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QUINOPROTEIN CHARACTERISTICS OF *N*-METHYLPUTRESCINE OXIDASE FROM TOBACCO ROOTS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; nicotine biosynthesis; *N*-methylputrescine oxidase; quinoprotein.

Abstract—*N*-Methylputrescine oxidase (MPO) was partially purified from *Nicotiana tabacum* roots by ammonium sulphate fractionation, anion-exchange chromatography, and metal chelate chromatography. Analytical-scale gel filtration HPLC in the presence of detergent reduced the composition of the final preparation to six protein species. Several amine oxidase inhibitors were screened for inhibition of partially-purified MPO. Phenylhydrazine and dinitrophenylhydrazine were the most inhibitory. Inactivation by phenylhydrazine had characteristics typical of 'suicide' inhibition, suggesting that MPO is a quinoprotein. Treatment of partially-purified MPO with [^{14}C]-labelled phenylhydrazine yielded a radiolabelled band of *ca* 70000 M_r on SDS-PAGE.

INTRODUCTION

Considerable evidence has accumulated for the biosynthetic route to nicotine in root tissue of *Nicotiana tabacum* (reviewed in ref. [1]). However, the enzymes of this pathway have yet to be purified to homogeneity and characterized in detail. Partial purification and limited characterization have been reported for putrescine *N*-methyltransferase (PMT) [2, 3] and *N*-methylputrescine oxidase (MPO) [4]. It has been suggested that *N*-methylputrescine oxidase is a copper-containing amine oxidase [4], and this raises the possibility that it also possesses a second, pyrroloquinoline quinone, prosthetic group. Although many quinoprotein enzymes have been demonstrated in bacteria and animals (reviewed in ref. [5]), there has only been one identification of a quinoprotein in plants [5]. Complete proof of quinoprotein identity requires dissociation, purification, and characterization (spectral and/or structural) of the pyrroloquinoline quinone (PQQ) cofactor, needing comparatively large amounts of the pure enzyme. For enzymes of plant secondary metabolism such as MPO this is impractical because they are only obtainable in very low yields. We have been able to assemble indirect evidence in support of a quinoprotein identity for MPO, which should be of value in further purification and characterization of the enzyme protein.

RESULTS AND DISCUSSION

Partial purification

In our hands the partial purification method of Mizusaki *et al.* [4] produced a low final yield of MPO. Furthermore we found the final preparation to be very heterogeneous when analysed by SDS-PAGE. Therefore we developed an alternative procedure that would yield the greatest specific activity using a minimum number of steps. Simplicity of the overall scheme was important in view of the substantial losses of activity occurring with each chromatographic technique (Table 1).

During the evolution of this scheme many chromatographic techniques were tested and the possibility of affinity chromatography was examined. The enzyme was bound by an immobilized substrate analogue (aminodecyl agarose) used for the purification of microbial diamine oxidases [6], but the elution profile from such a column was very similar to that obtained with anion exchangers. Higher salt concentrations were required to elute MPO from an immobilized imine (PEI-silica, Fig. 1) than from an immobilized quaternary amine (DEAE-cellulose), and the imine column effected better separation of MPO from other proteins. These observations led to the speculation that an affinity-based interaction might be taking place between MPO and immobilized imino groups. However, attempts to elute MPO from the imine column using substrate at the point of incipient salt elution were unsuccessful.

Polyols such as glycerol (e.g. at 20% v/v) completely prevented activity losses on freezing, but were unable to provide any protection during chromatography. 2-Mercaptoethanol was an essential component of all buffers, but the inclusions of BSA, extra mercaptoethanol, cupric ions, various protease inhibitors (leupeptin, pepstatin, PMSF), sodium chloride, or diamines (putrescine or *N*-methylputrescine (*N*MP); extraction buffer only) did not confer additional stability during chromatography or stimulate aged preparations. Our net conclusion from surveying a wide range of chromatographic systems was that MPO activity is least stable in the presence of highly charged supports and most stable in uncharged gel filtration media. Protease inhibitors were retained in the buffers as a precaution.

