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PURIFICATION AND PROPERTIES OF A NITRATE REDUCTASE-INACTIVATING ENZYME

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SUMMARY

The NADH nitrate reductase-inactivating enzyme in the maize root has been purified 460-fold. It was precipitated by 40–60% saturation with $(NH_4)_2SO_4$ and was soluble at pH 4.0. On a CM-32 cellulose column, equilibrated with 10 mM acetate (pH 5.0), it was eluted after a lag as a single peak with 10 mM acetate containing 50 mM NaCl (pH 5.0). It was then characterized on a Sephadex G-200 column and its molecular weight estimated to be 44 000. The inactivating enzyme, like nitrate reductase, has its optimum activity in the neutral pH range and appears to be located in the cytoplasm of the mature-root cell. It was more stable to heat treatment than nitrate reductase.

Inhibition of the inactivating enzyme by phenylmethylsulphonyl fluoride suggested the involvement of a serine residue at its active site. When phenylmethylsulphonyl fluoride was used in the extraction medium for the mature root, the in vitro inactivation of nitrate reductase was prevented. The fraction containing the inactivating enzyme degraded casein by a phenylmethylsulphonyl fluoride-sensitive reaction but it had no peptidase activity.

INTRODUCTION

The nitrate reductase (EC 1.6.6.1) in the mature-root region of the primary root of 3-day maize seedlings, in contrast to that in the younger root-tip cells, is relatively labile both in vivo and in vitro [1]. A protein fraction which mediates the in vitro inactivation of nitrate reductase has been identified in the mature-root extract and is referred to as a nitrate reductase-inactivating enzyme [2]. The purification and further characterization of this inactivating enzyme are described in this paper. Data are also presented from an investigation on the nature of its active site and a study of its peptidase and proteolytic activity.

Abbreviation: PMSF, phenylmethylsulphonyl fluoride.

MATERIALS AND METHODS

Plant material, harvest and extraction procedures

Seedlings of *Zea mays* L. (Hybrid variety DSCI supplied by the Dekalb Shand Seed Co., Tamworth, Australia) were grown, harvested and root extracts prepared as described previously [1, 2].

Assay of the nitrate reductase-inactivating enzyme

The apparent substrate nitrate reductase was prepared from the root tip (0–2 cm of the primary root) of 3-day maize seedlings grown on 5 mM nitrate. It was precipitated from the crude extract by 0–40 % saturation with $(NH_4)_2SO_4$ and assayed with NADH as electron donor [2]. Unless stated, 0.1 ml of this root-tip sample was used, containing approx. 0.20 mg protein and an initial nitrate reductase activity of about 50 nmoles NO_2^- produced/h (units). When incubated at 25 °C for 2 h, the loss of nitrate reductase was $<10\%$. The level of inactivating enzyme was estimated by the increase in the inactivation of nitrate reductase and is presented as units nitrate reductase inactivated per h.

Cellulose ion-exchange chromatography

Whatman CM-32 and DE-32 materials were prepared for column chromatography as described in the Whatman laboratory manual. Specific details are given in the results section. The column effluent was monitored with an LKB Uvicord unit at 280 nm, and 5.0-ml fractions were collected. A flow rate of approx. 80 $ml \cdot cm^{-2} \cdot h^{-1}$ was used.

Gel filtration and molecular weight estimation

Sephadex G-200 was employed as described by Reiland [3]. For molecular weight estimation the column was calibrated [4] with blue dextran (molecular weight $2 \cdot 10^6$) to give the void volume of the column, catalase (250 000), yeast alcohol dehydrogenase (150 000), bovine serum albumin (70 000), ovalbumin (45 000) and myoglobin (16 900).

Assay of peptidase and protease activity

The hydrolysis of α-benzoyl-L-arginine-*p*-nitroanilide was investigated by the procedure used by Kruger [5], while the substrate L-leucine-*p*-nitroanilide was tested by the method of Beevers [6]. In the assay for protease activity 1.0 ml of a 0.4 % casein solution in 25 mM phosphate, pH 7.0, was incubated with a 1.0-ml sample of the inactivating enzyme (0.1 mg protein) at 37 °C. Aliquots of 0.5 ml were removed and treated with an equal volume of 10 % trichloroacetic acid to precipitate protein. Amino acids released were tested for by a modified ninhydrin procedure [7] using alanine as a standard.

