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PROPERTIES OF NITRITE REDUCTASE FROM *CUCURBITA PEPO*

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Key Word Index—*Cucurbita pepo*; Cucurbitaceae; vegetable marrow; purification and properties; nitrite reductase.

Abstract—Nitrite reductase purified to homogeneity from vegetable marrow contains 2 atoms Fe/mol. Enzyme-bound iron exchanged extremely slowly with ^{59}-Fe in solution. Acid–acetone extracts of the enzyme have a spectrum which is consistent with the presence of a sirohaem prosthetic group. Inhibition by mersalyl, which partially bleaches the enzyme, is reversible by glutathione only if this is added within a few min of mersalyl. The absorption spectra of the reduced and autoxidised enzyme and of the nitrite, cyanide and CO complexes are described. Amino acid composition data are given. The hydroxylamine reductase activity of the purified enzyme was 0.2% of nitrite reductase activity.

INTRODUCTION

Nitrite reductase from the leaves of higher plants, together with nitrite and sulphite reductases from various other sources, comprise a group of enzymes which characteristically catalyse reactions involving the transfer of six electrons. In the case of nitrite reductase, ammonia is the reaction product. Chemically reduced viologen [1–7] or photosynthetically reduced ferredoxin [6, 8, 10] may serve as electron donors. Ferredoxin is the probable physiological electron donor. Hydroxylamine reductase, which was once thought to be an intermediary enzyme in the reduction of nitrite to ammonia, has been characterised from *Cucurbita pepo*, and the nitrite reductase from this species prepared to a high degree of purity [5, 10]. The present paper reports studies of physico-chemical properties of the homogeneous nitrite reductase from leaves of *C. pepo*, made possible by the refinement and extension of earlier purification methods. Particular attention has been given to the spectrum of the enzyme, amino acid composition and hydroxylamine reductase content.

RESULTS

Electrophoresis. Nitrite reductase running as a single protein band during electrophoresis on acrylamide gel was obtained from the second Sephadex G100 column (step 8, Table 1) and in some preparations from the second DEAE-cellulose column (step 7). The enzyme was visible as a red brown band during electrophoresis. Coomassie blue staining after electrophoresis revealed a band of the same mobility as the band visible after staining for nitrite reductase activity in gels run at the same time.

Iron content. Three colorimetric determinations with ferrozine gave 2.16, 1.82 and 2.00 atoms Fe/mol, assuming the MW of the enzyme to be 62 100. The presence of 2 atoms Fe/mol was found also for the spinach [8] and *Chlorella* [7] enzymes. The minimum MW based on Fe determination alone would be 29900.

Exchange of enzyme-bound iron. Purified nitrite reductase (0.103 mg) was incubated with bovine serum albumin (0.5 mg) at 1° for 54 days in the presence of $^{59}FeCl_3$ and EDTA (see Experimental). The enzyme albumin mixture acquired a radioactivity of 734 cpm compared with 162 cpm for an albumin control lacking enzyme. The difference represents an exchange of only 1.6% of the estimated Fe content of the enzyme.

Effect of mercury compounds. Nitrite reductase activity is more strongly inhibited by phenyl mercury acetate or sodium mersalyl than by *p*-chloromercuribenzoate [2]. The cause of these different responses to mercurials is not understood. Inhibition of activity by 0.5 mM mersalyl is fully reversible by 2 mM glutathione providing that the latter compound is added within 1–3 min of the mercurial. The inhibition becomes entirely irreversible after 30–60 min. Apparently two reactions between the enzyme and mersalyl are involved—an immediate glutathione-reversible coupling with sulphydryl groups, and a slow developing reaction which is not reversed by glutathione. The second of these may represent loss of Fe from the enzyme.

