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Purification of human placenta diamine oxidase

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Diamine oxidase (histaminase) is produced at very high levels by the decidual cells of the placenta. The presence of diamine oxidase has been demonstrated in human neutrophils. Purification of human placenta diamine oxidase was performed by four subfractionation steps and led to the isolation of one polypeptide whose molecular weight was 84 000, as assessed by SDS PAGE. Using polyclonal antibodies raised against the purified enzyme, we have demonstrated that the neutrophil diamine oxidase is immunochemically identical to the placental diamine oxidase. Development of immunological methods will be useful for detection and quantitation of diamine oxidase in neutrophils during the inflammation process.

Diamine oxidase [DAO] or histaminase catalyses the oxidative deamination of several substrates including putrescine, histamine and spermidine . Diamine oxidase is produced at very high levels by the decidual cells of the placenta (**1**). Plasma levels of this enzyme have been found to be elevated during pregnancy (**2**).

Its association with a number of human cancers has been recognized. Biochemical and immunological studies have shown that diamine oxidase in tumor tissues and malignant effusion fluids has the same properties as those of the placenta enzyme (**3**). Histamine is one of several well -

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Abbreviations: DAO, diamine oxidase oxidoreductase, EC 1.4.3.6; PMSF, phenylmethylsulfonyl fluoride; PMN, polymorphonuclear neutrophil; EGTA, ethylene glycol-bis (-amino-ethyl ether) N,N,N',N'-tetra-acetic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NBT, p-nitroblue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt.

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characterized mediators of allergic and immunologic reactions. The presence of diamine oxidase has been demonstrated in human eosinophils and neutrophils, but not in mononuclear cells (**4**) Eosinophil and neutrophil diamine oxidase was found to be physicochemically and functionally similar to diamine oxidase isolated from human placenta, and was predominantly localized in a granule rich fraction sedimenting at 27 000 g (**4, 5**). Here we report a new procedure for the purification of diamine oxidase from human placenta. We have demonstrated that the placental diamine oxidase is immunochemically identical to the neutrophil diamine oxidase and that antibodies raised against the placental enzyme can therefore be used for the study of the release of the neutrophil enzyme during the course of inflammation.

MATERIAL AND METHODS

Material

Reagents used in this work were obtained from the following sources : ^{14}C putrescine (the radiochemical center, Amersham, England) leupeptin, soybean trypsin inhibitor, Tween 20, phenylmethylsulfonylfluoride (PMSF), 3, 3', 5, 5' tetra methylbenzidine (Sigma chemical Co, St Louis,MO,USA). Affinity purified goat anti-rabbit IgG alkaline phosphatase conjugate, alkaline phosphatase color development reagent, BCIP (5 - bromo - 4 - chloro - 3 - indolyl phosphate p - toluidine salt) and NBT (p-nitroblue tetrazolium chloride) were from Biorad, Richmond CA USA. Ter-butyl -4 - phenyl) 2 (biphenylyl - 4) - 5 oxadiazole - 1, 3, 4, (butyl PBD) was from PROLABO, Briare, France.

Preparation of placenta extracts for diamine oxidase purification

The frozen placentae were thawed; the umbilical cords and the outer membrane were removed and the placentae were cut into small pieces, which were washed in 0.9 % NaCl and then homogenized in 10 mM NaK phosphate buffer pH 7.4 containing 1 mM PMSF, 1.5 mM EGTA, 5 µg/ml leupeptin and 1 mg/ml soybean trypsin inhibitor in a waring blendor at high speed. The homogenization was repeated twice, with 1 min cooling intervals. The slurry was centrifuged in 250 ml bottles at 10,000 g for 20 min at 4° C and the supernatants were assayed for protein concentration and DAO activity. Routinely, 8 to 10 placentae were processed at one time.