RESULTS

Initial purification of the inactivating enzyme with $(NH_4)_2SO_4$ and acid precipitation at pH 4.0

A 63-g sample of the mature-root region of 4-day maize seedlings (primary root

minus 0–2 cm tip region) was extracted in a mortar and pestle with 189 ml 50 mM phosphate, 0.5 mM EDTA and 5 mM cysteine (pH 7.5) and centrifuged at 27 000 × g (2 °C) for 30 min. The fractions precipitated by 0–40% and 40–60% saturation with $(NH_4)_2SO_4$ were prepared by the addition of solid $(NH_4)_2SO_4$ [8] to the enzyme sample at 0 °C with 0.05 M NaOH used to give a final pH of 7.5. After $(NH_4)_2SO_4$ addition, the suspension was allowed to stand for 15 min and then centrifuged at 12 000 × g for 15 min (2 °C). The fraction precipitated by 40–60% saturation with $(NH_4)_2SO_4$ was dissolved in 27 ml 15 mM phosphate, pH 7.0, and dialysed for 16 h (3 °C) against three changes of 15 mM phosphate, pH 7.0. The pH of this fraction was then adjusted to 4.0 (0 °C) and after standing for 15 min it was centrifuged at 12 000 × *g* for 15 min. The inactivating enzyme was recovered in the supernatant fraction.

With the $(NH_4)_2SO_4$ fractionation a 60–80% total recovery of the inactivating enzyme has been obtained, largely in the fraction precipitated with 40–60% saturation of the salt (Table I). In the second purification step, treatment at pH 4.0, a slight in-

TABLE I

SUMMARY OF THE PURIFICATION OF THE NITRATE REDUCTASE-INACTIVATING ENZYME

Full experimental details are given in Results.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units nitrate reductase inactivated × 10^{-3})	Recovery (%)	Specific activity (units nitrate reductase inactivated/mg protein × 10^{-3})	Purification factor
27 000 × *g* supernatant (30 min)	219	241.6	23.8	100	0.1	1
40–60% $(NH_4)_2SO_4$ precipitate	27	73.3	14.6	61	0.2	2
pH 4.0 supernatant	36	41.7	16.1	68	0.4	4
CM-32 fraction	20	3.9	33.5	141	8.6	86
Sephadex G-200 (A) fraction	30	1.3	18.6	78	14.3	143
Sephadex G-200 (B) fraction	30	0.25	11.5	48	46.0	460

crease in the activity of the inactivating enzyme was achieved, giving a total 4-fold increase in its specific activity in the first two purification stages. When the mature root was extracted at pH 4.0 (0.05 M citrate phosphate), the extract contained only 22% of the protein found with the standard procedure described above and no nitrate reductase activity was detected. The recovery of the inactivating enzyme was the same as that with the procedure described above.

Chromatography on CM-cellulose

A column of CM-32 cellulose was equilibrated with 10 mM acetate, pH 5.0, at room temperature, and the pH 4.0 supernatant containing 10 mM phosphate

loaded and washed in with the starting buffer. The data from a preliminary study with CM-32 cellulose are shown in Fig. 1, and for the main purification (Table I) the same procedure was followed with a 3.1 $cm^2 \times 4.5$ cm column. When the samples were loaded, each column was washed with the starting buffer until no further protein was detected in the eluant. Then a solution containing 10 mM acetate, pH 5.0, and 50 mM NaCl was used to elute the column. After a volume of 35 ml had been passed through

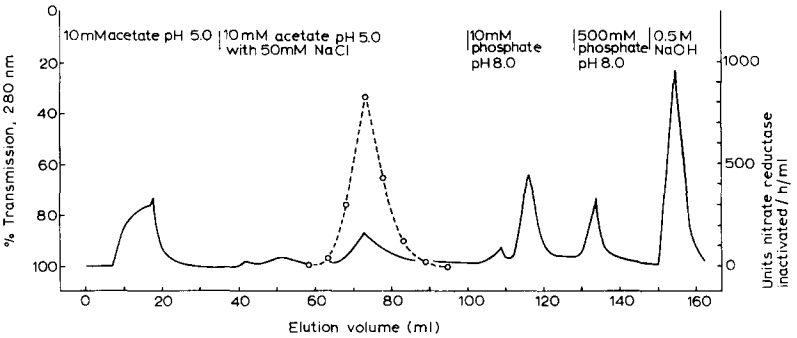

Fig. 1. Chromatography of the nitrate reductase-inactivating enzyme on CM-32 cellulose. A 12-ml sample of the pH 4.0 supernatant (see Table I), containing 15.5 mg protein, was loaded on a 0.78 $cm^2 \times 4.5$ cm column equilibrated with 10 mM acetate, pH 5.0, and eluted by the procedure indicated. The percent transmission of the column effluent was monitored (———) and 5-ml fractions were collected for assay of the inactivating enzyme (— — —) after adjusting the pH to 7.0.