Typical elution profiles from the PEI-silica module and chelated-zinc column are shown in Figs 1 and 2. Table 1 summarizes the results of a typical partial purification. The final preparation was seen by SDS-PAGE analysis to

Table 1 Partial purification of MPO from 2 kg of tobacco roots

Stage	Volume (ml)	Protein (mg)	Activity (nkat)	Sp activity (nkat/mg)	Yield (%)	Purification (fold)
Crude	3850	3800	127	0 033	100	1
$(NH_4)_2SO_4$	60	568	28	0 16	22	1 5
PEI-silica	12	80	22	0 28	17	8 3
Chelated Zn	2 2	1 07	2.9	2.7	2 3	81

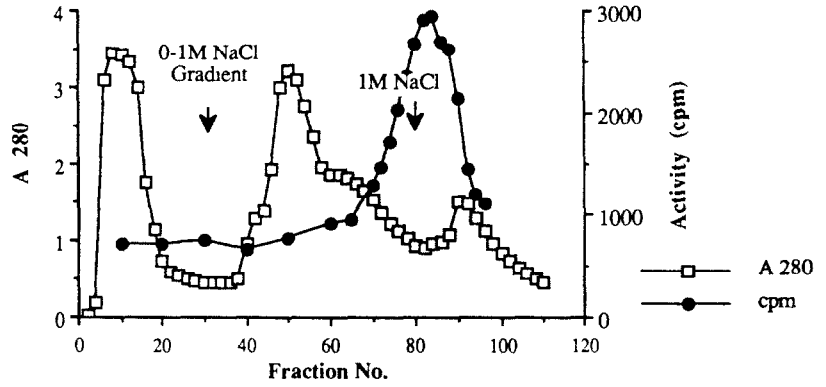

Fig 1 Anion exchange chromatography of ammonium sulphate fraction on 'Fastchrom' PEI-silica module. The ammonium sulphate preparation (125 ml, 2400 mg protein, 88 nkat) was applied to a 9.0 × 2 5 cm Kontes 'Fastchrom' PEI-module in buffer B at 600 ml/hr, collecting 25 ml fractions. Elution was by a 1 2 linear 0–1 M NaCl gradient in buffer B beginning at fraction 32 The module was washed with 1 M NaCl in buffer B beginning at fraction 83 Enzyme activity is shown as cpm/2 ml toluene layer in the radiometric MPO assay Fraction aliquots of 200 μl were assayed, using an incubation time of 20 min

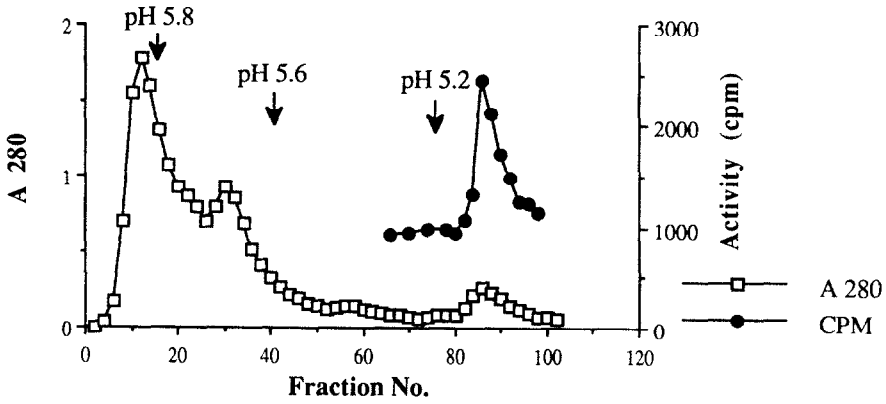

Fig 2 Chelated zinc chromatography of MPO from the PEI-silica module Twenty-six ml of sample (290 mg, 21 nkat) were applied to an equilibrated chelated zinc Sepharose-6B column (4 8 × 7.2 cm) Fractions of 8 ml were collected at a flow rate of 2 3 ml/min Activity is shown as cpm/2 ml toluene layer in the radiometric MPO assay Fraction aliquots of 50 μl were assayed, using a 30 min incubation time The MPO activity was eluted in the pH 5 2 wash, earlier fractions were shown to contain no enzyme activity

contain nine significant protein species and *ca* 12 minor ones.

