STUDIES ON DEHYDROASCORBIC ACID REDUCTASE IN *ESCHERICHIA COLI* K₁₂

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It was reported by Esselen and Fuller (1) that a certain strain of *Escherichia coli* has the activity of reducing dehydroascorbic acid to ascorbic acid. The same activity was assigned to *Staphylococcus albus* by Stewart and Sharp (2). Gunsalus and Hand (3), and Mapson and Ingram (4) used this reduction activity for quantifying dehydroascorbic acid. As long as glucose exists in the medium containing *E. coli*, dehydroascorbic acid is reduced. As this reducing activity is lost in one hour at 60°, the participation of an enzyme has been suspected (4). Dehydroascorbic acid reductase has hitherto been found in plants, but never been detected in animals and bacteria (5,6). The present report describes the partial purification of dehydroascorbic acid reductase isolated from *E. coli* K₁₂ and some properties of the enzyme.

EXPERIMENTAL

Materials

1. Chemicals

L-Ascorbic acid and D-araboascorbic acid were commercial products, and were recrystallized from methanol and dioxane. Dehydroascorbic acid solution was obtained by oxidation of ascorbic acid solution with bromine, the small amount of excess bromine being removed by vigorous aeration. It was kept at 0° at pH 2—3 (adjustment was not needed after aeration) and used within 5 minutes. This solution was used as the standard substrate (hydrogen acceptor). Glutathione, NADH₂ and NADPH₂ were obtained from C.F. Boehringer and Sone and Company, Ltd. DEAE-cellulose was obtained from Nutritional and Biochemicals Corporation.

2. Bacteria

A 20-hour culture of *E. coli* K₁₂ was added to 400 liters of broth containing 1 per cent peptone, 1 per cent glucose and 1 per cent meat extract, and was allowed to grow for 20 hours at 37°. The culture was centrifuged in a continuous centrifuge, and the precipitated cells were stored in a refrigerator after being washed twice with 0.9 per cent sodium chloride solution. No appreciable deterioration in the enzyme activity of the cells was detected in 6 months when they were kept

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at -20° .

Methods

1. Assay Method of Dehydroascorbic Acid Reductase

Dehydroascorbic acid ($4.0\ \mu\text{moles}$) was incubated in a Thunberg tube with the enzyme, glutathione ($8.0\ \mu\text{moles}$) and phosphate buffer, pH 6.8, ($120\ \mu\text{moles}$) in the final volume of 2.0 ml under anaerobic condition at 37° for 5 minutes. Dehydroascorbic acid should be added last of all. The reaction was stopped by the addition of trichloroacetic acid at 0° , and the mixture was filtered. The remaining dehydroascorbic acid and the total ascorbic acid in the filtrate were determined by the following procedure.

The determination of the remaining dehydroascorbic acid was carried out with 2,4-dinitrophenylhydrazine by the modified method of Roe *et al.* (7), the incubation period being 1 hour at 50° in a half scale of the original method (8). The total ascorbic acid was determined with 2,4-dinitrophenylhydrazine after oxidizing ascorbic acid with bromine, and the small amount of excess bromine having been removed by the addition of thiourea. The difference between total ascorbic acid and dehydroascorbic acid was concluded to be the amount ascorbic acid formed. The amount formed enzymatically was estimated by the substration of the amount formed without the addition of the enzyme.

2. Definition of Unit and Specific Activity of the Enzyme

One unit of the enzyme is defined as one μmoles of dehydroascorbic acid produced enzymatically in 5 minutes under the condition described above. The specific activity is the units per mg of protein. The amount of protein was determined with Folin-Ciocalteu's reagent (9).

3. Purification Procedure of Dehydroascorbic Acid Reductase

Step 1. *Preparation of Extracts*—20 g of cell paste was suspended in 50 ml of 0.02 M phosphate buffer, pH 7.4, containing 0.5 mg of glutathione per ml. The suspension was subjected to the action of 10-kc. oscillator for 20 minutes and the supernatant fraction was obtained after centrifugation at $105,000\times g$ for 1 hour at 0° . The supernatant fraction contained 29 mg of protein per ml. All subsequent steps are carried out in the cold.

