NITRATE REDUCTION BY HIGHER PLANT PEROXIDASE

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1. Introduction

Nitrate reductase in higher plants (EC 1.6.6.1.) responsible for the reduction of nitrate to nitrite is known to be a molybdoflavoprotein capable of accepting electrons from NADH or $FMNH_2$ ($FADH_2$) [1].

In our previous experiments cell-free extracts from different plants, unable to form nitrite in the presence of nitrate and any nitrate reductase donator, reduced nitrate when incubated with a specific donor-diethyldithiocarbamate and sulfite mixture [2, 3]. Since the preparations possessed high peroxidase activity, it appeared logical to study peroxidase preparations of various purity with respect to their ability to reduce nitrate. The present work has shown that it is a peroxidase enzyme that is responsible for nitrate reduction under the conditions described below.

2. Materials and methods

Commercial horseradish peroxidase preparations of rather low purity were used in the experiments. The enzyme was additionally purified by ammonium sulfate precipitation (56–65%) and column chromatography on Sephadex G-100. Preparation purity was expressed as A_{403}/A_{275} (RZ).

The standard assay system for nitrate reduction contained in a final volume of 1 ml of 0.05 M phosphate buffer, pH 8.0:160 μmoles KNO_3, 0.6–0.8 μmoles each Na-diethyldithiocarbamate and $K_2S_2O_5$ (or Na_2SO_3) and 0.1 ml peroxidase (0.2–0.4 μg). Incubation was for 5 min at 28°. The reaction was stopped by adding 3.0 ml of 95% ethanol, 0.4 ml of 0.6% sulfanilic acid and 0.2 ml of 0.6% α-naphthilamine. Absorption at 530 nm was measured. Specific nitrate reduction activity was expressed in nmoles (μmoles) of nitrite formed per mg protein per minute in the standard assay system.

Table 1
Enzymic activities of peroxidase preparations in relation to their purity.

Enzyme preparation	Purity (RZ)	Enzymic activity	
		Peroxidation (A_{470}/mg protein/min)	Nitrate reduction (μmoles NO_2^-/mg protein/min)
Preparation 1	0.6	70	41.4
from Beitch	1.3	219	97.5
(Japan)	1.5	266	106.2
	1.6	303	114.5
	1.8	583	150.0
Preparation 2	0.2	7.5	1
from Reanal	0.9	326	189
(Hungary)	1.3	383	420

The incubation mixture for the peroxidative activity determination contained in a final volume of 4 ml of phosphate buffer pH 6.8: 20 μmoles H_2O_2, 2.8 μmoles quaiacol and 2–10 μg enzyme. Optical density at 470 nm was measured after 2–3 min and the activity was defined as the increase in absorbance per min per mg protein. Protein was estimated by the method of Lowry et al [4]. The removal of hemin from the enzyme was performed by the acid acetone precipitation technique [5].

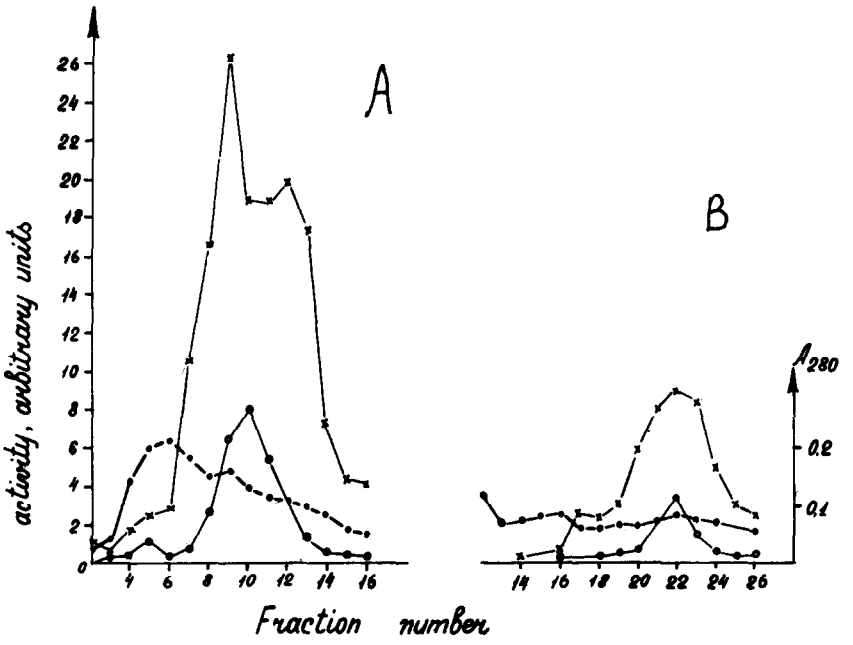

Fig. 1. Enzymic activity patterns. A) Eluted from Sephadex G-100. B) Acrylamide gel electrophoresis. Absorbance at 280 nm (•—•—•); peroxidative activity (o—o—o); nitrate reduction activity (x—x—x).