Diamine oxidase purification

Diamine oxidase was purified from the 20 000 g homogenate supernatant. The first step consisted of precipitation with ammonium sulfate; diamine oxidase activity was recovered between 40 and 60 % ammonium sulfate. The 60 % ammonium sulfate precipitate was collected by centrifigation for 20 min at 20 000 g and solubilized with 24 ml of 20 mM Na K phosphate buffer pH 7.4 (buffer A). Purification was continued by chromatography of the dialyzed protein solution on an A50 DEAE Sephadex column (3 cm x 30 cm) equilibrated in buffer A. Bound proteins were eluted with 400 mM Na K phosphate buffer pH 7.4. The diamine oxidase enriched fractions were pooled, dialysed against 10 mM Na K phosphate pH 7.4 and then loaded onto an hydroxylapatite column (1.2 cm x 10 cm). The column was eluted with 200 mM Na K phosphate pH 7.4. The active fractions were pooled and dialysed against a buffer consisting of 50 mM Na K

phosphate and 400 mM NaCl pH 7.4. This buffer was used to equilibrate a column of 4 B concanavalin A Sepharose (1.2 cm x 8 cm), which was used for the next chromatography step. The concanavalin A bound proteins were eluted by washing with the equilibration buffer supplemented with 0.2 M α-methyl mannoside. The final step was by FPLC using a Superose HR 10/30 column equilibrated with a solution of 50 mM Tris HCL, 300 mM NaCl pH 8. The active fractions recovered from concanavalin A Sepharose were concentrated under vacuum and dialysed against the Superose equilibration buffer. An aliquot of 200 µl of the fraction was loaded onto the Superose column. The column was eluted with the equilibration buffer and the diamine oxidase enriched fractions were pooled.
For production of polyclonal antibodies, diamine oxidase was electroeluted from a preparative SDS-PAGE carried out with a partially purified preparation obtained at the concanavalin A Sepharose step. After electrophoresis, the peptide band which corresponded to the 84000 dalton purified protein was cut out, and electroeluted in a 100 mM NH_4HCO_3 buffer pH 8.1 supplemented with 0.05 % w/v SDS for 15 hours at 80 V and at 4° C. The electroeluted protein was injected into rabbits.

Isoelectric focusing

A linear 0 - 40 % sucrose gradient containing 1.7 % (v/v) LKB ampholines pH 3.5 - 9.5 was made in water in a 110 ml column at 4° C. A protein solution containing 11.2 mg protein, recovered from the concanavalin-Sepharose step, was applied to the middle of the ampholine column. The electrode reservoir at the cathode (bottom of the column) was filled with 5 mM NaOH in 40 % sucrose (w/v) and the reservoir at the anode was filled with 5 mM sulfuric acid. Electrofocusing was carried out at 4° C for 24 h at 600 V at an intensity ranging between 7 and 0.3 mA. After completion of electrophoresis, 4 ml fractions (tubes, 1 - 16 and 23 - 32) and 2 ml fractions (tubes 17 - 22) were collected and the pH and protein content were determined. The diamine oxidase activity of the fractions was measured after adjustement of the pH to about 7.0.

Elisa

Aliquots of two hundred microliters of the purified DAO diluted in PBS were placed in the wells of the Elisa plate. After an overnight incubation at 4° C, the solution was removed and the wells were washed with PBS. They were then filled with 200 µl of a 3 % BSA solution in PBS and the plate was allowed to stand for 1 h at room temperature for saturation of unspecific sites. After washing with PBS containing 0.05 % (w/v) Tween 20, 200 µl of antiserum diluted in PBS-Tween was added to the wells and the plate was incubated at room temperature for 2 h. The plate was washed with PBS-Tween, and 200 µl of a solution of peroxidase conjugated anti-rabbit IgG diluted 2000 fold with PBS-Tween was added to each well. After another 2 h incubation at room temperature followed by washing, each well was filled with 200 µl of a solution of 3,3',5,5' - tetramethylbenzidine in DMSO (1% w/vol) diluted 100-fold with 0.1 M sodium acetate/citric acid pH 6 and supplemented with 3 µl of 30 % H_2O_2. The peroxidase reaction was allowed to develop for 10 min at room temperature; it was stopped by addition of 50 µl of 2 M H_2SO_4. The absorbance of the reaction medium was determined at 450 nm with an automatic reader.