Amino acid content. Analysis of the enzyme shows a predominance of acidic amino acids (Table 2); the amino

Table 1. Purification of nitrite reductase from *Cucurbita pepo*

Purification step	Specific activity	Purification factor	Yield (n kats)
Crude extract	1 15	—	67000
$(NH_4)_2SO_4$ [1]	4.78	4 16	48100
Sephadex G25 [2]	6.32	5.5	39700
DEAE-cellulose 1 [3]	17 7	15.3	26900
$(NH_4)_2SO_4$ [4]	124	108	5690
Sephadex G100 [5]	375	326	4540
Hydroxyl apatite [6]	588	511	1290
DEAE-cellulose 2[7]	1020	888	1100
Sephadex G100[8]	1420	1240	551

The enzyme was purified from 5 kg of leaf material. Sp. act. is expressed as nmol NO_2^- reduced/sec/mg of protein (nkats/mg of protein). Yield is expressed as nmol NO_2^- reduced/sec (nkats) for the total enzyme fraction.

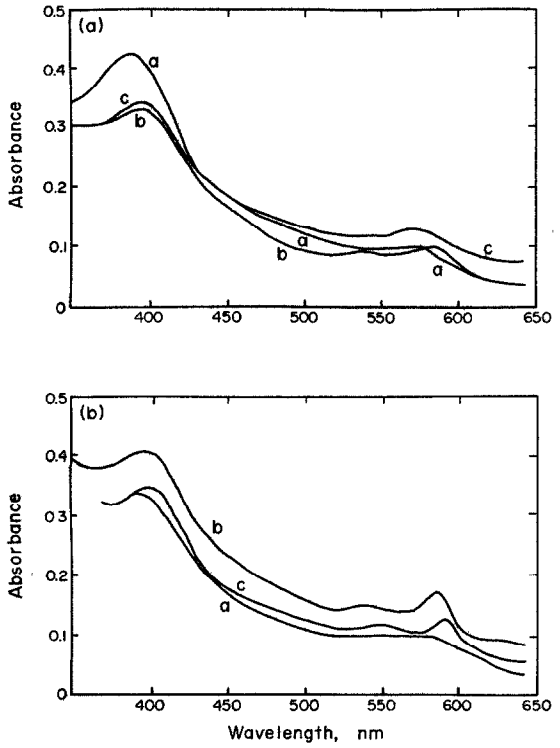

Fig. 1. Effect of nitrite (a) or carbon monoxide (b) on the absorption spectrum of nitrite reductase from *Cucurbita pepo*. a. (a) Native enzyme as purified (1.06 mg protein/ml). (b) Enzyme + 0.17 mg dithionite/ml and 0.24 mM nitrite. (c) Spectrum of (b) 3 hr after reoxidation of dithionite. b. (a) Enzyme (1.06 mg protein/ml) + 0.17 mg dithionite/ml. (b) Enzyme + dithionite + excess CO. (c) Autoxidation of (b) to remove dithionite. The spectra of the native enzyme (Fig. 1a) and the dithionite-reduced enzyme (Fig. 1b, 2b) are applicable to both Figs.

acid content shows general similarity to that of *Chlorella* nitrite reductase [7], but with a lower alanine and higher valine content. Calculated from the amino acid data, MW = 62 146.

Spectrum. The absorption spectrum of marrow nitrite reductase [22] has maxima at 280, 384 and 572 nm and smaller peaks at 532, 635 and 697 nm (Fig. 1). Maximum ratios obtained have been $E_{384}/E_{280} = 0.52$ and $E_{572}/E_{280} = 0.14$. Fall in ratio E_{384}/E_{280} often of 20–30% is observed during ultrafiltration, dialysis, etc. or upon storage. The absolute values for the molar extinction coefficients at 384 and 572 nm have therefore not yet been attained, apparently because of some lability in the prosthetic group. A minimum value of E $mM_{384} = 37.9$ was observed. The A at these two wavelengths is higher relative to the 280 nm maximum than has been reported for the spinach and *Chlorella* enzymes [1, 7, 21], and other minor differences occur in other species [9].

Reduction by dithionite, which alone is a relatively inefficient donor, causes a shift of the 384 and 572 nm maxima towards 400 and 590 respectively with a lowering in A (Fig. 2). A in the region of 700 nm was eliminated. Autoxidation restores the A at wavelengths higher than 450 nm, including the 572 nm maximum, but the peak at 384 nm is not restored.

