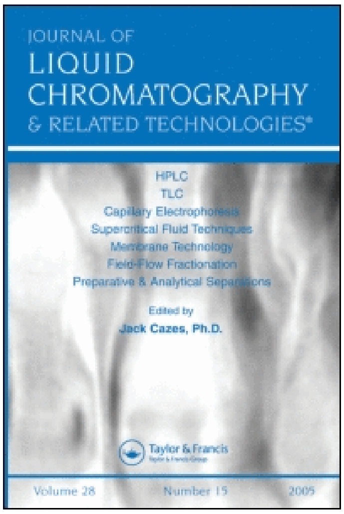

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PURIFICATION OF HUMAN PLATELET MONOAMINE OXIDASE B BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Human platelet monoamine oxidase (MAO B), a membrane bound enzyme was purified to homogeneity by DEAE-Sephacel column chromatography, chromatofocusing, and high performance liquid chromatography (HPLC). The crucial purification step was HPLC on a anion exchange column (SynChropak AX 300). The HPLC column was eluted initially with potassium phosphate buffer (100 mM, pH 7.4) for 10 min at a flow rate of 1.0 ml/min, followed by a gradient (0-1%) of octyl-β-D-glucopyranoside (octylglucoside) in the same buffer for 10 min, and finally with buffered octylglucoside (1%) for 40 min. The elution of pargyline-bound or active MAO was established by determining either radioactivity in each fraction when MAO B had previously been covalently labeled with [^{3}H]-pargyline [^{3}H(G)] or catalytic activity using [^{14}C-methylene]-benzylamine as substrate. [^{3}H]-pargyline-bound and active MAO B eluted from the column at approximately 34 min. The extent of homogeneity and the subunit M_r (approximately 59,000) of MAO B were determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining for proteins.

INTRODUCTION

Monoamine oxidase (amine: oxygen oxidoreductase, E.C. 1.4.3.4.) (MAO) is an intrinsic membrane flavoprotein localized in the outer mitochondrial membrane (1). This enzyme has an approxi-

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mate molecular weight of 120,000 and consists of two subunits, one of which contains covalently bound flavin adenine dinucleotide. MAO has been classified into two types, A and B, depending upon their differences in sensitivity to specific inhibitors and their preference for different amines as substrates. MAO A preferentially deaminates 5-hydroxytryptamine and norepinephrine and is sensitive to inhibition by clorgyline (2, 3), while MAO B deaminates benzylamine and β-phenylethylamine (4) and is preferentially inhibited by pargyline (5) and deprenyl (2). Both types of MAO are present in most tissues, each being expressed in varying proportions in different tissues (6-10). In human tissues, however, platelets contain mostly MAO B, whereas placenta has a high proportion of MAO A.

In this article, we describe the purification of MAO B from outdated human platelets, employing a three-step procedure which includes DEAE-Sephacel column chromatography, chromatofocusing, and high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals:

Octylglucoside (octyl-β-D-glucopyranoside) was obtained from Calbiochem-Behring Corp. [^{3}H]-pargyline (Pargyline-HCl, specific activity, 15 Ci/mmol) was purchased from New England Nuclear and [^{14}C]-benzylamine (Benzylamine-HCl [methylene-^{14}C], specific activity, 14 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc.

Purification of MAO from Human Platelets:

Platelet rich plasma (PRP) was obtained from The University of Texas Medical Branch Blood Bank immediately after they became out-dated (72 hours after blood drawing). The PRP was stored in a cold-room overnight. In each MAO preparation, a batch of 25 units (65 ml/unit) was used.

Pooled PRP was centrifuged at 600 x g for 3 min to remove contaminating red blood cells and lymphocytes. The supernatant was centrifuged at 2,500 x g for 20 min and the platelets collected. The platelets were washed by dispersing in 0.9% saline-5 mM EDTA (saline-EDTA), pH 7.4, and then centrifuged at 2,500 x g for 20 min. After two more washings with saline-EDTA solution, the washed platelets were suspended in cold distilled water to a final protein concentration of 5 mg/ml. The suspension was frozen at -20°C overnight, thawed, and centrifuged at 35,000 x g for 60 min. The pellet was suspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.1% Triton X-100 (freshly prepared). After the suspension was stirred for 60 min at 4°C, it was centrifuged at 35,000 x g for 30 min. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.5% Triton X-100, stirred for 60 min at 4°C and centrifuged at 150,000 x g for 60 min. The supernatant containing solubilized MAO was dialyzed against 3 x 6000 ml of 10 mM potassium phosphate buffer, pH 8.0, for 36-40 h.

