3-Nitropropionic acid oxidase from horseshoe vetch (*Hippocrepis comosa*): a novel plant enzyme

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A novel enzyme that catalyses the oxygen-dependent oxidation of 3-nitropropionic acid (3NPA) to malonate semialdehyde, nitrate, nitrite and H₂O₂ has been purified from leaf extracts of the horseshoe vetch, *Hippocrepis comosa*, and named 3NPA oxidase. The enzyme is a flavoprotein with a subunit molecular mass of 36 kDa containing 1 molecule of FMN and exhibits little specificity for all nitroalkanes tested other than 3NPA (apparent K_m 620 μ M). The maximum enzyme activity *in vitro* was expressed

at pH 4.8 and was inhibited strongly by the products nitrate and nitrite. 3NPA oxidase activity was detected in green shoots, which also contain high concentrations of 3NPA, from plants grown with nitrate, ammonium or N₂ as sources of nitrogen. Enzyme activity was absent from roots and cell cultures, neither of which accumulate high levels of 3NPA.

Key words: flavin, legume, nitrate, nitrification, nitrite.

INTRODUCTION

Free and esterified forms of 3-nitropropionic acid (3NPA) accumulate in large quantities in the shoots of certain leguminous plants [1–3]. As a potent inhibitor of succinate dehydrogenase [4], 3NPA is extremely toxic and probably serves as a powerful chemical defence against herbivory in plants that contain it. However, the inhibition of phloem mitochondrial function by 3NPA poses severe limitations on its translocation from senescing leaves and necessitates a detoxification process to conserve such a large resource of fixed carbon and nitrogen.

A number of prokaryotes and fungi contain oxidase enzymes that catabolize the breakdown of nitroaliphatic compounds to a residual carbon skeleton plus oxidized inorganic nitrogen [5–8]. In comparison, little is known about the metabolism of 3NPA in plants. Horseshoe vetch, *Hippocrepis comosa*, contains very high concentrations of 3NPA in its leaves [3]; here we characterize an enzyme from the leaves of this species that catalyses the oxidation of 3NPA, which might function as part of a detoxification mechanism. Significantly, the enzyme reaction also accomplishes a nitrification in which both nitrate and nitrite are generated as reaction products.

MATERIALS AND METHODS

Plant material

H. comosa L. was grown in water culture with an Arnon and Hoagland culture solution (diluted 1:5) [9]. Nitrogen in these cultures was supplied as potassium nitrate, ammonium tartrate or urea (2.8 mM) and iron was supplied as FeEDTA. For enzyme purification, fresh leaves were also collected from natural, limestone cliff populations near Mumbles (Swansea, U.K.).

3NPA oxidase assay

Enzyme activity was measured by the 3NPA-dependent production of nitrite or the consumption of oxygen. The reaction mixture consisted of 67.5 μ mol of 3NPA, 225 μ mol of Mes buffer, pH 5.0 (unless otherwise stated), and 0.1 ml of cell-free extract or purified enzyme, in a final volume of 1 ml. Assays were incubated for 5 min at 30 °C. Nitrite was estimated color-

imetrically [10]. Oxygen consumption was followed polarographically in a Hansatech oxygen electrode [11]. Protein was determined by the Bradford method [12].

Enzyme purification

Leaves (25 to 40 g fresh weight) were ground in 200 ml of 0.5 M Mes buffer. After centrifugation at 10000 *g* for 25 min at 2 °C, the supernatant was filtered through a 0.45 μ m Acrodisc syringe filter (Gelman). A 40–60 % satd. (NH₄)₂SO₄ fraction of this crude extract was then dialysed for 48 h against two changes of 1.5 litres of Mes buffer at 4 °C; 10 ml of dialysed extract was then subjected to FPLC (Pharmacia) anion-exchange chromatography at a flow rate of 1 ml/min with Q-Sepharose (Sigma) in a 6 cm $\times$ 1 cm column. The column was equilibrated with 90 ml of buffer A (0.05 M Mes buffer) before being loaded with a 10 ml superloop. After an initial 60 ml elution wash with buffer A, the column was eluted with a 20 ml 0–35 % gradient of buffer B (buffer A containing 1 M K₂HPO₄), followed by a 5 ml 35–100 % gradient of buffer B. Fractions (1 ml) with 3NPA oxidase activity were pooled and concentrated to a volume of approx. 500 μ l by ultrafiltration with an Amicon PM 30 filter system for 45 min at 2000 *g*. Ultrafiltered protein was then subjected to FPLC gel filtration, in 200 μ l batches, with a Superose 12 HR/30 column (Pharmacia). All active fractions were pooled and ultrafiltered as necessary.