Addition of nitrite to the dithionite-reduced enzyme immediately resulted in the appearance of small peaks at 582 and 544 nm (Fig. 1b). Exhaustion of the dithionite which, in the presence of excess nitrite, took place within 5 min, was not accompanied by an immediate change in spectrum. After 3 hr however, the 572 nm peak was found to have reappeared, but as in the case of autoxidation, the 384 nm peak was not restored (Fig. 1(a)c).

Cyanide, an inhibitor of nitrite reductase [2], reacts with dithionite-reduced enzyme to give two maxima (398 and 407 nm) in the Soret region. The peak at 572 nm is eliminated (Fig. 2c). Carbon monoxide, which also inhibits nitrite reductase activity [22], reacts with dithionite-reduced enzyme to give a derivative with peaks at 398, 589 and 543 nm (Fig. 1(b)b). During autoxidation, the peak at 589 nm shifts to 584 nm, while the other two peaks change slightly in magnitude but not wavelength (Fig. 1(b)c). Nitrite, carbon monoxide or cyanide treatment of the native enzyme without prior reduction causes a slight shift (4–6 nm) of the 384 nm peak to longer wavelength together with a small loss in extinction at this maximum.

As reported for the spinach [23] and *Chlorella* [7] enzymes, the nitrite reductase from vegetable marrow possesses a prosthetic group which can be removed by treatment with an appropriate organic solvent and acid. Treatment with acetone–HCl followed by centrifugation to remove precipitated protein, gave a supernatant solution with an absorption spectrum with maxima at 384 and 583 nm closely resembling that of the "sirohaem" from spinach nitrite reductase [23]. The pyridine derivative of the extracted haem has maxima at 400 and 556 nm.

Hydroxylamine reductase. Assays of the hydroxylamine reductase activity of purified nitrite reductase using the dithionite–methyl viologen assay showed that the capacity of the enzyme for hydroxylamine reduction is 0.2% of that for nitrite reduction with equimolar quantities of substrates; this concentration was saturating for nitrite.

Table 2. Amino acid composition of nitrite reductase from *Cucurbita pepo*

Amino acid	No. of residues/mol
Asp	63
Thr	27
Ser	32
Glu	64
Pro	31
Gly	48
Ala	39
Val	53
½ Cys	10
Met	10
Ileu	20
Leu	56
Tyr	21
Phe	19
His	11
Lys	31
Arg	30
Total	565

The figures given are the mean of 3 determinations. Analyses were made on 75–100 μg of protein.

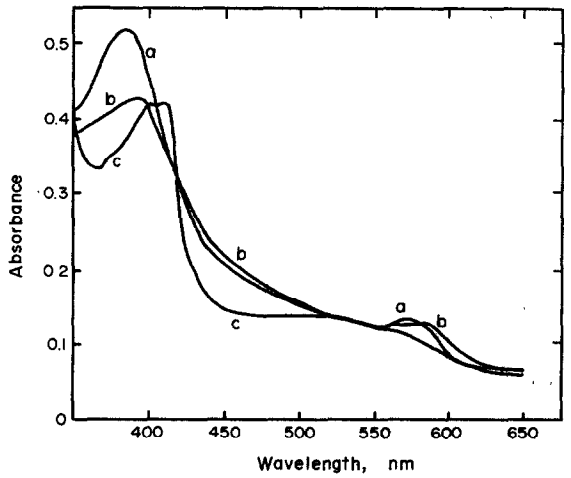

Fig. 2. Effect of cyanide on the absorption spectrum of nitrite reductase from *Cucurbita pepo*. (a) Native enzyme. (b) Enzyme + 0.17 mg dithionite/ml. (c) Enzyme + dithionite + 0.5 mM KCN. The enzyme soln contained 0.95 mg protein/ml.