When this preparation was subjected to analytical-scale gel filtration HPLC, MPO activity was eluted from the column at an early retention time corresponding to the void volume, with an apparent M_r in excess of 220 000. Analysis of the active fractions by SDS-PAGE showed the presence of many protein species over a range of M_rs. These results suggested to us that we had purified protein aggregates in which the MPO protein was bound, and that further purification of MPO would require aggregate dissociation.

Our strategy to alleviate this aggregation problem comprised detergent treatment followed by HPLC Several ionic, nonionic and zwitterionic detergents were tested for their effects on MPO activity by inclusion in the

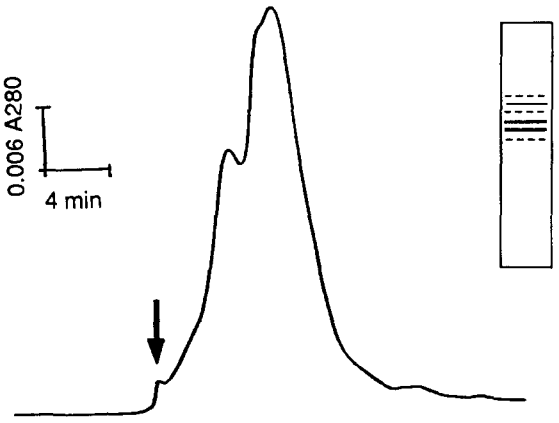

Fig. 3 Gel filtration HPLC of MPO in the presence of SDS. The HPLC column was BioRad BioSil TSK 400 and the run buffer comprised 100 mM Tris–HCl pH 7.0, 10 mM 2-mercaptoethanol, 0.1 mM SDS. The MPO sample was obtained by partial purification and dialysed against buffer B prior to loading. The arrow shows the position of MPO activity in the eluted fractions Most of the fractions showed large numbers of bands when analysed by SDS-PAGE except the early, active fractions which showed only six, weakly-staining, significant bands These are diagrammed in the inset.

assay. Tween 20 and Triton X-100 up to 0.5% (v/v) did not affect enzyme activity, and neither did SDS up to 1% (w/v). There was a 20% reduction of MPO activity by 0.75% (w/v) CHAPS. Each of these detergents was then added to a partially purified MPO sample immediately prior to HPLC. The only detergent to change the HPLC profile significantly was SDS (results not shown).

Inclusion of 0.0015% w/v SDS (0.1 mM) in the HPLC mobile phase provided useful resolution (Fig. 3). Detectable MPO activity was recovered from the column under these conditions (*ca* 20% yield). The eluted fractions from several identical HPLC runs were correspondingly combined to obtain sufficient material for SDS-PAGE analysis. Six bands were visible in the eluted fractions that contained MPO activity (Fig. 3). Three of these bands were of similar M_r to lentil seedling diamine oxidase subunit (*ca* 75 000) [7]. This HPLC step was not used in routine partial purification because the overall yield for the complete scheme was too low.

Sensitivity to amine oxidase inhibitors

Two principal classes of amine oxidase have been recognized, i.e. flavin-containing 'monoamine oxidases' and copper-containing 'diamine oxidases' (reviewed in ref. [8]). Methylputrescine oxidase is thought to belong to the latter category because of sensitivity to certain carbonyl reagents and indirect evidence that it contains copper [4]. Many inhibitors have been described for both types of oxidase [8] and some of these have been shown to bind to the enzyme protein covalently, e.g. 2-aminopropionitrile inhibition of aortic lysyl oxidase [9]. We tested the sensitivity of MPO to several amine oxidase inhibitors (Table 2).