SDS PAGE

Protein fractions were subjected to SDS-PAGE (**6**) in a 5 to 15 % acrylamide gel with a 5 % stacking gel. Separated proteins were stained with Coomassie brilliant blue R 250.

Production of antisera

Purified diamine oxidase (50 µg - 100 µg) in PBS (1 vol) was mixed with Freund's complete adjuvant (1vol) and injected into rabbits. Booster injections starting on day 15 using Freund's incomplete (1vol/1vol) adjuvant were repeated every 15 days for 2 months. Blood was taken 15 days after the last booster injection and allowed to clot at 37°C. After centrifugation (10 min at 500 g), the serum was decanted, diluted

with 1 vol glycerol and stored at -20°C. IgG was recovered from the antiserum using Protein A sepharose, as described (**7**).

Immunoblotting

Proteins were subjected to SDS-PAGE. Following electrophoresis, the gel and the nitrocellulose membrane were rinsed in cold electro-transfer buffer consisting of 15 mM Tris-HCl, 129 mM glycine, 0.01 % w/v SDS and 10 % methanol, final pH 8.3. Electrotransfer was performed for 5 h, at 0.8 mA/cm^2 and at 4° C (15 - 40 V) (**8**).

After blotting, the membrane was incubated for 60 min in the 50 mM Tris-HCl, 0.2 M NaCl, 0.02 % NaN_3, 1 mg/ml BSA, 1 mg/ml polyethylene glycol pH 7.6 (Tris-buffered saline) containing 3 % (w/v) serum albumin and then for 2 h with the appropriate specific antisera. After three washes in Tris-buffered saline, alkaline phosphatase-conjugated goat anti-rabbit IgG was added. After 45 min, the membrane was washed three times and color development was initiated by incubation with the NBT/BCIP reagent.

Purification of neutrophils and exocytosis experiments

Neutrophils purification and exocytosis experiments were recently described (**9**).

Biochemical assays

The diamine oxidase activity was assayed as described (**10**) using [^{14}C] putrescine 0,5 µCi/mmole as substrate. The assay was carried out in 15 mM Na K phosphate buffer pH 7.4 with a [^{14}C] putrescine concentration of 13 µM. The reaction was carried out at 37° C for 60 min and terminated by the addition of 1 mM aminoguanidine, a specific inhibitor of diamine oxidase. The product of the reaction was separated from the unused substrate by extraction with 10 ml of liquid scintillation solution containing 0.5 % (ter-butyl-4 phenyl) 2 (biphenyl-4) - 5 oxadiazole-1, 3,4 (butyl PBD) in toluene. The organic phase which contained the labeled reaction product was poured out and the radioactivity counted in a liquid scintillation counter. Activity was expressed as ng of putrescine hydrolyzed per h at 37° C.

RESULTS AND DISCUSSION

The described procedure consists of five steps and yielded 2 mg of homogeneous active enzyme from eight human placentae (Table I). The first step (ammonium sulfate subfractionation) eliminated the majority of low molecular weight proteins (figure 1). The subsequent chromatography

TABLE I Purification of diamine oxidase

STEPS	volume ml	Protein mg/ml	Total Protein mg	Total Activity ng (^{14}C) degraded putrescine	Specific Activity ng (^{14}C) degraded putrescine /h/mg	Recovery %	Purification Factor
HOMOGENATE	2030	13,2	26796	801850	30	100	1
$(NH_4)SO_4$ 40 % / 60 %	51	27,2	1387	127500	92	16	3
DEAE A 50	10	16,8	168	25672	152	3	5
Hydroxylapatite	11,5	9,1	105	15939	120	2	4
Concanavalin A	24	0,5	12	3888	324	0,5	11
FPLC Superose 12	21	0,09	1,85	3171	1714	0,4	57