DISCUSSION

Evidence from several laboratories has shown that nitrite reductases from spinach [1, 4] and *Chlorella* [7] contain 2 atoms/mol Fe. No problem was encountered in showing this relationship to hold for *Cucurbita* nitrite reductase. The low rate of exchange of enzyme bound Fe with ^{59}-Fe in solution indicates high stability of Fe in the prosthetic group, and is consistent with the resistance of the enzyme to attack by organic chelators [2]. The absorption spectrum of the enzyme has features which are unusual for a haem-protein; in particular, the maximum at 384 nm occurs at lower wavelength than is usual for a Soret peak. However, treatment with acetone–HCl followed by centrifugation removes a prosthetic group with a spectrum resembling those which were observed after similar treatment of spinach nitrite reductase and sulphite reductase from *E. coli* [23]. This type of spectrum has been attributed by Murphy *et al.* [24] to an unusual haem type (sirohaem); their studies with *E. coli* sulphite reductase identified this haem as a tetrahydroporphyrin of the isobacteriochlorin type with eight carboxylate side-chains. Spectrophotometric data therefore suggest that the marrow nitrite reductase also contains at least one sirohaem group.

Reduction of the enzyme with dithionite causes a shift of the main absorbance peaks (384 and 572 nm) to longer wavelength (Fig. 2b). Restoration of the 572 but not the 384 nm peak by autoxidation may indicate that reduction with dithionite produces a mixture of reduced forms, only one of which is autoxidisable. Alternatively, the enzyme may be damaged by reduction and autoxidation in a way which affects only the structures absorbing at 384 nm. Although dithionite is able to reduce the enzyme, it is a very inefficient electron donor for nitrite reduction in the absence of ferredoxin or viologens; the spectrum of the dithionite-reduced enzyme is therefore probably not that of the physiologically reduced enzyme.

The effects upon the spectrum of carbon monoxide in the presence of dithionite closely resemble those described for bisulphite reductase from *Desulfatomaculum nigrificans* [25], which also has a similar native absorption spectrum but with peaks at longer (8–10 nm) wavelength. The loss of the CO spectrum after oxidation is typical of haem proteins, but the reaction with cyanide is less characteristic in that it is observed after reduction by dithionite whereas haem proteins generally react in the oxidised form. However, haem from *Aerobacter aerogenes* which yields a chlorin upon removal of Fe [26] forms a relatively stable ferrous cyanide complex as well as a carbon monoxide derivative. The green haem of the dissimilatory nitrite reductase from *Micrococcus denitrificans* also forms a cyanide derivative in the reduced state [27]; moreover, this complex like that of the *Cucurbita* nitrite reductase (Fig. 2c) shows a split Soret band. Double Soret bands are unusual, but have been reported in *Rhodopseudomonas* haem protein [28] and desulfoviridin [29, 30] after reduction. Lack of visible absorption bands is another curious feature of the cyanide complex of *Cucurbita* nitrite reductase (Fig. 2c). Absence of charge transfer bands and sometimes of the γ-band of ferric derivatives is well-established [31].

The very low hydroxylamine reductase activity of the purified enzyme emphasizes earlier conclusions [5] that hydroxylamine is not a free intermediate in the reduction of nitrite to ammonia. The cause of the declining ratio of hydroxylamine reductase/nitrite reductase during purification has yet to be satisfactorily explained. Removal of another hydroxylamine reductase as described by Hucklesby and Hewitt [5] early in the purification does not seem to be the sole cause; rather some change in the nitrite reductase itself may be responsible.

The spectral studies of the enzyme from *C. pepo* support the view that the higher plant nitrite reductase is a haem protein having features in common with several other enzymes concerned with the reduction of nitrite or sulphite compounds and with various chlorin-containing proteins. The nature of the relationship between this haem prosthetic group and the labile sulphide of the enzyme [7, 20] is likely to prove of unusual interest.

EXPERIMENTAL

Plant material. Vegetable marrow plants (Sutton's Green Bush) were grown in sand culture in a greenhouse under natural light conditions. The plants were irrigated 1–2 times each day with nitrite-containing Long Ashton nutrient soln [11]. Partially expanded leaves from plants 3–5 weeks old were harvested and stored immediately at −15° for periods of up to 1 yr before use.