the column (Fig. 1), a fraction was eluted which contained the inactivating enzyme. A 2-fold increase in the total activity of the inactivating enzyme was obtained, resulting in a 21-fold increase in its purification (Table I). Other fractions were eluted with 10 and 500 mM phosphate, pH 8.0, but the bulk of the protein was only recovered from the column with 0.5 M NaOH. When the fraction containing the inactivating enzyme was re-chromatographed on CM-32 cellulose with a gradient of 0–100 mM NaCl in 10 mM acetate, pH 5.0, a single broad peak was obtained. If the elution step with 50 mM NaCl was omitted, the inactivating enzyme was eluted with 10 mM phosphate, pH 8.0.

The inactivating enzyme (40–60% $(NH_4)_2SO_4$ fraction prepared as above), when loaded on a DE-32 column at pH 7.0 (10 mM phosphate), was largely recovered (80%) in the fraction not retained by the column and the remainder eluted with 50 mM phosphate, pH 7.0. No activation occurred as with chromatography on the CM-32 column (Table I). Under the same conditions nitrate reductase 0–40% $(NH_4)_2SO_4$ fraction of the root tip was retained by the DE-32 column and eluted with 200 mM phosphate, pH 7.0.

Gel filtration with Sephadex G-200

A 2 $cm^2 \times 67$ cm column of Sephadex G-200 at room temperature was used with 10 mM phosphate, pH 7.0, as eluant. The CM-32 fraction was concentrated approx. 10-fold by enclosing in a dialysis sac surrounded by Sephadex G-25 and a 2.0-ml final sample applied to the column. The bulk of the activity of the inactivating enzyme was recovered in a fraction eluted just after the main protein peak (Fig. 2).

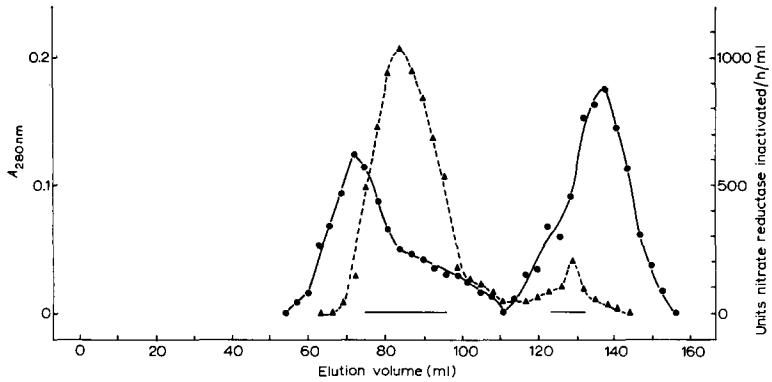

Fig. 2. Separation of the nitrate reductase-inactivating enzyme on Sephadex G-200. A 2-ml sample of pooled CM-32 eluates, containing 3.8 mg protein, was loaded on a 2 cm^2 × 67 cm column with 10 mM phosphate, pH 7.0, as buffer. Fractions of the column effluent (3 ml) were collected and their absorbance (●—●) and content of inactivating enzyme (▲---▲) estimated. The solid bar designates the fractions retained for further study. The column volume was 138 ml and the void volume 47 ml.

A low molecular weight fraction which strongly absorbed at 280 nm also had a low activity of the inactivating enzyme. This fraction, like the main peak of the inactivating enzyme, was heat labile. It was not an amino acid but is possibly a phenolic compound. When the main fraction of the inactivating enzyme (see Fig. 2) was re-chromatographed, a single fraction was found with the same elution volume. No low molecular weight fraction was detected. From the elution volume of the inactivating enzyme and that for known proteins (see Materials and Methods), the molecular weight of the inactivating enzyme was found to be approx. 44 000 (Fig. 3). It had a similar elution pattern to ovalbumin. A loss of activity of the inactivating enzyme occurred in both Sephadex steps but the further purification obtained (Table I) resulted in a final 460-fold purification of the inactivating enzyme.