The results indicated a considerable sensitivity of MPO to phenylhydrazine and dinitrophenylhydrazine (Table 2). Some of the other compounds tested (e.g. aminoacetonitrile, 2-aminopropionitrile, propargylamine) were also inhibitory, but less effective than dinitrophenylhydrazine. Several of the less effective compounds

Table 2. Inhibition (%) of MPO activity by various amine oxidase inhibitors

	Concentration in assay (μM)						
Inhibitor	1	10	50	100	150	500	1000
Aminoacetonitrile				38		75	84
2-Aminopropionitrile				2		4	7
Quinacrine				41		73	86
Pargyline			<1		<1	2	
Phenylcyclopropylamine					22	47	64
Phenylhydrazine			52		77	91	
Phenylhydrazine (a.s.)	26	90		99			99
Phenylhydrazine (PEI)	22	74	95	98			
2,4-Dinitrophenylhydrazine (a.s.)		20	85	100			
Propargylamine				72		94	96
Semicarbazide				12		25	42
Sodium azide				5		3	6
Sodium azide (a s.)		4		9			16
2-Bromoethylamine (a s.)		11		21			82

Inhibitors were included in the standard MPO assay, without substrate, for a 30 min preincubation at 30°. Radiolabelled *N*-methylputrescine was then added and after a further 30 min incubation at 30° the reaction was stopped and the product extracted as usual.

The MPO had been partially purified through the complete scheme (i e. with chelated zinc chromatography as the final step) except where indicated as 'PEI' (post PEI-silica column) or 'a.s.' (ammonium sulphate fraction).

are potent inhibitors of monoamine oxidases (e.g. phenylcyclopropylamine, pargyline), so our results suggest that MPO is of the diamine oxidase type. Furthermore phenylhydrazine has been shown to bind irreversibly to lentil seedling copper-containing amine oxidase [10]. Phenylhydrazines have been used by J. A. Duine to identify the organic prosthetic group of microbial and animal diamine oxidases as pyrroloquinoline quinone (PQQ) [11], a cofactor which has also been shown to occur in plants [5] Duine's group has shown that these compounds function as 'suicide' inhibitors and bind irreversibly to the covalently linked PQQ-enzyme complex. We examined the phenylhydrazine inactivation of MPO to see if it had characteristics of 'suicide' inhibition, which we defined as irreversibility, a relatively low inactivation rate suggestive of an involvement of the catalytic mechanism, and a susceptibility to competition by substrate (*N*MP).

An uninhibited control preparation of MPO lost only 15% of its activity when dialysed for 21 hr against a large excess of buffer B. Preparations which had been inhibited 14 and 62% with phenylhydrazine also retained their corresponding activities, showing that the inhibition could not be reversed by dialysis. Phenylhydrazine inactivation took place relatively slowly, e.g. 60% inhibition in 30 min by 3 μM phenylhydrazine.

Evidence of competition between *N*MP and phenylhydrazine is shown in Table 3. Incubation of MPO with (unlabelled) *N*MP, followed by rapid gel filtration chromatography to remove the substrate, resulted in some loss of activity relative to control samples which had been incubated without *N*MP and processed identically As expected, there was also a reduction in activity if the enzyme was incubated with sufficient phenylhydrazine for partial inhibition and then subjected to gel filtration chromatography. The effect of incubation with *N*MP and phenylhydrazine together depended on the degree of purity of the MPO preparation. Using the most purified enzyme (Table 3, 'Zn stage') the combined incubation resulted in an activity drop that was considerably less than the sum of these two separate effects of *N*MP and phenylhydrazine. This result would be expected if substrate protected the enzyme against phenylhydrazine inhibition. It was reproducible using different substantially purified MPO preparations. It is interesting to note that with less pure preparations the protective effect was much less apparent (e.g. Table 3, 'PEI stage'). Purification might therefore have resulted in an alteration of the relative affinities for *N*MP and phenylhydrazine, or in the differential loss of a sub-population of MPO molecules having greater affinity for phenylhydrazine than for *N*MP