Then the dialyzed-extracted MAO was fractionated on a DEAE-Sephacel (Pharmacia) column (2.6 x 40 cm) which had been previously equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The column was developed by stepwise elution with 10 mM and 100 mM potassium phosphate buffer, pH 8.0, and MAO was eluted with 100 mM potassium phosphate buffer, pH 8.0, containing 0.25% Triton X-100. The fractions containing high MAO activity were pooled together and the active protein was precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. The mixture was centrifuged at 30,000 x g for 60 min and the precipitate which floated on the surface was collected. The precipitate was dissolved in 50 mM potassium phosphate, pH 8.0, containing 1% octylglucoside and then dialyzed against 3 x 6000 ml of 0.025 M Tris-acetate buffer, pH 7.4, for 36-40 h. Solid octylglucoside was added to the dialyzed solution to a final concentration of 1%. This preparation was further fractionated on a Polybuffer Exchanger 94 (Pharmacia) chromatofocusing column (0.9 x 27cm) according to the instructions from Pharmacia Fine Chemicals. The pH gradient was developed by elution with 200 ml of eight times-diluted Polybuffer 74 (Pharmacia), pH 4.0, (adjusted with 1 M HCl) containing 1% octylglucoside. The 2.8

ml fractions were collected and were assayed for absorption at 280 nm, pH, and MAO activity. The fractions showing high MAO activity near pH 5.3 were pooled together and the active protein fraction was precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation. The floating precipitate collected after centrifugation at 30,000 x g for 20 min was washed once with 80% saturated solution of $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 7.4. The washed precipitate was dissolved in 50 mM potassium phosphate buffer, pH 8.0, containing 1% octylglucoside and dialyzed against 6000 ml of 10 mM potassium phosphate buffer, pH 7.4, for 24 h. This dialyzed, chromatofocused fraction was further fractionated by HPLC.

HPLC was performed on a Beckman model 334 Gradient Liquid Chromatograph using a SynChropak AX 300 column (4 x 300 mm), a Beckman model 153 analytical UV detector containing 280 nm filter, and an Altex C-R1A processor. One ml of the chromatofocused fraction, (0.5-0.7 mg protein) in 10 mM potassium phosphate buffer, pH 7.4, was injected and eluted with potassium phosphate buffer, (100 mM, pH 7.4) for 10 min, followed by a gradient (0-1%) of octylglucoside in the same buffer for an additional 10 min. Catalytically active MAO was eluted with phosphate buffer containing 1% octylglucoside for 40 min. A flow rate of 1.0 ml/min was maintained throughout the elution profile.

Fractions containing catalytically active MAO were pooled together and dialyzed against 10 mM potassium phosphate buffer, pH 7.4, for 24 h. The dialyzed fraction (2.0 ml) was rechromatographed on the HPLC column as described above. The active fractions were pooled, dialyzed, and lyophilized before electrophoresis.

MAO Activity Determination:

MAO activity was assayed by the basic procedure of Wurtman and Axelrod (11). The assay mixture contained 2 mM $[^{14}C]$-benzylamine (specific activity, 2 mCi/mmole), 50 mM potassium phosphate buffer, pH 7.4, and the enzyme in a total volume of 15 µl. The mixture was incubated at 37°C for 30 min. The reaction was terminated by transferring the mixture to an ice bath and adding 3 µl of 6 M HCl.

TABLE 1

Purification of Human Platelet MAO

FRACTION		TOTAL PROTEIN (mg)	PROTEIN RECOVERY (%)	ENZYME SPECIFIC ACTIVITY (nmol/h/mg prot)	TOTAL ENZYME ACTIVITY (nmol/h)	ENZYME ACTIVITY RECOVERY (%)	PURIFICATION
A.	Crude	1349	100	90.8	123,000	100	-
B	Dialyzed 0.5% TX-100 Extract	121	8.97	526	60,900	49.4	5.8
C	DEAE-Sephacel Fraction 0-50% $(NH_4)_2SO_4$ precipitate	27	2.00	1330	35,700	28.9	14.7
D	Chromatofocused Fraction	1.8	0.13	1290	2,320	1.9	14.2
E	HPLC-Fraction	0.08	0.006	3920	314	0.25	43.2

