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PURIFICATION AND PROPERTIES OF DEHYDROASCORBATE REDUCTASE FROM SPINACH LEAVES

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Key Word Index—*Spinacia oleracea*: Chenopodiaceae; ascorbate; dehydroascorbate; glutathione; chloroplasts; dehydroascorbate reductase.

Abstract—Dehydroascorbate reductase was detected in the leaves of several plants and has been partially purified from spinach leaves. The enzyme has a MW of *ca* 25000, a pH optimum of 7.5, a K_m for glutathione (GSH) of 4.43 ± 0.4 mM and a K_m for dehydroascorbate of 0.34 ± 0.05 mM. High concentrations of dehydroascorbate inhibit the enzyme. Cysteine cannot replace GSH as a donor. The purified dehydroascorbate reductase is extremely unstable and also inhibited by compounds which react with thiol groups. Dehydroascorbate does not protect the enzyme against such inhibition. GSH reduces dehydroascorbate non-enzymically at alkaline pH values.

INTRODUCTION

Ascorbic acid and its oxidised product, dehydroascorbic acid, are present in the leaves of green plants, although the amount of ascorbate usually greatly exceeds that of dehydroascorbate [1]. Ascorbate is essential for plant metabolism and inhibition of its synthesis prevents growth [2]. It reacts with H_2O_2 and also with the phytotoxic free-radical superoxide ($O_2^{\cdot-}$); H_2O_2 and $O_2^{\cdot-}$ are generated in plant tissues [3, 4] so that ascorbate may well play an important role in protecting the plant against them [4].

Reaction of ascorbate with either $O_2^{\cdot-}$ or H_2O_2 produces dehydroascorbate [4]. This can decompose to yield, among other products, oxalic acid, and part of the oxalate formed in plants appears to arise from ascorbate [5]. However, it has long been known that crude leaf homogenates contain a heat-labile activity which catalyses reduction of dehydroascorbate back to ascorbate in in the presence of glutathione (GSH) [1] or cysteine [6] and it was suggested that a dehydroascorbate reductase enzyme is present, catalysing the reaction

$$\underset{\text{(2 cysteine)}}{2GSH} + \text{dehydroascorbate} \rightarrow \text{ascorbate} + \underset{\text{(cystine)}}{GSSG}$$

No studies have been reported on the properties of the purified enzyme from leaves. Yamaguchi and Joslyn [7] examined the properties of a similar enzyme in crude $(NH_4)_2SO_4$ fractions made from extracts of pea seeds. However, the dialysis step used by them to remove $(NH_4)_2SO_4$ from the enzyme caused considerable loss of activity, so it is not clear to what extent the enzyme had been purified, if at all. Because of our interest in the metabolic roles of ascorbate in plant tissues [4, 8, 9], we have purified the dehydroascorbate reductase activity from leaves of spinach (*Spinacia oleracea*).

RESULTS

Purification

High activities of dehydroascorbate reductase were detected in extracts from mature leaves of several plants.

Table 1. Purification of dehydroascorbate reductase

Stage	Total protein (mg)	Total enzyme activity (mol ascorbate produced/sec 10^6).	% recovery of activity	Specific activity (nkat/mg protein)	Purification factor
Crude homogenate	1390	2.72	100	2.0	1
Supernatant after centrifugation at 20000 *g*	1130	2.67	98	2.3	1.2
Second $(NH_4)SO_4$ ppt.	97	2.60	96	26.8	13
Gel filtration (the 3 most active fractions were pooled)	25	2.35	86	93.3	47

The purification procedure was as described in the Experimental section.