Derivatization with radiolabelled phenylhydrazine

These results resemble those obtained by Duine and other groups investigating the inhibition of diamine oxidases by phenylhydrazine and dinitrophenylhydrazine. We therefore suggested that tobacco root MPO is a PQQ-containing diamine oxidase and that it should be possible to radiolabel the protein with labelled phenylhydrazine as described for other such enzymes. To test this possibility, an MPO preparation at the PEI-silica stage was incubated with [^{14}C]-phenylhydrazine. Any unreacted inhibitor was then removed by rapid gel-filtration chromatography, and the macromolecular products were lyophilized and analysed by SDS-PAGE The gel was sectioned and the radioactivity of each section determined by liquid scintillation counting The slice profile (Fig. 4a) showed three peaks of apparent M_r 70, 50 and 30 000 (section numbers 5, 8, and 14 + 15 respectively), as well as a large peak that had migrated with the tracking dye The area of radioactivity in the M_r 70 000 region was of particular interest in view of the similar size of another plant diamine oxidase, that from lentil seedlings (*ca* 75 000/subunit; [6]).

Subsequent labelling experiments were done using enzyme purified through the chelated zinc column (Fig 4b). There was a reduction in the radioactivities of the M_r 50 and 30 000 regions, and an increase in radioactivity in the 70 000 region These results were reproduced with separate MPO preparations. The evidence suggesting that this radiolabelled band of M_r *ca* 70 000 represents MPO can be summarized as follows. (i) enzyme preparations taken through the entire purification procedure gave a simpler pattern of radiolabelled products in the gel than preparations taken only through to the PEI-silica

Table 3 Effect of substrate on inhibition of MPO by phenylhydrazine

	PEI stage			Zn stage		
Treatment	cpm 1	cpm 2	% Inhib	cpm 1	cpm 2	% Inhib
Control	4440	4560	—	1910	1920	-
NMP	3110	3290	29	1430	1480	24
PH	2960	3050	33	944	839	53
NMP + PH	1840	1850	59	1350	1320	39

An MPO preparation at either the PEI-silica column stage ('PEI stage') or the chelated-zinc column stage ('Zn stage') in purification was incubated at 30° for 30 min with no additions (control), 2 μM phenylhydrazine, 0.7 mM unlabelled *N*-methylputrescine (*N*MP), or phenylhydrazine (PH) + *N*MP at the above respective concentrations Radioactive *N*MP was then added to provide the same specific radioactivity as that used in the standard activity assay, and incubation was continued for a further 30 min The radioactive product formation therefore indicated the MPO activity in this second stage of the procedure Activity was determined as background-corrected radioactivity in 3 ml of the toluene layer, duplicate incubations are shown ('cpm 1' and 'cpm 2') Mean percentage inhibitions are also indicated