3. Results and discussion

As shown in table 1 no separation of nitrate reduction and peroxidative activities could be achieved in the course of purification of commercial peroxidase preparations. Both activities enhanced with the increase of RZ value. Apparent K_m values for nitrate in nitrate reduction reaction with the enzyme of RZ = 1.8 was 9 mM and for dithiocarbamate donator, 0.37 mM. Optimum pH ranged from 7.8 to 9.2.

Table 2
The role of heme in peroxidase activities.

	Relative activity (%)			
	Experiment 1		Experiment 2	
	Nitrate reduction	Peroxidation	Nitrate reduction	Peroxidation
Original enzyme	100	100	100	100
Apoenzyme	4	2	37	16
Reconstituted enzyme (apoenzyme + crystalline hemin)	14	40	60	100

No separation of activities studied was achieved when fractioned either by Sephadex G-100 chromatography or by preparative acrylamide gel electrophoresis. As shown in fig. 1 both activities ran parallel and their peaks coincided. This suggests that both activities belong to the same enzyme, namely, horseradish peroxidase. The following experiment with hemin extraction confirmed this suggestion. The removal of hemin (table 2) caused inactivation of both activities. Recombination of apo-peroxidase with crystalline bovine hemin led to a partial restoration of the enzyme. Thus both activities appear to involve the heme prosthetic group which seems to be the binding site for both H_2O_2 and NO_3^- substrates.

As heme proved to be essential for nitrate reduction we compared some other heme-containing proteins in their ability to reduce nitrate. The data obtained show (table 3) that peroxidase seems to be the

Table 3
Nitrate reduction and peroxidative activities of peroxidase as compared to those of some other heme-containing proteins.

	μg Fe in incubation medium	Enzymic activity		Rate of nitrate reduction as related to non-enzymic Fe^{2+} action
		Nitrate reduction (nmoles NO_2^- × μg Fe^{-1} × min^{-1})	Peroxidation (A_{470} × μg Fe^{-1} × min^{-1})	
$FeSO_4$	20	0.5	0	1
Horseradish peroxidaze (RZ = 1.3)	0.0003	400,000	360	800,000
Catalase from bovine liver (Reanal)	0.07	0	Trace	–
Lupine root nodule hemoglobin	0.04	162	0.3	324
Hemoglobin from horse heart (Reanal)	0.34	4.4	0.1	8.8
Crystalline bovine hemin (Reanal)	8.6	2.9	0.006	3.8
Cytochrome *c* from horse heart (Reanal)	0.4	0	Trace	–

only heme-protein capable of reducing nitrate. The root nodule hemoglobin possessed very low activity and other proteins tested were completely ineffective. As was reported in our previous paper [2] Fe^{2+} is capable of catalyzing a non-enzymic reaction of nitrate reduction by dithiocarbamatesulfite under the conditions described above. However the non-enzymic reaction needs a much higher iron concentration than that present in the assay medium for the peroxidase reaction. Therefore, when calculated per iron unit, the rate of the enzymic reaction is 800,000 times greater than that of a non-enzymic one.

It is worth emphasizing that the enzymic reaction under study is of very high activity compared to that of NADH-dependent nitrate reductase reaction. Maximum activity of nitrate reductase given by several authors for various plant species [1] is about 600–700 nmoles NO_2^- produced × min^{-1} × mg $protein^{-1}$ compared to 420,000 for the peroxidase preparation in our experiments. This peroxidase property appears not to be restricted to the horseradish enzyme. Wherever peroxidative activity has been found, either in leaves, roots, seeds, bleeding sap of various plant species or in protein preparations, dithiocarbamate nitrate reduction activity has also been revealed.

Besides the classic peroxidative reaction, peroxidase is known to catalyze the oxidative type of reaction using O_2 as the electron acceptor. Such oxidizing agents as ClO_2, $KBrO_3$, KIO_4, $K_2S_2O_8$, HOCl, $NaClO_2$ can also form complexes with peroxidase, some of which are capable of oxidizing phenols [6–8]. The present work suggests that nitrate may be the substrate for a peroxidase reaction where a specific substance, dithiocarbamate is oxidized.

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