Experimental conditions are detailed in Material and Methods

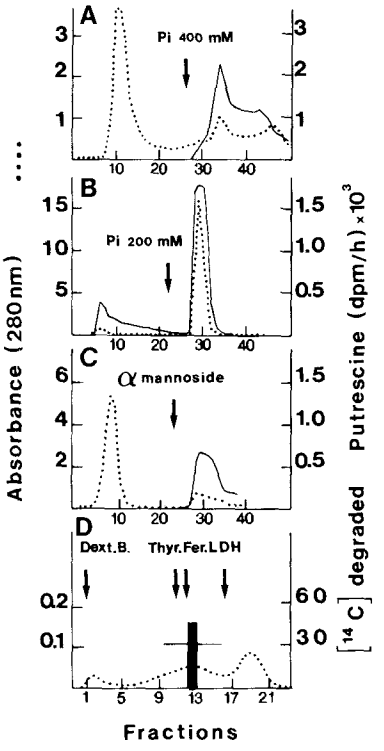


Figure 1. **Diamine oxidase purification**

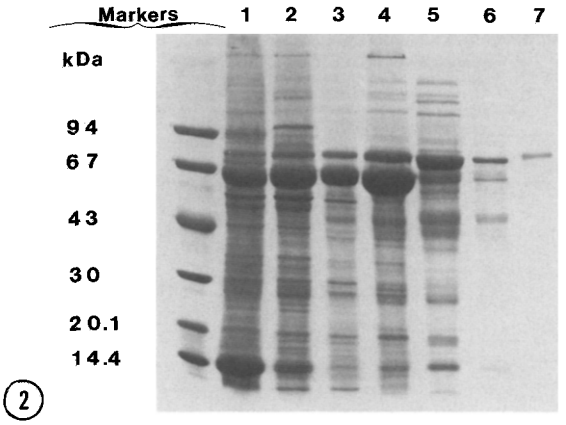
A. DEAE-Sephadex anion exchange chromatography of the 40-60 % $(NH_4)_2SO_4$ precipitated placenta homogenate proteins. The precipitated proteins were dissolved in 20 mM NaK phosphate buffer pH 7.4 and 20 ml of sample was applied to a A 50 DEAE Sephadex column (3 cm x 30 cm) equilibrated in the same buffer. Bound protein was eluted with 400 nM NaK phosphate pH 7.4 (arrow). Fractions of 10 ml were collected.

B. Hydroxylapatite chromatography.
Pooled active diamine oxidase fractions (10 ml) when applied to a hydroxylapatite column (1.2 cm x 10 cm) equilibrated with 10 mM NaK phosphate,pH 7.4. Elution (arrow) was performed with 200 mM NaK phosphate,pH 7.4. Fractions of 4 ml were collected.

C. Chromatography of diamine oxidase activity on concanavalin A Sepharose column (1.2 cm x 8 cm). Pooled active diamine oxidase fractions recovered from the hydroxylapatite step were applied to the column, which was washed with column buffer and then eluted in the same buffer containing 0.2 M methyl α-D-mannoside. Fractions of 3.5 ml were collected. Elution was initiated at the fraction indicated by the arrow.

D. Gel filtration of diamine oxidase activity on a Superose 12 HR 10/30 FPLC column. Fractions of 250 µl were collected. Standardization of the gel filtration column with globular protein markers as cytochrome c 12.5 kDa, bovine serum albumin 67kDa, lactate dehydrogenase 148 kDa, ferritin 440 kDa, tyroglobulin 669 kDa, dextran blue 2000 kDa, indicated that the molecular mass of the enzyme in the major peak was about 180 kDa.

