analyses. Further confirmation of the high degree of purity of the enzymes came from analyses by polyacrylamide gel gradient electrophoresis (PAGGE). The purified XO showed only a single band (Fig. 3), corresponding to approximately 300 kDa, indicating that the XO preparation was homogeneous.

On polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol (SDS–PAGE) the purified XO was dissociated into one major band of approximately 150 kDa and three bands of 100, 60, and 40 kDa (Fig. 4). This suggests that the 150-kDa band was proteolyzed into 100- and 40-kDa bands and that the 100-kDa fragment was further hydrolyzed into two polypeptides of 60 and 40 kDa. In addition, a small band of approximately 135 kDa was sometimes identified close to the band of 150 kDa.

This electrophoretic pattern is very similar to that observed by Krenitsky *et al.* (27) with the human liver XO, where five bands of 150, 135, 95, 55, and 38 kDa were identified. Those bands, with the exception of the 40-kDa band, have also been described in several other preparations of rat liver XO (28), bovine milk XO (29), and human milk XO (30). The electrophoretic pattern observed under denaturating conditions results from a limited proteolysis of XO, which is easily hydrolyzed by proteases of different specificities (3, 28–31). It is, however, important to remark that this proteolysis does not reduce the high XO-specific activity and that the enzyme displays the molecular weight characteris-

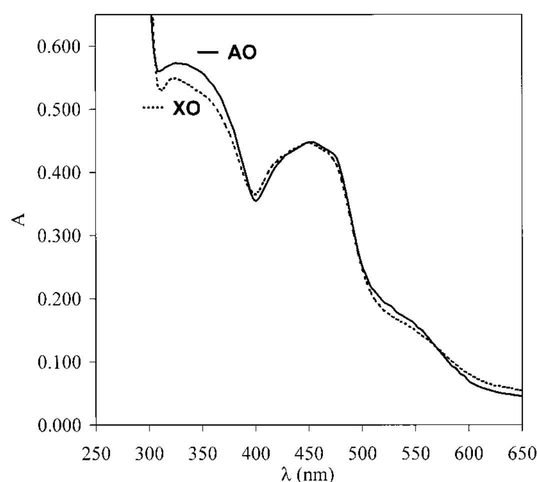

FIG. 2. Absorption spectra of purified rat liver XO (· · ·) and AO (—).

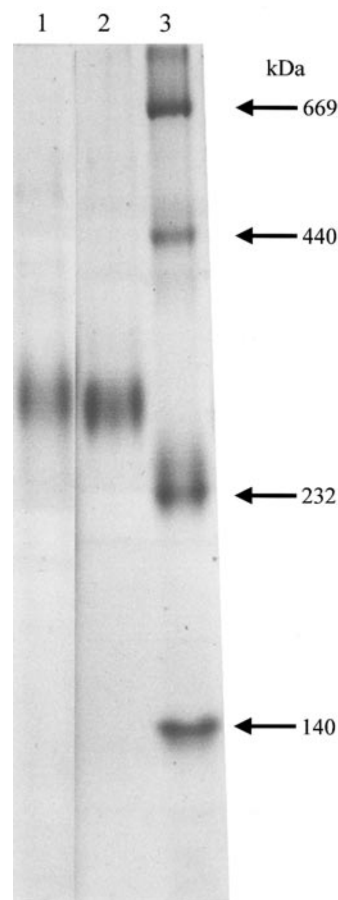

FIG. 3. PAGGE of purified XO and AO. Samples of purified XO and AO were electrophoresed on 7.5–20% gradient gels (14 × 16 cm) and stained for protein with Coomassie Brilliant Blue. Lane 1, 20 μg of purified XO; lane 2, 20 μg of purified AO; and lane 3, molecular weight standards. The molecular weight of the standards are indicated by the arrows.