Analytical methods

3NPA was determined by the method described by Matsumoto et al. [13], nitrate was measured by the cadmium reduction method [14] and malonic semialdehyde was measured by the method described by Porter and Bright [8].

Enzyme and flavin fluorescence spectra were recorded with a Perkin-Elmer LS-5B luminescence spectrometer and absorption spectra were observed with a Cecil CE 6602 Scanning UV Spectrophotometer. FMN was dissociated from the enzyme after incubation at 100 °C for 20 min and identified by a combination of paper chromatography (Whatman no. 4) with 5 % (w/v) Na₂HPO₄ as the developing solvent at 20 °C, apoenzyme reconstitution [7] and measurement of the change in fluorescence at

Abbreviation used: 3NPA, 3-nitropropionic acid.

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525 nm (excitation at 445 nm) before and after the addition of phosphodiesterase in *Naja naja* venom [15]. The molar absorption coefficient (ϵ_{446}) of the flavoprotein was estimated after comparison of the spectra of free ($\epsilon_{445}^{\text{FMN}}$ 12.5 mM⁻¹·cm⁻¹) and bound FMN.

Subunit size was determined by SDS/PAGE with the use of standard methods [16] and matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) MS with a Kratos Kompact MALDI III system in linear, positive-ion mode and a sinapinic acid matrix. 3NPA oxidase was prepared for MS in a solution containing 50 µg/ml protein.

N-terminal amino acid sequence analysis was performed in the School of Biological Sciences (Royal Holloway College, University of London, London, U.K.) with an Applied Biosystems 477A amino acid sequencer.

Nitrogen fixation was measured by the acetylene reduction technique [17].

RESULTS AND DISCUSSION

Purification and composition of 3NPA oxidase

Cell-free extracts of *H. comosa* contained large amounts of nitrite, which hampered initial investigations of the enzyme *in vitro*. However, partly purified extracts [40–60%–satd. (NH₄)₂SO₄ fractionation, followed by dialysis], yielded preparations in which a 3NPA-dependent nitrite-generating/oxygen uptake activity could be demonstrated. Tentatively, this enzyme activity was named 3NPA oxidase.

Purification was achieved after FPLC (Pharmacia) employing Q-Sepharose anion exchange, ultrafiltration and Superose-12 gel filtration, yielding a pale yellow protein preparation. Details of a purification starting with 25 g of fresh leaves are shown in Table 1. Precipitation of the crude cell-free extract with (NH₄)₂SO₄ followed by dialysis resulted in an activation of enzyme activity. The final yield (calculated on the basis that 100% total activity was expressed at the dialysis step) was 23%. Pure enzyme preparations exhibited a maximum activity at 40–50 °C and lost more than 50% of activity after being boiled for 5 min and more than 90% activity after being boiled for 25 min. The enzyme activity of pure preparations was not activated by the addition of 5 µM FAD or FMN. SDS/PAGE indicated one protein band at 37 kD (Figure 1a) and a MALDI–TOF mass spectrum of a separate preparation indicated a major peak at 35.4 kDa (Figure 1b).

A partial N-terminal amino acid sequence analysis (20 cycles) for the enzyme was obtained, suggesting that the N-terminus was not blocked. The sequence obtained was S¹KE/PFLLYFWY-GILQAXXGPD²⁰. The average amino acid repetitive yield during this analysis was 80.6%, with a theoretical initial yield of

Figure 1 Subunit size of *H. comosa* 3NPA oxidase

(a) SDS/polyacrylamide gel showing: lane 1, marker proteins [A, BSA (66 kDa); B, ovalbumin (45 kDa); C, glyceraldehyde-3-phosphate dehydrogenase (36 kDa); D, carbonic anhydrase (29 kDa); E, trypsin (24 kDa)]; lanes 2 and 3, purified 3NPA oxidase showing a band at 37 kDa; lane 4, dialysed 40–60%–satd. (NH₄)₂SO₄ fraction before Mono-Q anion-exchange chromatography. (b) MALDI–TOF mass spectrum of 3NPA oxidase showing a major peak at 35.4 kDa.