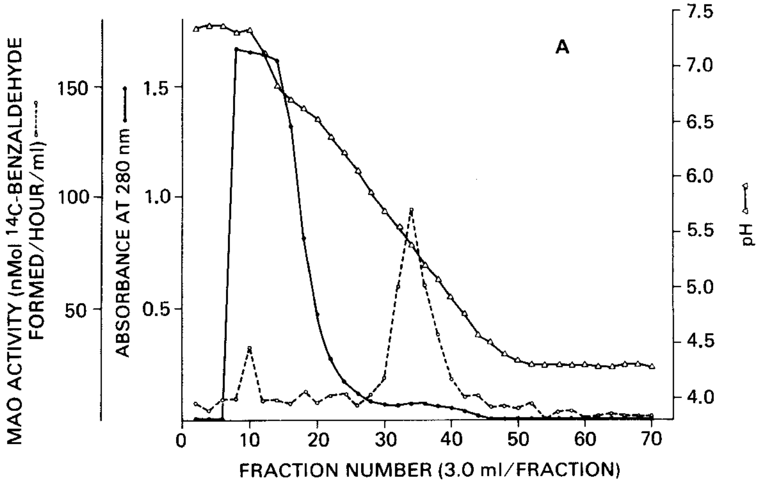

FIGURE 1

Chromatofocusing of DEAE-Sephacel fraction proteins on Polybuffer Exchanger 94 column. Column size: 0.9 x 30 cm. Bed height: 27 cm. Elution conditions: Starting buffer -- 0.025 M Tris-acetate, pH 7.4, containing 1% octylglucoside; Elution buffer -- Polybuffer 74 (8 times diluted), pH 4.0, containing 1% octylglucoside; Flow rate: 32 mL/h.

A. Sample: 48 mg of DEAE-Sephacel fraction protein in 0.025 M Tris-acetate, pH 7.4, containing 1% octylglucoside applied on the column. Each individual fraction was assayed for pH, absorbance at 280 nm, and catalytic activity.

Two types of blanks were used: a tissue blank, in which 3 μl of 6 M HCl was added to the complete reaction mixture before incubation; and a water blank, in which the enzyme was replaced by an equivalent volume of water. The [^{14}C]-benzylaldehyde formed was extracted with 120 μl of toluene and centrifuged. Sixty μl of the toluene layer was mixed with 3.0 ml of ScintiVerse I (Fisher Scientific Co.) cocktail and counted. The specific activity of the

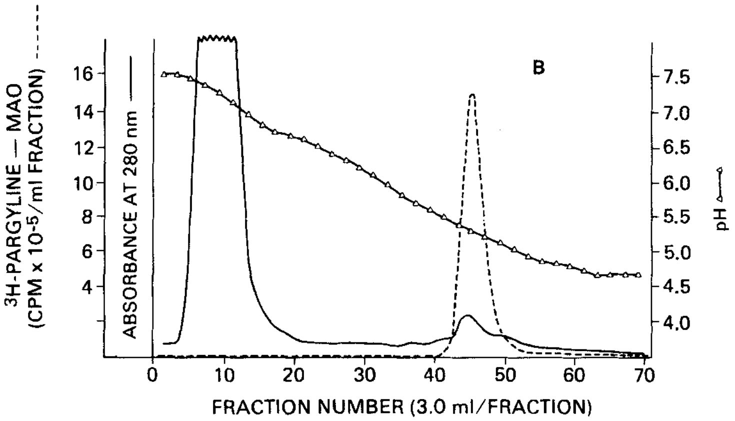

FIGURE 1 (continued)

B. Sample: 35 mg of [^{3}H]-pargyline treated DEAE-Sephacel fraction proteins in 0.025 M Tris-acetate, pH 7.4, containing 1% octylglucoside applied on the column. Absorbance profile at 280 nm was obtained by coupling LKB Uvicord II to the column. Each individual fraction was assayed for pH and [^{3}H] activity (10 µl aliquot from each fraction was mixed with 4.0 ml PCS counting fluid from Amersham Corporation and counted in Beckman LS 8000 scintillation counter).

enzyme was expressed as nmoles of [^{14}C]-benzylaldehyde formed/h/mg protein.

[^{3}H]-Pargyline Binding to MAO:

The DEAE-Sephacel fraction (22.6 mg protein/8.0 ml) was mixed with 0.96 ml of 0.5 M potassium phosphate buffer, pH 7.4, and 0.097 ml of [^{3}H]-pargyline solution (specific activity, 15.3 Ci/mMole) in a total volume of 9.6 ml. The mixture was incubated at 37°C for 60 min, cooled on ice, and then dialyzed against 3 x 6000 ml of 25 mM Tris-acetate buffer (pH 7.4) for 60 h. SDS-polyacrylamide gel electrophoresis showed that the [^{3}H] activity was concentrated in the protein band having a M_r of about 59,000, demonstrating specific labeling of MAO.