Purification of nitrite reductase. The procedure described yielded homogeneous enzyme. Increase in sp. act. ranged from 800- to 1200-fold, and yields were 1–5% (Table 1). A preparation *ca* 60% pure can be obtained by a sequence of 3 steps (nos 1, 5 and 7 of Table 1) with a 35% yield. Frozen marrow leaves (5–10 kg) which had been stored at −15° for at least 2 weeks were homogenised in a mincer. For each kg of leaf material minced, 36 g of Norit A charcoal and 100 ml of 1M KPi buffer pH 7.7, containing 5 mM EDTA and 50 mM cysteine hydrochloride, were mixed with the still-frozen homogenate immediately after mincing. The vessel containing the homogenate was placed in H_2O at about 40° and the homogenate was thawed to 3–5° with constant stirring. All subsequent operations were carried out at 0–3°. Crude cell debris was removed by centrifugation in a basket centrifuge at 3000–4000 rpm, using nylon filter bags. Further insoluble material was removed by centrifugation at 5600 *g* for 45–60 min. The extract still contained some suspended insoluble material after this treatment.

$(NH_4)_2SO_4$ precipitation. The pH of the extract was adjusted to 7.5 and the concentration of EDTA to 10 mM. For each l. of extract 205 g of $(NH_4)_2SO_4$ was added and after 30 min the ppt. removed by centrifugation at 5600 *g* for 60 min. This treatment removed the remaining insoluble material, leaving a completely clarified supernatant. The ppt. was rejected and a further 235 g/l. $(NH_4)_2SO_4$ added to the supernatant. After 30 min standing the ppt. formed was removed by centrifugation at 5600 *g* for 30 min, and redissolved in a minimum vol of 20 mM KPi buffer, pH 7.7 containing 0.1M KCl. This soln, and all the buffers used at subsequent stages in the purification, contained 1 mM cysteine–HCl and 0.1 mM EDTA.

Sephadex G25 chromatography. A column of Sephadex G25 (height 70 cm, 2½ l bed vol.) was used to remove $(NH_4)_2SO_4$, phenolic materials and other low MW contaminants from the extract. Equilibration of the column and elution of the enzyme was carried out with buffer of the same composition as that used for redissolving the $(NH_4)_2SO_4$ ppt. The vol. of enzyme collected from the column was 1.5 times the vol. of applied sample.

DEAE-cellulose chromatography. For each 2.5 kg of leaf homogenised, one DEAE-cellulose column (bed vol. 300 ml, height 15 cm) was used for further purification of the nitrite reductase enzyme. After adsorption of the enzyme the column was washed for 18 hr with 6–10 bed vols of the buffer used for Sephadex G25; the running rate was *ca.* 150 ml/hr. Nitrite reductase was eluted with 20 mM Pi buffer containing 0.2M KCl. Fractions (150 × 10 ml) were collected and the nitrite reductase activity determined. The more active fractions were combined. This soln, which usually contained 1–2 mg/ml of protein, was adjusted to a Pi buffer concentration of 0.1M and to pH 7.5.

$(NH_4)_2SO_4$ precipitation [2]. To each l. of enzyme soln 480 g of $(NH_4)_2SO_4$ was added. After 30 min the ppt. was removed by centrifugation (5600 *g* for 30 min). $(NH_4)_2SO_4$ (180 g/l.) was added to the supernatant. The ppt. was removed after 30 min standing period by centrifugation at 5600 *g* for 60 min, and then dissolved in minimum vol. of the buffer used for chromatography on Sephadex G100.

Sephadex G100 [1]. The column used was of 4 l. bed vol., height 95 cm, and was equilibrated and eluted with the buffer described above for the Sephadex G25 step. The enzyme after elution for 18 hr at 150 ml/hr (15 ml fractions) was assayed by the dithionite–benzyl viologen method and the active fractions bulked.