27.94 pmol. Residues 16 and 17 could not be assigned with any certainty.

In addition to its colour, a number of observations indicated that the enzyme was a flavoprotein. The absorption spectrum of the enzyme exhibited a maximum at 274 nm and a broad peak centred at 328.4 nm. The visible spectrum showed a peak at 446 nm and a shoulder at 470 nm (Figure 2a). The fluorescence excitation spectrum exhibited a maximum at 440 nm (results not shown) and the protein emitted fluorescence on irradiation at 440 nm, with a maximum emittance at 520 nm (Figure 2b). Flavin that was dissociated from the enzyme after boiling did not reconstitute the apoenzyme of D-amino acid oxidase; neither did it show stimulated fluorescence after treatment with snake venom phosphodiesterase, suggesting that the enzyme contained FMN. In addition, dissociated flavin co-migrated with FMN (R_F 0.5) on paper chromatography (Whatman no. 4) at room temperature with 5% (w/v) Na₂HPO₄ as solvent. Assuming a subunit molecular mass of 36 kDa, we estimated the presence of 0.93 mol of FMN per mol of subunit, implying one FMN per subunit. The

Table 1 Purification of 3NPA oxidase from leaves of *H. comosa*

Enzyme activity was measured by nitrite production.

Step	Volume (ml)	Total activity (µmol/min)	Total protein (mg)	Specific activity (units/mg)
Crude extract	125	4.8	36.2	0.13
(NH ₄) ₂ SO ₄	125	11.5	27.9	0.41
Dialysis	210	67.8	27.5	2.46
Mono Q ion exchange	5.5	25.0	5.9	4.24
Superose 12 gel filtration	8.5	15.8	1.1	14.4

Figure 2 Absorption and fluorescence spectra of *H. comosa* 3NPA oxidase

(a) Visible absorption spectrum. (b) Fluorescence spectrum expressed as relative intensity. Spectra were obtained at 20 °C with 5 μ M enzyme in 0.5 M Mes buffer.

Figure 3 Effect of pH on *H. comosa* 3NPA oxidase activity

Enzyme activity (100% = 4.9 μ mol oxygen uptake/min per mg) was measured in assays buffered at 0.5 M with phosphate (○), citrate (●), phthalate (□) or Mes buffer (■). Experiments with Mes buffer were performed with a separate enzyme preparation.

Table 2 Nitroalkane specificity of *H. comosa* 3NPA oxidase

Nitroalkane substrates were supplied at 67.5 μ mol per assay. Specific activity is nmol of O_2 /min per mg of protein. Results are the means for two separate determinations.

Substrate	Specific activity (nmol/min per mg)
3-Nitropropionic acid	1458
1-Nitropropane	21
2-Nitropropane	43
2-Nitroethanol	0
2-Nitro 1-propane	30
3-Nitro 2-propanol	5
3-Nitro 2-butanol	26
Nitromethane	7
Nitroethane	0
3-Nitrocyclohexane	0

Table 3 Stoichiometry of 3NPA oxidation catalysed by *H. comosa* 3NPA oxidase

Assays were performed by following oxygen consumption polarographically at 20 °C and were left to proceed to completion. H_2O_2 was determined as oxygen after the addition of catalase. Results are means for two separate experiments.

Assay	Substrates and products (nmol)	
	Without catalase	With catalase
3NPA consumed	150	150
O_2 consumed	123	161
Nitrite formed	53	26
Nitrate formed	96	138
Aldehyde formed	130	130
H_2O_2 formed	25	0

molar absorption coefficient (ϵ_{446}) of the bound FMN was estimated to be 11.6 $\text{mM}^{-1} \cdot \text{cm}^{-1}$.