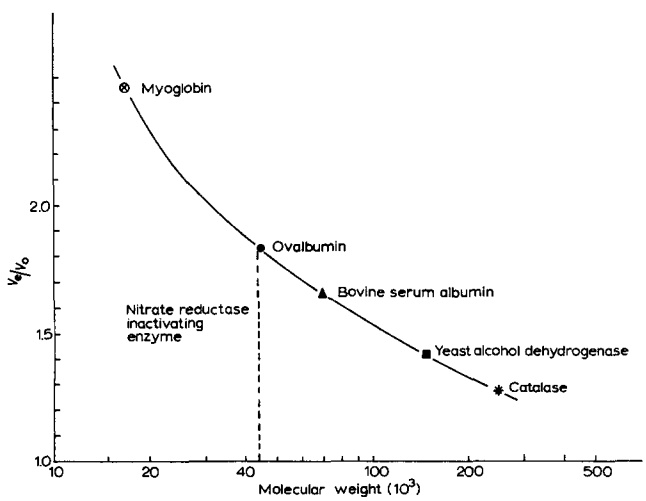

Fig. 3. Plot of ratio V_e/V_0 (elution volume/void volume) against log molecular weight for proteins on a Sephadex G-200 column (2 cm^2 × 67 cm) at pH 7.0.

Influence of enzyme level, substrate level and pH on the activity of the inactivating enzyme

The activity of the nitrate reductase-inactivating enzyme was dependent on the level of its apparent substrate nitrate reductase and the concentration of the inactivating enzyme (Fig. 4). The range of enzyme levels which allowed for a linear relationship between enzyme concentration and activity increased with the purity of the enzyme, especially after chromatography on CM-32 cellulose. This is further evidence for some inhibitor of the inactivating enzyme in the mature-root extract. As shown (Table I) a 2-fold increase in activity of the inactivating enzyme was obtained during the CM-32 purification step.

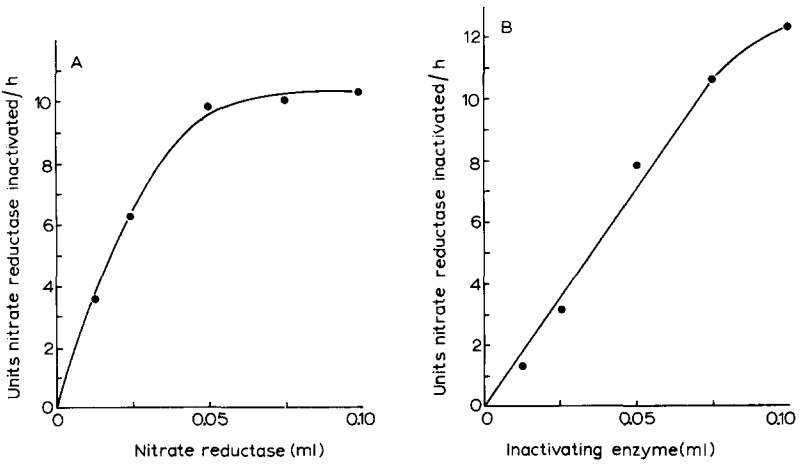

Fig. 4. Influence of the level of the substrate nitrate reductase (A) and the concentration of inactivating enzyme (B) on the rate of nitrate reductase inactivation. The nitrate reductase sample, 0–40% $(NH_4)_2SO_4$ precipitate of the root tip, contained 440 units nitrate reductase activity/ml and 2 mg protein/ml. The inactivating enzyme was a 40–60% $(NH_4)_2SO_4$ fraction of the mature root containing 1.5 mg protein/ml. In the study on effect of nitrate reductase level, 0.05 ml of inactivating enzyme was used. Similarly in the investigation on the effect of the concentration of inactivating enzyme on its activity, the level of nitrate reductase was 0.05 ml.

Characterization of the pH optimum of the inactivating enzyme was complicated by the fact that the stability of its substrate nitrate reductase was decreased during incubation at pH values below or above 7.0 (Table II). Therefore, the assay of the inactivating enzyme at each pH was conducted with a range of nitrate reductase levels so that the V could be established. A higher activity of the inactivating enzyme was indicated at pH 7.0 than at pH 6.0 or 8.0 (Table II).