The enzyme activity of *S. oleracea* leaf extracts, measured as GSH-dependent ascorbate formation, was 60 nkat/mg chlorophyll; for *Beta vulgaris* 13; for *Brassica oleracea* (cabbage) 315 and for *B. oleracea* (cauliflower) 68 nkat/mg chlorophyll. Activity was destroyed by heating these extracts at 100° for 10 min. We decided to purify the enzyme from *Spinacia* leaves, since these were readily available throughout the year and several other studies of ascorbate metabolism have been carried out on this plant [1, 5, 8]. Table 1 summarises the purification achieved by the techniques described in the Experimental section: a *ca* 50-fold purification, with 70–90% recovery of the starting activity, was routinely obtained. However, the activity eluted from the Sephadex column was extremely unstable at 4°, losing 50% of its activity in 24 hr. Addition of bovine serum albumin (1% w/v) or GSH (1 mM) did not increase stability. Attempts at further purification using ion-exchange polymers (cellulose, Sephadex and Sepharose) were unsuccessful; there was a rapid loss of enzyme activity on the columns, so that the enzyme eluted had no greater sp. act. than the material applied. Inclusion of GSH, ascorbate or 2-mercaptoethanol in the elution buffers did not prevent loss of activity. Thus spinach-leaf dehydroascorbate reductase becomes increasingly unstable as the degree of purification becomes greater. This is not due to loss of a metal ion cofactor, as incubation of the enzyme with EDTA (1–2 mM) did not decrease activity and attempts to increase activity by incubation of the enzyme with several metal ions, and other possible cofactors, were unsuccessful. In view of the high sp. act. achieved by gel filtration (Table 1), we thought it justified to study the properties of the enzyme using the most pure fractions from the Sephadex column. These could be stored frozen at −20° for up to 1 week without significant loss of activity. Disc electrophoresis, using 12.5% polyacrylamide gels [10] showed that only 2 protein bands were present (as compared to at least 30 in the supernatant from which the enzyme was purified), although it was not possible to stain these gels for enzyme activity.

Properties

The enzyme activity eluted from the Sephadex column as a symmetrical peak and there was no evidence for more than one protein species with activity. The elution volume corresponded to a MW of *ca* 25000 using gels calibrated as described by Andrews [11]. Heating the enzyme at 50° for 10 min caused 35% loss of activity whereas heating at 60° for 10 min caused complete inactivation.

Table 2. Stoichiometry of dehydroascorbate reductase

Batch of enzyme	μmol of GSH consumed	μmol of GSSG formed	μmol of ascorbate formed
A	1.53	—	0.87
B	1.59	—	0.74
C	—	0.68	0.84
D	—	0.58	0.59
E	—	0.83	1.02

Five separate batches of purified enzyme were assayed for activity by measuring either dehydroascorbate-dependent oxidation of GSH, dehydroascorbate-dependent formation of GSSG or GSH-dependent formation of ascorbate. Appropriate controls were used in each case to correct for non-enzymic rates. All assays were carried out in 0.1 M KH_2PO_4 adjusted to pH 6.3 with KOH. GSSG was assayed by the sensitive and specific enzymic procedure described by Tietze [12] and GSH by using Ellman's reagent [13].

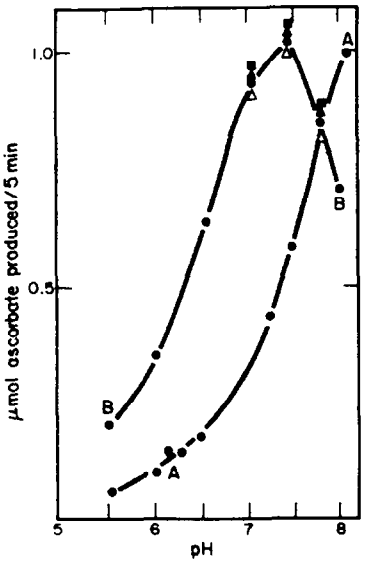

Fig. 1. Effect of pH on dehydroascorbate reductase activity. Assays were carried out as described in the Experimental section. Graph A shows the rate of the non-enzymic reaction and graph B the rate of the enzyme-catalysed reaction (corrected for non-enzymic rates). ● Phosphate buffer (0.05 M KH_2PO_4 adjusted to the required pH with KOH); △ Triethanolamine buffer (0.05 M adjusted to the required pH with HCl); ▲ TES buffer (0.05 M adjusted to the required pH with KOH); ■ HEPES buffer (0.05 M adjusted with KOH).

All previous workers have assayed dehydroascorbate reductase by GSH-dependent ascorbate formation [1, 6, 7] and we have also adopted a modification of this method for routine use [8]. In several experiments, however, we also assayed the enzyme by following dehydroascorbate-dependent loss of GSH or formation of GSSG. The results obtained agree fully with the predicted stoichiometry of the reaction (Table 2).