Step 2. *Removal of Nucleic Acid with Protamine*—The supernatant fraction was adjusted to pH 6.0 with 1 N acetic acid. Protamine sulfate solution, 2 %, pH 5.0, was added with vigorous stirring until the 280/260 $m\mu$ absorption ratio of 1.0 was obtained in a centrifuged aliquot. The supernatant fraction was obtained after centrifugation.

Step 3. *First Ammonium Sulfate Fractionation*—The supernatant fraction was added with solid ammonium sulfate, and the precipitate between 0 and 0.9 saturation was dissolved in distilled water. The solution was dialysed against 0.02 M phosphate buffer, pH 7.4. The precipitate appearing on dialysis was centrifuged and discarded.

Step 4. *Second Ammonium Sulfate Fractionation*—The dialysed fraction was added with solid ammonium sulfate and the precipitate obtained from 0.5 to

0.9 saturation was dissolved in 0.005 *M* tris (hydroxymethyl) aminoethane-HCl buffer³ (pH 8.0). The solution was dialysed against the same buffer.

Step 5. *Cellulose Column Chromatography*—DEAE-cellulose bufferized with 0.005 *M* tris(hydroxymethyl)aminoethane-HCl buffer, pH 8.0, was packed in column of 2×10 cm. The dialysed fraction was adsorbed on the column and eluted with 0.1 *M* NaCl in 0.005 *M* tris(hydroxymethyl)aminoethane-HCl buffer, pH 8.0. Analysis of the effluent fraction is shown in Fig. 1.

FIG. 1 *Chromatographic Purification of the Enzyme on DEAE-Cellulose Column*

Eluant: 0.005 *M* Tris-buffer (pH 8.0) containing NaCl at various concentrations. Fraction volumes: 10 ml.

—●—, enzyme activity (unit/ml); ---○---, protein (mg/ml)

The specific activity, the yield, and the total protein of each step are summarized in Table 1.

TABLE 1
Summary of Purification of the Enzyme

Fraction	Specific activity	Recovery	Total protein
	<i>unit/mg protein</i>	<i>per cent</i>	<i>mg</i>
Supernatant fraction	0.05	100	1,677
First ammonium sulfate fraction	0.10	87	718
Second ammonium sulfate fraction	0.14	64	387
DEAE-cellulose column treatment (Fraction No. 15)	1.0	5.7	4.8

³ In this paper the molar concentration of tris(hydroxymethyl)aminoethane-HCl buffer is shown by its Cl⁻ concentration.

RESULTS

1. Time Course of Reduction in the Enzymatic Reaction

The amount of dehydroascorbic acid reduced was proportional to the incubation period in the presence of the enzyme, as long as the substrate remained was large enough to be indicated by the zero order reaction (0 to 5 minutes). As the non-enzymatic reduction under the same condition was appreciable, it was subtracted.

2. Effect of the Enzyme Concentration

The substrate reduced during incubation was proportional to the enzyme concentration in the assay method. To observe this proportionality, the enzyme solution had to be diluted to the appropriate activity, even when non-purified supernatant fractions were used as the enzyme solution.

3. pH Optimum

The pH-activity curve is shown in Fig. 2, in which non-enzymatic reduction was subtracted. The optimum pH was at 6.8–7.0. On account of the non-enzymatic hydrolysis of dehydroascorbic acid, the enzyme activity at pH values higher than 7.2 was unable to be measured.