Intracellular distribution of nitrate reductase and the inactivating enzyme

The extraction medium used (Table III) was based on that employed by Gibson and Paleg [9] in studies on the lysosomal nature of enzymes in wheat aleurone cells. It was found that both nitrate reductase and its inactivating enzyme were not sedimented by centrifugation at 150 000 $\times$ g_{av} for 45 min. Two-thirds of the total NADH cytochrome c reductase recovered was in the 150 000 $\times$ g microsomal pellet.

TABLE II

INFLUENCE OF pH ON THE IN VITRO STABILITY OF NITRATE REDUCTASE AND THE ACTIVITY OF THE INACTIVATING ENZYME

The root-tip nitrate reductase used as substrate for the inactivating enzyme (Materials and Methods) was adjusted to the required pH. After incubation and before assay of nitrate reductase, 1.0 ml of 0.2 M Tris, pH 7.0, was added which allowed re-adjustment of the pH to 7.0 where necessary. Under these assay conditions the initial nitrate reductase activity was 32 units. A CM-32 fraction containing 1 μg protein was used as inactivating enzyme.

pH	Nitrate reductase level after incubation for 2 h at 25 °C (units)	V for inactivating enzyme (units nitrate reductase inactivated/h)
6.0	21	5.8
7.0	29	10.5
8.0	23	7.9

Heat stability of the inactivating enzyme

As reported previously [2], the nitrate reductase-inactivating enzyme is inactivated by treatment for 5 min in a boiling water-bath. It is, however, more stable to heat treatment than nitrate reductase. From the data shown in Fig. 5 it can be seen that 10 min at 43 °C would give 50% inactivation of nitrate reductase, while a similar treatment at 65 °C would be necessary to give a 50% inactivation of the inactivating enzyme. The inactivating enzyme was quite stable at room temperature and on long-term storage at both 0 and −15 °C.

Use of active-site inhibitors to characterize the inactivating enzyme

Proteases active in the neutral pH range can usually be classified as

TABLE III

INTRACELLULAR DISTRIBUTION OF NITRATE REDUCTASE AND THE INACTIVATING ENZYME IN THE MAIZE ROOT

The extraction medium was 0.05 M Tris containing 0.5 M mannitol, 5 mM EDTA, 5 mM cysteine, 2.5% Ficoll and 0.1% bovine serum albumin (pH 7.5). The ratio sample fresh-weight to volume of extraction medium was 1:2 (w/v). The samples were extracted gently with a mortar and pestle. The extract was passed through Miracloth and subjected to a preliminary centrifugation of 1000 × g (10 min). The fractions indicated were then collected and the pellet fractions washed once with 0.05 M Tris (pH 7.5) containing 0.5 M mannitol. For nitrate reductase and NADH cytochrome c a root-tip sample was used and the enzymes isolated in a 0–40% $(NH_4)_2SO_4$ precipitate. The inactivating enzyme (40–60% $(NH_4)_2SO_4$ precipitate) was obtained from the mature root. The results are given for 1 ml original extract in each case.

Fraction	Nitrate reductase (units/h)	Nitrate reductase inactivating enzyme (units nitrate reductase inactivated/h)	NADH cytochrome c reductase (nmoles cytochrome c reduced/min)
20 000 × g_{av} pellet (10 min)	2	0	2
150 000 × g_{av} pellet (45 min)	4	0	69
150 000 × g_{av} supernatant (45 min)	132	33	34

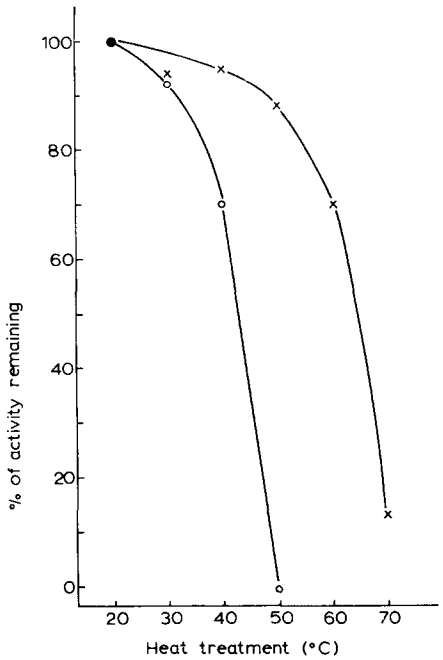

Fig. 5. Influence of heat treatment on the activity of nitrate reductase and the inactivating enzyme. Aliquots of root-tip nitrate reductase (○—○) and mature-root inactivating enzyme (×—×) (see Fig. 4) were incubated at the temperatures shown for 10 min before determination of their activity.