Fig. 1 shows the effect of pH on the enzyme-catalysed reaction. At pH values above 7, there is a rapid non-enzymic reduction of dehydroascorbate by GSH. Above pH 8, this reaction uses up substrates so rapidly that it is difficult to measure the enzyme-catalysed rate. Although the pH optimum of the enzyme appeared to be 7.5 (Fig. 1), we routinely assayed it at pH 6.3, when the non-enzymic reaction was very small. The type of buffer used did not markedly affect the reaction rates.

Fig. 2a shows the effect of dehydroascorbate concentration on the enzyme activity at a fixed concentration of GSH. High concentrations of dehydroascorbate inhibited the enzyme. However, a K_m could be deduced from the linear portion of the Lineweaver–Burk plot (Fig. 2b): this was 0.34 ± 0.05 mM (mean ±S.D. of 5 determinations on different batches of enzyme) in the presence of saturating (11–17 mM) concentrations of GSH.

When the concentration of GSH was varied in the presence of fixed concentrations of dehydroascorbate, the enzyme obeyed the Michaelis–Menten equation over a wide range of GSH concentrations. The K_m for GSH, determined using Lineweaver–Burk plots and also

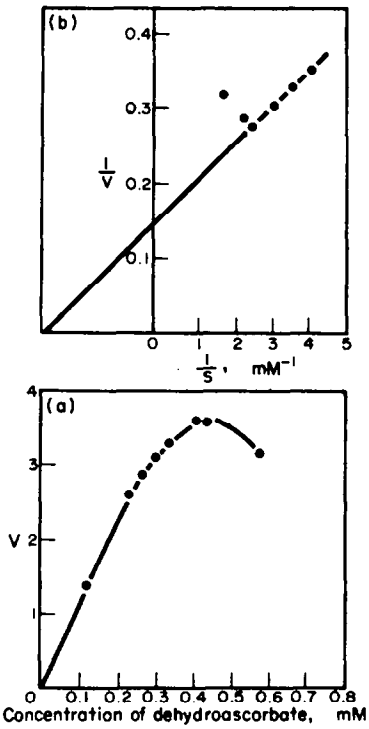

Fig. 2. Effect of dehydroascorbate concentration on dehydroascorbate reductase activity. Activity was assayed at pH 6.3 in phosphate buffer [8]. Corrections were made for any non-enzymic reduction at each concentration of substrates used. (a) shows the results as a plot of initial reaction rate against dehydroascorbate concentration and (b) shows the data as a Lineweaver–Burk plot, indicating how K_m was determined. The concentration of GSH used in this experiment was 15 mM but similar results were obtained using GSH concentrations from 11–17 mM.

the direct linear plots introduced by Eisenthal and Cornish-Bowden [14], was 4.32 ± 0.4 mM (mean ± S.D. of 5 determinations) at pH 6.3 in the presence of 0.3 mM dehydroascorbate. Raising the dehydroascorbate concentration to 1–2 mM, which inhibits the enzyme, did not alter the K_m for GSH. Thus the enzyme seems to have a much greater affinity for dehydroascorbate than for GSH. Cysteine, however, could not replace GSH in the enzyme-catalysed reaction.

Table 3 Effect of inhibitors on dehydroascorbate reductase activity

Compound present during preincubation	Concentration (mM)	Activity in subsequent assay (nkat)	% Inhibition
None	—	7.2	0
N-ethylmaleimide	0.1	6.0	16
	1.0	3.8	47
p-Hydroxymercuri-benzoate	0.1	4.3	40
	1.0	2.2	70
Iodoacetic acid	0.1	2.5	65
	1.0	1.7	77
$CuSO_4$	0.1	0.17	98

The enzyme was pre-incubated in phosphate buffer (50 mM, pH 6.3) with the compound at the concentration stated for 5 min. It was then assayed for dehydroascorbate reductase by GSH-dependent ascorbate formation. The amount of inhibitor carried over into the assay mixture was not sufficient to decrease the concentration of GSH significantly.

Pre-incubation of dehydroascorbate reductase with compounds that react with thiol groups severely inhibited its activity in a subsequent assay (Table 3). Inclusion of dehydroascorbate in the pre-incubation mixture did not significantly protect the enzyme. The enzyme was also inhibited when exposed to heavy metal ions, such as Cu^{2+}.