Substrate specificity, stoichiometry and inhibitor studies

We considered the possibility that the true substrate for the enzyme is propionate-3-nitronate, an ionization state of 3NPA that is deprotonated at the nitroalkyl group [8]. However, maximum 3NPA oxidase activity was obtained when assays were performed in Mes buffer at pH 4.8 (Figure 3). In addition, the rate of nitrite generation and oxygen consumption in assays supplied with 3NPA as substrate were limited by enzyme concentration at pH 5 and pH 7. In contrast, with 3NPA as substrate, the activity of propionate-3-nitronate oxidase from *Penicillium atrovenetum* seemed to be rate-limited by the availability of propionate-3-nitronate at acid pH and not increased by further additions of enzyme [8]. Our results indicate a clear difference between the plant and fungal enzymes and suggest that propionate-3-nitronate is not as effective as 3NPA as a substrate for plant 3NPA oxidase.

Substrate specificity was tested with ten nitroalkanes (Table 2). Nitrite production and oxygen consumption were significant only in assays supplied with 3NPA as substrate. The apparent K_m for 3NPA as substrate at pH 5.0 was 620 μ M.

The stoichiometry of the reaction was determined in assays supplied with 150 μ M 3NPA and saturating oxygen at 20 °C, in the presence and the absence of catalase. Assays were followed polarographically and left to proceed to completion. A summary of the results is given in Table 3. Virtually all (98%) of the

Table 4 Effects of anions on *H. comosa* 3NPA oxidase activity

Enzyme activity was measured as oxygen consumption; 100% activity = 8.3 units/mg. Results are means for two separate experiments.

Addition	Concentration (mM) ...	Activity (% of control)		
		1	10	100
KCl		74.5	69	50
NaCl		80	48.5	35.5
Na ₂ SO ₄		78	66	50
K ₂ SO ₄		87	86	67
Na ₂ HPO ₄		73	64	61
K ₂ HPO ₄		80	78	73
KH ₂ PO ₄		92	89	82
CH ₃ CO ₂ Na		98	81	71
NaNO ₂		61	53	21
KNO ₂		85	62.5	47
NaNO ₃		67	50	20
KNO ₃		59	43	13

oxidized nitrogen produced in the reaction was accounted for as nitrate and nitrite. In assays that did not include catalase, nitrate production exceeded nitrite production by up to 2-fold. With catalase present, the proportion of nitrate increased significantly. The carbon product could be accounted for (at least 86%) as an aldehyde that served as a substrate for β -hydroxybutyrate dehydrogenase and was presumed to be malonate semialdehyde. Variable but significant amounts of H₂O₂ were generated in all experiments, indicating that the enzyme is an oxidase and not a dioxygenase. Inclusion of superoxide dismutase in assays (results not shown) resulted in a 60% increase in H₂O₂ production, suggesting that superoxide is also a product of the reaction. In this the plant enzyme differs from the nitroalkane-oxidizing activity reported from the yeast *Hansenula mrakii* [7], which is described as a dioxygenase. Overall, the observed stoichiometry of the reaction catalysed by the *H. comosa* enzyme seems to be complex and non-integral in the production of H₂O₂ and the consumption of oxygen. One possibility is that an enzyme-catalysed oxidation of 3NPA to nitrite and semialdehyde is accompanied by a non-enzymic oxidation of nitrite to nitrate involving peroxide or superoxide. However, results obtained with assays including catalase seem to rule out a direct role for peroxide in such a scheme. A free-radical chain reaction that results in a non-enzymic autoxidation of substrate has been proposed to explain the non-integral stoichiometry observed with the propionate-3-nitronate oxidase reaction from *P. atrovenerum* [8].

Enzyme activity (oxygen consumption) was not inhibited by 8-hydroxyquinoline, mercaptoethanol, cysteine, glutathione, *p*-chloromercuribenzoate or EDTA (results not shown), which are all inhibitors of 2-nitropropane dioxygenase from *H. mrakii* [7]. Similarly, the addition of FMN, FAD, NADH or NAD to assay mixtures had no effect on enzyme activity. In contrast, enzyme activity increased in assays containing 2,4,6-tripyridyl-s-triazine and was inhibited completely in the presence of HgCl₂. The addition of non-substrate nitroalkanes (listed in Table 1) to assays had no effect on 3NPA-dependent oxygen consumption or nitrite generation (results not shown).