sulphydryl, serine or metal dependent [10]. A range of specific inhibitors (Table IV) was tested to examine the nature of the active site of the inactivating enzyme. The sulphydryl group inhibitor *p*-chloromercuribenzoic acid at a concentration of 0.1 mM had no effect on the activity of the inactivating enzyme. This concentration of *p*-

TABLE IV

INFLUENCE OF ACTIVE-SITE INHIBITORS ON THE NITRATE REDUCTASE-INACTIVATING ENZYME

Details of the root-tip nitrate reductase used as substrate are given in Materials and Methods. The CM-32 fraction of the inactivating enzyme (5 μg protein) was used. *p*-Chloromercuribenzoic acid was prepared in 0.1 M glycylglycine (pH 7.5). All other inhibitors were prepared in 0.025 M phosphate (pH 7.5). PMSF was first dissolved in a small volume of isopropanol (final concentration 1% when PMSF 0.25 mM).

Inhibitor	Concentration	Inhibition (%)
p-Chloromercuribenzoic acid	0.10 mM	0
PMSF	0.25 mM	100
N-α-*p*-Tosyl-L-lysine chloromethyl ketone	0.10 mM	28
Trypsin inhibitor (soybean)	0.25%	11
Trypsin inhibitor (ovomucoid)	0.50%	26
EDTA (Na_2)	10.00 mM	47
o-Phenanthroline	1.00 mM	20

chloromercuribenzoic acid caused a high level of inhibition of nitrate reductase which was reversed by the inclusion of 1 mM cysteine in the medium used to assay it. Phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine-dependent enzymes, e.g. trypsin [11], when tested at 0.25 mM did not have any influence on nitrate reductase but completely inhibited the inactivating enzyme. The inhibition by PMSF was not influenced by the inclusion of either mercaptoethanol or glutathione (25 mM) in the incubation medium, so PMSF is not reacting with a thiol group as in papain [12].

The other active-site inhibitors tested (Table IV) did give some inhibition of the inactivating enzyme but only at relatively high concentrations. It is doubtful therefore if such inhibition is due to a specific inhibition at the active site. Thus the maize-root inactivating enzyme, while apparently having a reactive serine group, is distinguished from trypsin [13] by the absence of significant inhibition by *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and hence non-involvement of a histidine residue at its active site. Similarly, high levels of trypsin inhibitors, which give 100% inhibition of trypsin [14], had only small inhibitory effects on the inactivating enzyme.

The metal-chelating agents, *o*-phenanthroline and particularly EDTA, inhibited the inactivating enzyme. However, because of the high concentrations used it cannot be concluded that a metal is involved at the active site of the enzyme. A slight inhibition (16%) of the inactivating enzyme was observed with 100 mM phosphate, but the same concentration of NaCl had no effect.

Use of PMSF to prevent the in vitro loss of nitrate reductase

The inhibitor of an active-site serine, PMSF, was shown above to prevent the inactivation of root-tip nitrate reductase by the inactivating enzyme. If the loss of nitrate reductase activity in crude extracts of the mature root is due to this inactivating enzyme, as has been suggested [2], it should be prevented by the inclusion of PMSF in the extraction medium. It is shown in Fig. 6 that when 1 mM PMSF was used in the extraction medium, the mature-root nitrate reductase was virtually stable during 80 min incubation at 25 °C.

Investigation of peptidase and protease activity of the inactivating enzyme

The CM-32 fraction of the inactivating enzyme did not give any significant hydrolysis of α-benzoyl-L-arginine-*p*-nitroanilide or L-leucine-*p*-nitroanilide. Both the CM-32 fraction and the most highly purified sample of the inactivating enzyme obtained from Sephadex G-200 do exhibit protease activity when incubated with casein (see Materials and Methods). The level of amino acid release from casein (μg amino acid N/h per mg protein in the sample of inactivating enyzme) was 35 for the CM-32 fraction and 241 for the Sephadex fraction. The degree of purification of protease activity, with casein as substrate, obtained with Sephadex treatment was the same as for the inactivating enzyme (Table I). The protease activity was eliminated by heating the inactivating enzyme sample in a boiling water-bath for 5 min or incubating with PMSF (0.5 mM). Further, no protease activity was detected in two other fractions of the mature-root extract isolated from the CM-32 column (Fig. 1), one eluted by 10 mM acetate and 50 mM NaCl prior to the inactivating enzyme and the other eluted with 10 mM phosphate, pH 8.0.