steps were DEAE Sephadex anion exchange chromatography (step A), hydroxylapatite chromatography (step B), concanavalin A Sepharose chromatography (step C) and gel filtration onto Superose 12 (step D) (figure 1). Steps A and C were particularly efficient in removing contaminant proteins (figure 1). Following hydroxylapatite chromatography (step B) proteolysis of diamine oxidase was markedly diminished. Binding



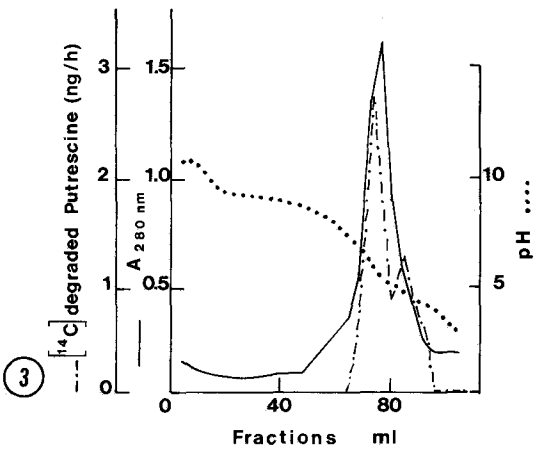


Figure 2. **SDS-PAGE (5 - 15 % gel) of the purified diamine oxidase**
Lane 1 contains the proteins of homogenate. Lane 2 and 3 contain the proteins precipitated by 40 % and 60 % $(NH_4)_2$ SO_4 respectively. Lane 4 was the same as lane 3 after dialysis. Lane 5 and 6 contain the active diamine oxidase fraction recovered after A 50 DEAE sephadex and concanavalin A Sepharose chromatography respectively. Lane 7 was the electroeluted purified diamine oxidase. Fifty µg of each fraction was loaded on the gel except for lane 7 where the amount used was 25 µg. On the left, the molecular mass standard proteins from Pharmacia are phosphorylase b 94 kDa, albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 20.1 kDa and α lactalbumin 14.4 kDa. Staining was performed with Coomassie blue.

Figure 3. **Electrofocusing of human placental diamine oxidase**
Isoelectric focusing was carried out at 4° C on a diamine oxidase enriched fraction (11.2 mg protein), recovered from the concanavalin Sepharose column, in a 110 ml sucrose gradient (0 - 40 % sucrose) containing 1.7 % v/v LKB ampholines. Conditions are described under Material and Methods. The protein content of the eluate was monitored at 280 nm. The enzyme activity was assayed on 0.2 ml of each fraction.

of diamine oxidase to concanavalin A Sepharose and elution by 0.2 M methyl α-D- mannoside (step D) was in accordance with the glycoprotein nature of the enzyme (**11**). In the purification experiment shown in Table I, the specific activity of purified diamine oxidase was 1714 ng [^{14}C] putrescine degraded per hour and per ng protein; the purification factor was 57 and the yield 0.4 %.

Purified placenta diamine oxidase had a molecular mass of 84, 000 dalton as determined by SDS-PAGE (figure 2) which is in the range of the published values (**3, 12**). Upon isoelectric focusing, placental diamine oxidase focused in two peaks at pH 4.9 and 5.5 (figure 3). The western blot illustrated in figure 4 B shows that anti-diamine oxidase antibodies reacted with the placenta purified protein (lane 1), with the amniotic fluid diamine oxidase (lane 2) and with the diamine oxidase present in a neutrophil supernatant recovered from exocytosis (lane 3). The data presented in figure 5 A illustrate the potent reactivity of the antisera