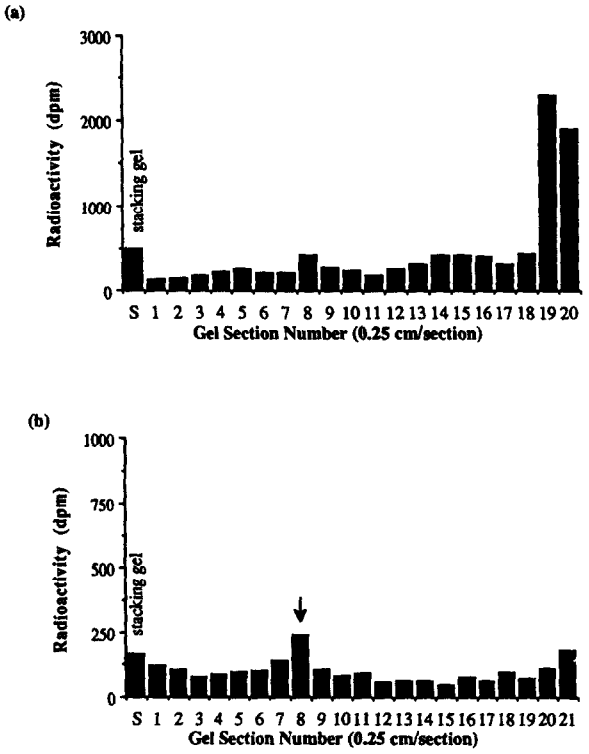

Fig. 4. Analysis of [^{14}C]-phenylhydrazine-derivatized MPO preparations by SDS-PAGE. (a) A preparation of MPO at the PEI-silica purification stage (0.31 nkat) was incubated for 30 min at 30° with [U-^{14}C]-phenylhydrazine (0.86 mM, 9.64 μCi/μmol) in buffer A containing 1 μM pepstatin (total volume 960 μl). Another aliquot of MPO preparation was incubated in parallel with unlabelled phenylhydrazine and assayed to verify 100% inhibition. The labelled sample was then subjected to rapid gel filtration chromatography. The macromolecular fraction was lyophilized, redissolved in SDS-PAGE sample buffer and electrophoresed. Each lane was then cut from the gel and divided into 0.25 cm sections. Each section was ground to fine particles with a glass rod in the scintillation vial and scintillation fluid was added. Counting efficiency, typically around 95%, was determined by the 'sample channels ratio' method (b) A similar experiment performed with MPO at the chelated zinc chromatography stage in purification, incubating 1.7 nkat of activity with phenylhydrazine in a total reaction volume of 1170 μl. The SDS-PAGE conditions were modified to provide increased resolution; the resolving gel bis-acrylamide concentration was lowered from 0.8% (w/v) to 0.6%, and the run-time was increased. These changes resulted in altered mobilities, and electrophoresis of dye front and its associated radioactivity out of the gel. The arrow marks the position corresponding to an apparent M_r of 70 000.

stage. The M_r 70 000 labelled region was the only significant radioactive area of the gel to remain after the MPO preparation was taken from the PEI-silica stage through the chelated zinc column. (ii) The radioactive region of interest is in the same area of the gel as the three lower M_r bands obtained from additional gel filtration HPLC of uninhibited MPO (Fig. 3). (iii) The radioactive region of interest has an apparent M_r very similar to that of the lentil seedling diamine oxidase subunit.

We take these results, together with the above characteristics of phenylhydrazine inhibition, as evidence that MPO is a quinoprotein. Assuming a subunit M_r of 70 000, we calculated from the phenylhydrazine radioactivity in that region of the SDS gel that 1 kg fresh weight of root tissue yielded *ca* 50 μg of this protein before purification, 1 μg at the end of our routine procedure. Additional purification of the enzyme protein should now be possible via excision of the phenylhydrazine-labelled region from an electrophoresis gel.

EXPERIMENTAL

Chemicals. *N*-Methylputrescine and [^{14}C]-*N*-methylputrescine (1.3 mCi/ml, 22 mCi/mmol) were gifts. Phenylhydrazine was purchased from Sigma, and [U-^{14}C]-phenylhydrazine from ICN (9.64 mCi/mmol).

Buffers. (A) 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 10 mM 2-mercaptoethanol, 2% (w/v) polyethyleneglycol 400, 0.5% (w/v) Na ascorbate, 0.5 mM PMSF, 1 μM leupeptin and 1 μM pepstatin. (B) 10 mM Tris–HCl pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM PMSF, 1 μM leupeptin and 1 μM pepstatin. (C) 10 mM MES–NaOH pH 6.5, 0.5 mM NaCl and 10 mM 2-mercaptoethanol. (D) 25 mM MES–NaOH pH 5.8, 0.5 mM NaCl and 10 mM 2-mercaptoethanol. (E) buffer D at pH 5.6 (F) buffer D at pH 5.2.