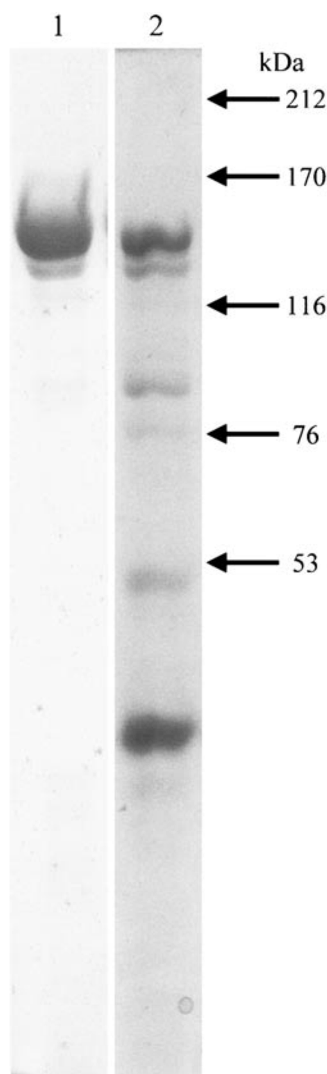

FIG. 4. SDS–PAGE of purified XO and AO. Samples were electrophoresed on 7.5% gels (14 × 16 cm) and stained for protein with Coomassie Brilliant Blue. Lane 1, 10 μg of purified AO; lane 2, 7.5 μg of purified XO. The migrating positions of the molecular weight standards are indicated by the arrows.

tic of pure XO (3), indicating that proteolyzed-formed polypeptides do not dissociate, but remain bound within the native molecule.

The PAGGE electrophoretogram of purified AO (Fig. 3) showed only a single protein band, corresponding to 300 kDa. This result illustrates the high degree of purification achieved and suggests that the purified AO was nearly free from proteic impurities.

Analysis of the purified AO by SDS–PAGE (Fig. 4) showed one band of approximately 150 kDa. A small band of approximately 135 kDa and two additional bands of 100 and 40 kDa were sometimes identified. This last electrophoretic pattern suggests that AO, like XO, is also prone to proteolyses.

Conversion of XO form to XD form. XOR was purified in its XO form. Nevertheless, the XD form can be obtained through incubation of XO with sulphydryl reducing reagents. Following DTT treatment, XO was 80–85% converted to XD in a time-dependent manner (Fig. 5). The XOR purified herein was, accordingly, 15–20% in its irreversible XO form. The achieved dehydrogenase to oxidase (D/O) ratios of 4–6 are quite similar to that obtained by Della Corte and Stirpe (32), Waud and Rajagopalan (18) or McManaman *et al.* (26). These results demonstrate that the proteolysis observed in the XO preparation (Fig. 4) does not affect greatly the interconversion between XO and XD.

Enzyme stability. AO and XO in buffer solutions at pH 7.8 were found to be highly stable, with no significant activity loss for 6 months either at 5°C or −10°C. On the contrary, XD solutions were not very stable, being slowly converted to XO, even in the presence of DTT. The conversion can be, however, reversed by incubation with fresh DTT.

Conclusion. The simultaneous purification of XOR, in its XO form, and AO from rat liver have been achieved by means of a simple, rapid, and effective procedure. XD, the other functional form of XOR, was also easily obtained from XO. This is important, because, due to the broad and complementary substrate specificity of these enzymes, they are essential when investigating the metabolic fate of any new therapeutic agent or chemical present in the environment.

Besides the simplicity of the method described herein, it should be emphasised the advantage of time and raw material saving without compromising the final specific activity of each enzyme. Both enzymes are

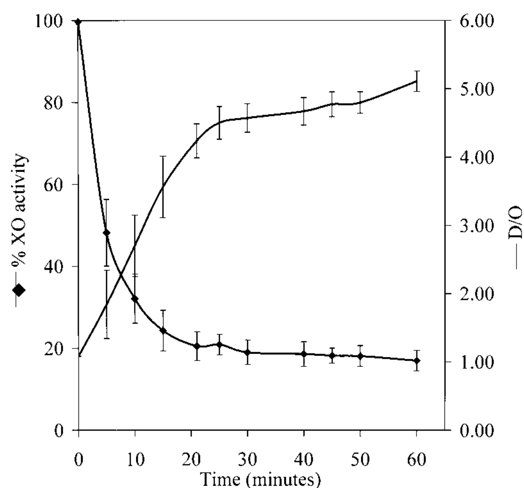

FIG. 5. Time-dependent conversion of XO form into XD form. Purified XO was incubated with 5 mM DTT, at 30°C, and, at indicated times, XO and XD activities were measured and the D/O ratios determined. The D/O ratios (—) and the remaining XO activity (◆) are shown. The values represented are means (± standard deviation) of three independent experiments.

copurified in a three-step process, that involves heat desnaturation, ammonium sulfate fractionation and adsorption chromatography on a HTP column that ensures the AO separation from XO. Further purification of AO and XO is achieved by affinity and ion exchange chromatography, respectively.

The purified AO and XO preparations show the absorption spectrum, specific activity, and the native electrophoretic behavior characteristic of highly purified enzymes.

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