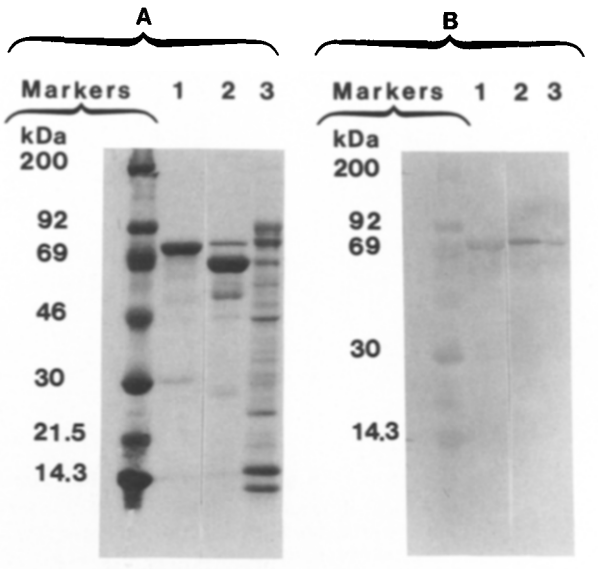


Figure 4. **SDS-PAGE and immunoblots of diamine oxidase**

A. SDS-PAGE : 50 µg of enzyme preparation was loaded on a 5-15 % SDS-PAGE gel adjacent to molecular weight markers under reducing conditions. Lane 1, partly purified diamine oxidase; lane 2, amniotic fluid; lane 3, exocytosis supernatant. The gel was stained with Coomassie Blue and destained.

B. WESTERN-BLOT : electroblotting onto nitrocellulose of the separated proteins. The membrane was incubated with preimmune or immune anti serum against diamine oxidase with a 1:200 dilution and with a goat anti rabbit IgG alkaline phosphatase, 1:1000. Staining was performed with NBT-BCIP reagent.

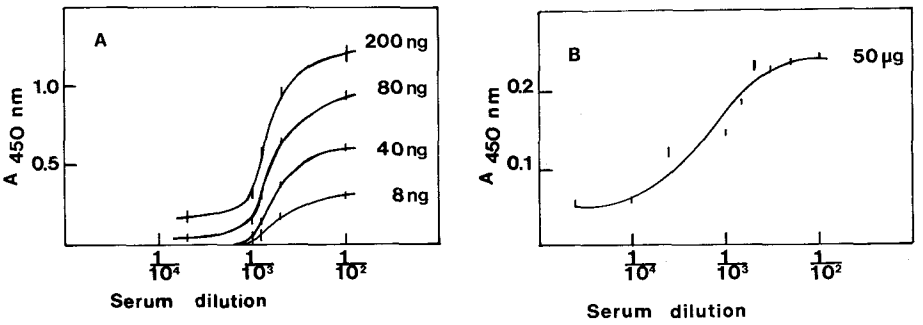


Figure 5.

Reactivity of anti-diamine oxidase antiserum against placenta purified diamine oxidase and secreted diamine oxidase from neutrophils assessed by ELISA.

Microtiter plates were coated with (A) partially purified diamine oxidase recovered from concanavalin Sepharose (8-200 ng/ml in PBS) or (B) with exocytosis supernatant of FMLP stimulated neutrophils (50 µg in PBS). The immobilized proteins were incubated with the corresponding antisera added at different dilutions. This was followed by addition of peroxidase-conjugated rabbit antibody, hydrogen peroxide as substrate and TMB as chromogenic indicator (cf. material and methods).

against the antigen. No reaction was found with the preimmune serum. The ELISA was also used to investigate the diamine oxidase activity of the supernatant recovered from exocytosis experiments carried out with FMLP stimulated neutrophils. The results (figure 5 B) corroborated immunoblotting experiments (figure 4) showing immunoreactivity of specific antiserum with the purified placenta diamine oxidase and the secreted neutrophil enzyme.

In this paper, we have been described a convenient procedure for the purification of diamine oxidase from human placenta and the isolation of specific antibodies against this enzyme. Although several reports have appeared on diamine oxidase from different sources (see Introduction), very few studies on neutrophil diamine oxidase have so far been made. This is probably due to the low activity of diamine oxidase in neutrophils and to the lack of sensitive methods for assaying the enzyme. The observation that antibodies against the placental diamine oxidase cross-react with the neutrophil enzyme points the way to an immunochemical analysis of the neutrophil diamine oxidase.

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