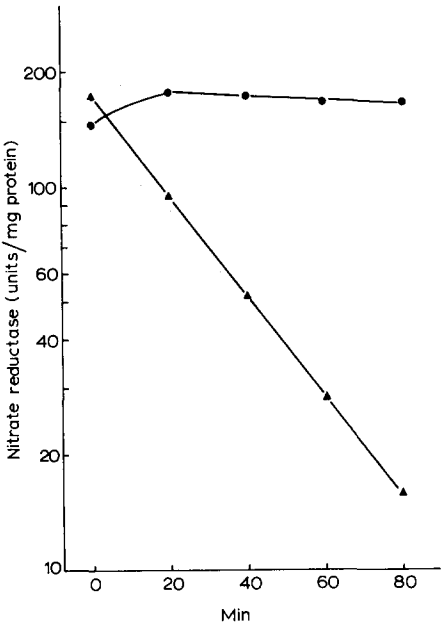

Fig. 6. Use of PMSF to prevent the in vitro inactivation of nitrate reductase. A sample of the mature root of 3-day maize seedlings was extracted either with standard extraction medium of 0.05 M phosphate, 0.5 mM EDTA and 5 mM cysteine (▲—▲) or with the addition of 1 mM PMSF (●—●), both at pH 7.5. The crude extracts (27 000 × *g* supernatant) were incubated at 25 °C for the times shown.

DISCUSSION

The investigation described in this paper confirms [2] that a specific protein from the mature region of the primary root of maize seedlings mediates the in vitro inactivation of NADH nitrate reductase. It has been purified 460-fold and its molecular weight estimated to be approx. 44 000. Nitrate reductase in maize tissues has been estimated to have a molecular weight of 160 000 [15]. The inactivating enzyme could be chromatographed on a cation-exchange cellulose at pH 5.0 but, unlike nitrate reductase, was not retained on an anion-exchange cellulose at pH 7.0. At this pH, which is apparently below the isoelectric point of the inactivating enzyme, it would be positively charged, while the nitrate reductase exchanged is negatively charged. Thus, the binding of nitrate reductase and its inactivating enzyme at neutral pH could be maintained by electrostatic forces.

The experimental procedure used to identify lysosomes in maize-root cells [16] was employed in an investigation of the intracellular location of the inactivating enzyme. It was not found to be associated with a lysosomal or particulate fraction of mature-root cell and, like nitrate reductase, appears to be localized in the cytoplasm. Both enzymes have optimal activity in the region of pH 7.0. Thus, where nitrate reductase inactivation by the inactivating enzyme can be demonstrated in vitro, as in the mature-root extract, a similar in vivo relationship between the two enzymes is suggested. The inactivating enzyme, in contrast to nitrate reductase, was stable over a greater pH and temperature range. No evidence of its autolysis was obtained, as

reported for the phytochrome-degrading protease in etiolated oat shoots [17].

A study with active-site blocking agents indicated that the inactivating enzyme was inhibited by PMSF, suggesting the involvement of a serine residue at its active site. Unlike trypsin, it was not inhibited by *N*-α-*p*-tosyl-L-lysine chloromethyl ketone or the trypsin inhibitors from soybean or ovomucoid. As has been demonstrated for other enzymes [18, 19], PMSF included in the extraction medium can prevent the inactivation of nitrate reductase in the mature-root extract.

The demonstration of the degradation of casein by the inactivating enzyme fraction suggests that the inactivation of nitrate reductase may be due to its proteolytic degradation. Such a mode of action has been clearly demonstrated for specific inactivating enzymes in the small intestine of rats [20]. Since the nitrate reductase in higher plants has not yet been highly purified [15], the actual mode of action of the nitrate reductase-inactivating enzyme cannot be ascertained.

A protease in *Neurospora* which inactivates tryptophan synthase [19] has very similar properties to the nitrate reductase-inactivating enzyme. It is precipitated by higher concentrations of $(NH_4)_2SO_4$ than required to precipitate tryptophan synthase, has a neutral pH optimum, is inhibited by PMSF, has a molecular weight of 40 000 and accumulates as the cells age. The activity of both these inactivating enzymes, as has been reported for other inactivating enzymes, increases with purification, suggesting the presence also of a protease inhibitor in the root extracts such as that which has been identified in *Neurospora* [19].

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