DISCUSSION

We have partially purified a dehydroascorbate reductase from spinach leaves and studied some of its properties. We have also confirmed the stoichiometry of the reaction. The enzyme is unstable and seems to require one or more thiol groups for activity, as evidenced by the inhibition by reagents that react with such groups. It is not known if these groups are present at the active site of the enzyme, as dehydroascorbate did not protect against inhibition.

The pH optimum of the enzyme was 7.5 (Fig. 1), but the non-enzymic reaction occurred at comparable rates at this pH. The enzyme therefore reduces the pH at which GSH-dependent dehydroascorbate reduction will occur. Subcellular fractionation studies have shown that the enzyme in spinach leaves is located in the cytosol [8]. The pH of the cytosol may be insufficiently high to allow the non-enzymic reduction to occur. In contrast, chloroplasts contain GSH and ascorbate, but no dehydroascorbate reductase [8]. However, the pH in the stroma of illuminated spinach chloroplasts is close to 8, when the non-enzymic reduction can occur rapidly [8].

The K_m of dehydroascorbate reductase for GSH seems to be high. However, mM concentrations of GSH are present in spinach leaves [8]. The K_m for dehydroascorbate is much lower and this would account for the observation that concentrations of dehydroascorbate in leaves are always much lower than those of ascorbate. GSH for the continued operation of the enzyme may be regenerated by the action of the $NADP^+$-linked glutathione reductase activity of spinach leaves [1]. It is difficult to attribute any metabolic significance to the inhibition of dehydroascorbate reductase by high concentrations of dehydroascorbate. It is possible that the inhibition represents formation of some kind of 'abortive complex', e.g. if the enzyme normally binds two molecules of GSH followed by one of dehydroascorbate to give an active complex, it might bind only one molecule of GSH before binding dehydroascorbate when the latter is present at high concentrations.

EXPERIMENTAL

Materials. Dehydroascorbic acid was purchased from Koch-Light Ltd. Spinach, cabbage, cauliflower and spinach-beet were obtained from a local market or grown in a greenhouse. Only undamaged mature leaves were used.

Purification of enzyme. All operations were carried out at 0–4°. Washed, de-ribbed spinach leaves (150 g) were homogenised in 400 ml of KH_2PO_4 buffer (0.1 M, adjusted to pH 6.3 with KOH). The homogenate was squeezed through two layers of muslin and centrifuged at 20000 *g* for 15 min. The supernatant was brought to 50% satn by addition of solid $(NH_4)_2SO_4$ and allowed to stand for 10 min, with stirring. It was then centrifuged at 20000 *g* for 15 min, and the pellet discarded. The supernatant was brought to 80% satn with $(NH_4)_2SO_4$, allowed to stand

for 10 min and then centrifuged at 20000 *g* for 15 min. The supernatant was discarded and the pellet dissolved in 5 ml of 10mM Pi buffer, pH 6.3. This soln was applied to a column of Sephadex G100 (35 × 2.4 cm) which was eluted with 10mM KH_2PO_4, adjusted to pH 6.3 with KOH and 10 ml fractions were collected. The bulk of enzyme activity eluted between 100 and 130 ml and fractions were stored at −20°.

Preparation of leaf extracts. Mature leaves were washed, de-ribbed and 30 g of the lamellar tissue homogenised in 100 ml of ice-cold 0.1 M KH_2PO_4–KOH buffer, pH 6.3. The homogenate was squeezed through two layers of muslin and assayed as described below. Enzyme activity was proportional to the amount of homogenate assayed.

Assays. Dehydroascorbate reductase was assayed by measuring GSH-dependent production of ascorbic acid [8]. Conditions for all assays were chosen so that the rate of reaction was constant in the time used and proportional to the amount of enzyme added. Controls without enzyme and without GSH were carried out to correct for any non-enzymic reduction and for any ascorbate present in the fraction being assayed (this was especially important with crude leaf extracts). Chlorophyll was measured by the method described in ref. [15]. Protein was pptd from soln with TCA and assayed by the method of ref. [16]. Crystalline BSA, desiccated before use, was used as a standard. The protein content of column eluates was determined spectrophotometrically [17].

MW determination. A column of Sephadex G200 was prepared and calibrated as described in ref. [11]. The calibration proteins used were catalase (MW 240000), ferritin (540000), cytochrome *c* (12500), aldolase (158000) ovalbumin (45000) and BSA (67000). The dehydroascorbate reductase corresponded to a MW of 25000.

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