Analytical Methods:

The protein concentration was determined by the method of Lowry *et al*. (12), using bovine plasma gamma globulin as standard. SDS-polyacrylamide gel electrophoresis analyses of the fractions were carried out according to Fairbanks (13), with 2.5% stacking gel and 7.5% running gel. Proteins were stained with silver as described by Merrill *et al*. (14). Commercial Triton X-100 was purified before use by the procedure described by Chang and Bock (15).

RESULTS AND DISCUSSION

A three step method including DEAE-Sephacel column chromatography, chromatofocusing on Polybuffer Exchanger 94 (Pharmacia) column, and HPLC employing an anion exchange column has been used to purify human platelet MAO to a single band on SDS-polyacrylamide gels. Treatment of the membrane fraction obtained after centrifugation of a freeze-thawed platelet suspension with buffered detergent (0.1% Triton X-100) did not release MAO activity into the wash. Further treatment with buffered 0.5% Triton X-100 solubilized 45-55% (4 preparations) of the platelet MAO activity. This extraction step increased the specific activity 5.8 times (Table 1, Fraction B). On further fractionation of the dialyzed Triton X-100 solubilized extract on a DEAE-Sephacel column, MAO activity was eluted with 100 mM potassium phosphate buffer, pH 8.0, containing 0.25% Triton X-100. The enzyme from the pooled active fractions after precipitation with 50% ammonium sulfate saturation showed a 2.5 fold increase in specific activity (total purification: 14.7 fold; Table 1, Fraction C). Upon chromatofocusing, which separates proteins on the basis of differences in isoelectric pHs, MAO activity was eluted as a sharp peak at pH 5.3 (Figure 1A and 1B). Proteins are eluted from the column by pH gradients generated by interaction of polybuffer exchanger equilibrated at one pH with polybuffer (eluent) which has been adjusted to a second, lower pH value. This step of purification did not result in an increase in

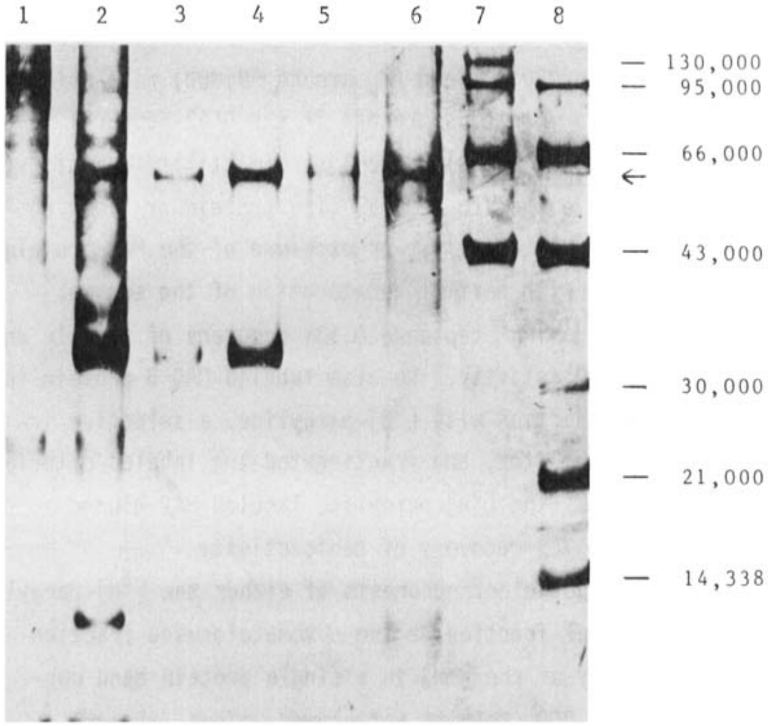

Figure 2

SDS-polyacrylamide gel profile of various fractions. Electrophoresis was run and stained for proteins with silver as described in Materials and Methods. Lane 1: DEAE-Sephacel fraction (10 µg protein); Lane 2: Chromatofocused fraction (10 µg protein); Lane 3 and 4: HPLC fraction after first run (2.6 and 5.2 µg protein); Lane 5 and 6: HPLC fraction after second run (1.0 and 2.0 µg protein); Lane 7: BioRad high molecular weight markers (0.3 µg protein per band--from top to bottom, β-galactosidase, 130,000; phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000); Lane 8: BioRad low molecular weight markers (0.3 µg protein per band--from top to bottom, phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,338). The arrow indicates the position of MAO, corresponding to M_r 59,000.

specific activity of the MAO (Table 1, Fraction D), but SDS-polyacrylamide gel electrophoretic analysis showed increased concentration of the MAO protein (M_r around 59,000) with elimination of several other proteins present in the DEAE-Sephacel fraction (Figure 2, lanes 1 and 2). The loss of activity at this stage could be attributed either to removal of a protein or lipid component required for MAO activity, or exposure of the MAO protein to its isoelectric pH, with partial denaturation of the enzyme.