During preliminary experiments designed to optimize the anion-exchange step of the purification procedure, we observed large losses of enzyme activity when NaCl or KCl was used in gradients. Therefore we tested the effect of a number of Na⁺ and K⁺ salts at concentrations between 1 and 100 mM on enzyme activity (Table 4). All salts tested had an inhibitory effect, particularly at high concentrations, but nitrate, nitrite and chloride were the most inhibitory. The addition of nitrate or nitrite, which are reaction products, resulted in inhibitions that varied between 15% and 40% at 1 mM, depending on the cation. With the exception of nitrates, Na⁺ salts were more inhibitory than K⁺ salts. Acetate, sulphate and phosphate were the least inhibitory. Potassium phosphate was chosen as the most suitable salt for anion gradients on the basis of its low inhibition at high concentrations. These results also suggest that enzyme activation observed after dialysis (Table 1) is due to the removal of inhibitory anions, and provide an explanation for the suppression of enzyme activity observed in assays containing non-zwitterionic buffers (Figure 3).

Distribution of 3NPA oxidase in *H. comosa*

High levels of 3NPA oxidase activity were detected in extracts from leaves of plants grown in water culture with ammonium, urea or nitrate as nitrogen sources (Table 5). Leaves sampled from natural populations contained similar levels of enzyme activity (not shown); roots of these plants exhibited acetylene reductase activity and were presumably using N₂ as a nitrogen source. In contrast, enzyme activity was detected neither in roots nor in suspension cultures, whether they contained chloroplasts (cultures derived directly from sterile leaves) or not (derived from callus). High levels of enzyme activity (113–166 nmol of NO₂/min per mg of protein) in leaves correlated with the presence of high concentrations of 3NPA (116–138 μ mol/g). In contrast, roots contained very low concentrations of 3NPA and none could be detected in suspension culture. In some experiments we were unable to detect any 3NPA in water-cultured roots.

Table 5 Distribution of 3NPA and 3NPA oxidase in *H. comosa* grown in water culture with different nitrogen sources

Results are means $\pm$ S.E.M. for three determinations. Enzyme activity was determined in 40–60%-satd. (NH₄)₂SO₄ fractions and is expressed as nmol of NO₂/min per mg of protein. 3NPA is expressed as μ mol/g fresh weight. Abbreviation: n.d., not determined.

Nitrogen source	Component	Leaves	Roots	Suspension culture
Ammonium	3NPA (μ mol/g)	121.8 $\pm$ 5.9	5.4 $\pm$ 0.3	n.d.
	3NPA oxidase (nmol/min per mg)	132.0 $\pm$ 1.1	0	n.d.
Urea	3NPA (μ mol/g)	138.0 $\pm$ 2.8	3.0 $\pm$ 0.1	n.d.
	3NPA oxidase (nmol/min per mg)	166.0 $\pm$ 2.6	0	n.d.
Nitrate	3NPA (μ mol/g)	116.0 $\pm$ 2.3	9.7 $\pm$ 0.3	0
	3NPA oxidase (nmol/min per mg)	113.3 $\pm$ 1.5	0	0

The occurrence of an enzyme in *H. comosa* that oxidizes 3NPA to nitrate, nitrite and a semi-aldehyde carbon skeleton provides a substrate-specific detoxification mechanism that conserves fixed carbon and nitrogen. The flavoprotein enzyme, which we have tentatively named 3NPA oxidase, has not previously been described in plants. The enzyme catalyses the following reaction:

It differs in several of its properties from most microbial nitroalkane oxidases that have been described but resembles the propionate-3-nitronate oxidase from *P. atrovenetum* [8]. It differs from the *Penicillium* enzyme in its low pH optimum and its apparent preference for 3NPA. We suggest that the enzyme is normally sequestered in a compartment separate from 3NPA. Possibly, 3NPA is stored in vacuoles and is released after damage and during senescence. In either of these events, two important consequences would be a mingling of enzyme with a large pool of substrate and an acidification of the reaction matrix, resulting in a large production of nitrite and nitrate in leaves.

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