Plant material. Nicotiana tabacum (cv Samsun) plants were germinated in a growth chamber, then transferred 3–4 weeks later to the greenhouse. At 6–7 weeks from germination they were transplanted into individual 1-gallon pots. The soil mix comprised sand, peat moss, perlite, and vermiculite (2:5:6:5 v/v), and the plants were watered with Plantex 15-15-18 fertilizer (Plantco Inc., Brampton, Ontario) diluted to provide 120 ppm nitrogen. Greenhouse conditions were 24–30° maximum, 16–21° minimum temp., with a 16 hr photoperiod. Roots were harvested 2–3 months from germination. In accordance with the observations of ref. [3] we observed no increase in enzyme activity after topping the plants, but we routinely removed 15–20 cm of the apex 18–28 hr prior to the root harvest. The detached roots were cleaned by rinsing with deionized H_2O, and stored at −70°.

Enzyme extraction. All extraction and purification steps were performed at 0–4°. 2 kg of tissue were homogenized in a Waring blendor with 4 l buffer A. The homogenate was filtered through 2 layers of cheesecloth, brought to 40% satn with $(NH_4)_2SO_4$, then centrifuged at 10 000 *g* for 30 min. The supernatant fraction was recovered and $(NH_4)_2SO_4$ added to 65% satn. After centrifugation as above the pellet was resuspended in buffer A with the Tris at 10 mM and polyethylene glycol omitted. The resuspended pellet was dialysed overnight against buffer B.

Partial purification. A Kontes 'Fastchrom' PEI-silica anion exchange module (2.5 × 9.0 cm) was equilibrated to pH 7.5 with 100 mM Tris–HCl pH 7.5 then washed with buffer B. The dialysed enzyme preparation was centrifuged at 27 000 *g* for 30 min and applied to the module, which was then washed with buffer B until the A_{280} of the effluent fell below 0.5. A 0–1 M NaCl gradient (total vol. 1.2 l) in buffer B was then applied. Additional 1 M NaCl in buffer B was used to remove remaining MPO activity which eluted near the end of the gradient. The active fractions were pooled and brought to 65% satn $(NH_4)_2SO_4$. After centrifugation as described above the pellet was redissolved in buffer C. This preparation was loaded onto a 4.8 × 7.2 cm Pharmacia Chelating Sepharose-6B column which had been charged with Zn^{2+} per the manufacturer's instructions and equilibrated with buffer C. The column was then washed successively with buffer C, buffer D, buffer E, buffer F, and 50 mM

EDTA The MPO activity eluted in the pH 5 2 wash. Active fractions were pooled, and concd using an Amicon Centriprep-30 filter unit The pH was adjusted to 7 5 using 0.42 M Tris–HCl pH 8.0. This preparation was dialysed against 10 mM Tris–HCl pH 7 5, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, 0 5 mM PMSF, 1 μM leupeptin and 1 μM pepstatin, and stored at $-20°$ or used immediately.

Enzyme assay. The MPO activity was determined by the method of ref [12], the reaction product (1-methyl-2-cyanopyrrolidine) being partitioned into toluene for liquid scintillation counting. Protein was estimated by the procedure of ref [13]

Batchwise gel filtration chromatography. In phenylhydrazine derivatization experiments a modification of the method of ref [14] was used to remove unbound labelled phenylhydrazine. *Ca* 5 ml of slurried Sephadex G25-80 (5 g/50 ml swollen in 10 mM Tris–HCl pH 7.5) were pipetted into Fisher disposable filter columns and packed by centrifugation at 1 400 *g* for 3 min to give a final bed vol of 3 ml. The columns were then washed twice with 3 ml 1 mM Tris–HCl pH 7 5, 10 mM 2-mercaptoethanol, 0 5 mM PMSF, 1 μM pepstatin and 1 μM leupeptin, centrifuging each time exactly as above The sample was applied and filtration effected by the same centrifugation procedure

Electrophoresis. For SDS-PAGE the standard Laemmli procedure was used Gel dimensions were $5 \times 8 \times 0.15$ cm, and compositions 10% (w/v) acrylamide (5% in the stacking gel), 0.6% (w/v) bis-acrylamide

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