The chromatofocusing step gave 0.13% recovery of protein and 1.9% recovery of MAO activity. We also labeled MAO B protein in the DEAE-Sephacel fraction with [^{3}H]-pargyline, a selective irreversible MAO B inhibitor, and fractionated the labeled material by chromatofocusing. The [^{3}H]-pargyline labeled MAO eluted at pH 5.3 (Fig. 1B), with 70% recovery of radioactivity. SDS-polyacrylamide gel electrophoresis of either the [^{3}H]-pargyline labeled DEAE-Sephacel fraction or the chromatofocused fraction resulted in recovery of the [^{3}H] in a single protein band corresponding to M_r 59,000 (results not shown). Thus, the [^{3}H]-pargyline labeling technique provided a convenient way to follow the purification of MAO.

In the next step of purification, an HPLC with an anion exchange column (SynChropak AX 300) was employed using a gradient of buffered octylglucoside (Fig 3, A and B). The catalytically active MAO or [^{3}H]-pargyline labeled MAO was consistently eluted from the HPLC column in fraction 34. The electrophoretic patterns of this fraction on SDS-electrophoretic gels showed two major bands with M_r 59,000 and 28,000 (Figure 2; lanes 3 and 4). The 28,000 M_r protein was easily removed by repeating the HPLC step (Figure 2; lanes 5 and 6). This step of purification resulted in a further 3 fold increase in specific activity (total purification: 43.2 fold; Table 1, Fraction E). The final preparation of purified MAO B from human platelets is suitable for production of antibodies and protein characterization.

The extensive purification of MAO B using this procedure demonstrates that this purification scheme can be successfully applied to the purification of membrane bound proteins, although we

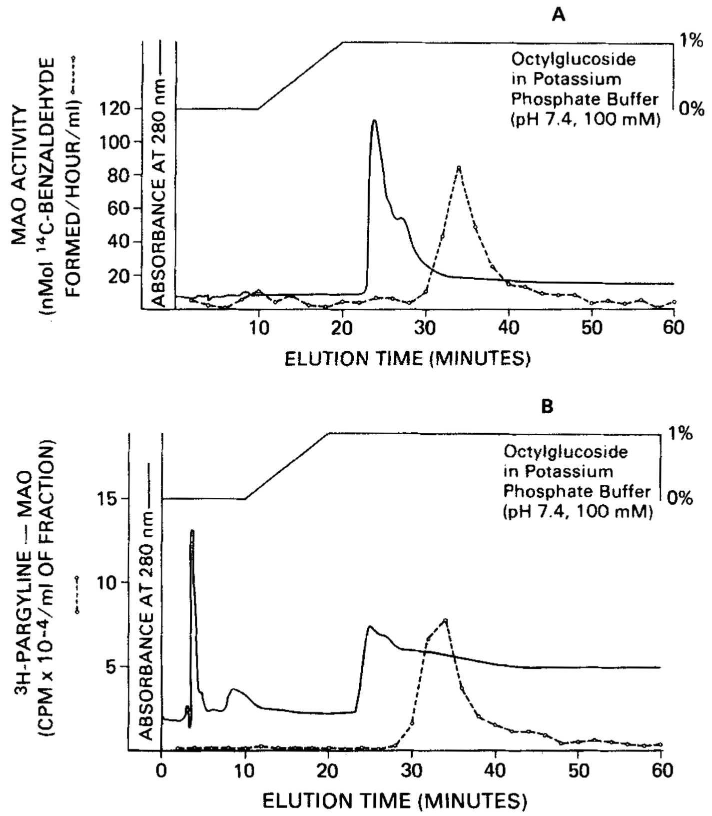

Figure 3

Separation of MAO protein from chromatofocused fraction by HPLC. The sample (0.5 - 0.7 mg protein) in 10 mM potassium phosphate buffer, pH 7.4, was injected on SynChropak AX 300 column (4 x 300 mm).

A. Catalytically active chromatofocused fraction: Alternate fractions were assayed for MAO activity.

B. $[^3H]$-pargyline treated chromatofocused fraction: 10 µl aliquots from the alternate fractions were mixed with 4.0 ml of PCS counting fluid (Amersham Corporation) and $[^3H]$ activity determined in Beckman LS 8000 scintillation counter.

lost considerable enzyme activity during the chromatofocusing step. In such purification schemes, octylglucoside represents the detergent of choice because it does not interfere with UV detection, it is a relatively mild nonionic detergent permitting retention of catalytic activity, and it is easily removed by dialysis.

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