Hydroxyl apatite. The enzyme soln was adjusted to pH 7 with HCl and adsorbed on to a hydroxyl apatite column (bed vol. 25 ml, height 3.1 cm) at the natural running rate of the column. The column was washed with 3 bed vols each of KPi, pH 6.8, of increasing concentration, viz. 40, 60 and 80 mM, followed by 4 bed vols of 80 mM KPi, pH 7.7. Most of the enzyme activity was eluted at this concn. A second washing of the column with 40 and 80 mM KPi, pH 7.7 sometimes yielded additional enzyme. Fractions (8 ml) were collected at all stages following the wash with 40 mM Pi; these were assayed for nitrite reductase by the column eluate method, and fractions of high activity were combined for chromatography on DEAE-cellulose.

DEAE-cellulose [2]. A column of DEAE-cellulose, bed vol. 40 ml, height 14 cm, was equilibrated with 20 mM Tris–HCl buffer, pH 7.7. After adsorption of the enzyme, the column was washed with 2 bed vols of the same Tris buffer containing successively 0.14M NaCl (2 bed vols), 0.16M NaCl (3 bed vols) and 0.18M NaCl (3 bed vols). Nitrite reductase was eluted at the last of these concns. Fractions (8 ml) were collected and assayed for nitrite reductase by the column eluate method. Active fractions were combined and concentrated to 5 ml by ultra-filtration through an Amicon PM 30 membrane. The extract was passed in most cases through another Sephadex column for final purification.

Sephadex G100 [2]. The column was of 600 ml bed vols and 80 cm in height. Equilibration and elution were as described for the previous Sephadex steps. The enzyme was eluted at 20 ml/hr for 18 hr (8 ml fractions). After location of the enzyme by the column eluate method, the active fractions were combined and concentrated as previously (after DEAE-cellulose 2).

Enzyme assays. Nitrite reductase was assayed at 27° with dithionite and methyl viologen [12] except where otherwise stated. A rapid method of assay [12] involving dithionite and benzyl viologen was used to locate the enzyme in column eluates. Hydroxylamine reductase was determined with the same assay mixtures used for nitrite reductase, substituting equimolar quantities of hydroxylamine hydrochloride for nitrite. Activity was measured as hydroxylamine disappearance [2].

Fe estimation. The enzyme was digested to release Fe by a procedure adapted from refs [13] and [14]. The technique devised gave quantitative recovery of Fe from myoglobin. The Fe released was determined colorimetrically with ferrozine [15]. Acid ferrozine reagent and NH_4 OAc buffer, which also contains hydroxylamine, were prepared as described in ref. [15]. Other details were as follows: 0.3 ml of the protein sample was evaporated to dryness in a test-tube at 100°. 0.1 ml of HCl (Aristar grade) followed by 0.1 ml of 100 vol. H_2O_2 were added, and evaporated to dryness for 2 hr at 100°. The tube was then heated at 110° to remove condensed H_2O. After cooling, 0.1 ml of ascorbic acid (10 mg/ml of aq. soln) and 0.5 ml of 1.5% H_2SO_4 were added, and the tube heated at 100° for 10 min. After the addition of acid ferrozine reagent (60 μl), the mixture was again heated at 100° for 10 min. The tube was cooled and 0.12 ml of NH_4OAc buffer added. The soln was diluted to 2.5 ml with H_2O and its *A* at 560 nm measured against a control. Standards were prepared from spectroscopically pure $FeSO_4$.

Protein. Estimations were made colorimetrically [16]. For purified enzyme, a relationship was established between *A* at 278 nm of a nitrite reductase soln and its dry wt determined on an electrobalance, after dialysis against 1 mM NH_4HCO_3 and freeze-drying.

Spectrophotometric studies. These were made in 4 mm wide cells of 10 mm path length.

Amino acid analysis. Enzyme samples were hydrolysed *in vacuo* [17] for 24 and 72 hr and analyses were made in an amino acid analyser. Corrections were made for threonine, serine and tyrosine levels, assuming first-order decay during hydrolysis.

Electrophoresis. Acrylamide gels (11.25%) were used for electrophoresis. Conditions of electrophoresis and location of nitrite reductase were as described in ref. [18]. The enzyme was applied as 50–100 μg protein/gel. Coomassie Blue was used as an *in situ* protein stain [10].

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