FIG. 2 *Effect of pH on Enzyme Activity*

4. Reaction Product

After incubation, the reaction product was oxidized by bromine, and identified in the form of bis-2,4-dinitrophenylhydrazone (2,4-dinitrophenylosazone). Dehydroascorbic acid and glutathione (10 times the standard condition) was incubated with excess enzyme until the remaining dehydroascorbic acid was not detectable at all with 2,4-dinitrophenylhydrazine, and then the reaction mixture was deproteinized. The product was oxidized to dehydroascorbic acid with bromine, the small amount

of excess bromine being removed by the addition of thiourea. The reaction product oxidized was treated with 0.4% 2,4-dinitrophenylhydrazine in $N H_2SO_4$ at 50° for 1 hour. The precipitate obtained was washed with distilled water, and recrystallized from hot methanol, and methanol-acetone. The resulting orange needles showed mp. 281° — 282° . The authentic bis-2,4-dinitrophenylhydrazone of dehydroascorbic acid gave mp 282° — 283° , and the mixed mp was 281° . The R_f values of bis-hydrazone are shown in Table 2. There were no difference in the R_f values of bis-hydrazone between the reaction product oxidized and dehydroascorbic acid.

TABLE 2
Thin Layer Chromatography of Reaction Product

	R_f value using the developing solvent system		
	A	B	C
Dinitrophenylhydrazine derivative of dehydroascorbic acid	0.16	0.30	0.48
Dinitrophenylhydrazine-treated oxidized product of reaction product	0.16	0.31	0.49

A, cyclohexane-acetone (7 : 3); B, chloroform-acetone (9 : 1);
C, toluene-acetone-5% acetic acid (2 : 1 : 1).

The infrared spectrum of bis-hydrazone from the product oxidized of the reaction product was identical to that from the authentic sample of dehydroascorbic acid.

5. Enzyme Stability

The activity was completely lost on heating in a boiling water bath for 5 minutes. The purified enzyme was stable at 5° for a week in the eluate obtained from DEAE-cellulose column chromatography.

6. Metal Requirement

The purified enzyme was active without Mn^{2+} ($10^{-3}M$), Mg^{2+} ($10^{-3}M$), or Co^{2+} ($10^{-3}M$). There was no effect in EDTA.

7. Stoichiometry

The reaction assayed under the standard condition proceeded almost stoichiometrically with the oxidation of glutathione (Table 3).

TABLE 3
Stoichiometry

Exp. No.	DAsA ^a added	AsA ^b formed	DAsA remained	Glutathione ^c oxidized
	$\mu moles$	$\mu moles$	$\mu moles$	$\mu moles$
1	4.0	0.9	3.1	1.9
2	4.0	0.4	3.5	0.7

^a Dehydroascorbic acid; ^b Ascorbic acid;

^c Glutathione was determined with the nitroprusside method (10).

8. *Hydrogen Acceptor*

The enzyme hydrolysed also dehydro-D-arboascorbic acid at the rate of 70 % of dehydro-L-ascorbic acid. 2,3-Diketogulonic acid was not reduced by the enzyme.

9. *Hydrogen Donor*

The enzyme was effective on other hydrogen donors such as homocysteine, cysteine and thioglycolate, but not effective on NADH₂, NADPH₂, CoA and lipoic acid (Table 4).

TABLE 4
Hydrogen Donor Specificity

	Activity
	<i>unit</i>
Glutathione	0.50
Homocysteine	0.50
Thioglycolate	0.25
Cysteine	0.20
NADPH ₂	0
NADH ₂	0
CoA	0
Lipoic acid	0

10. *Contamination of Other Enzymes in the Preparation*

Glyoxalase I activity was observed in the initial extract of *E. coli* but not observed in the purified enzyme, showing that the enzyme was different from glyoxalase I. The activity of gluconolactonase, which is known to delactonize dehydroascorbic acid (11-13), was observed in the initial extract of *E. coli*, but not observed in the purified enzyme.

11. *Intracellular Distribution*

There was an activity on dehydroascorbic acid in the soluble fraction, but no activity was found in the particulate fractions.

DISCUSSION

Kendall *et al.* (15), confirmed the fact that *Mucosus capsulatus* and enterococci have a strong property of destroying ascorbic acid and that the acid is destroyed prior to dehydroascorbic acid. The decomposition by these intestinal bacteria can bring about the deficiency of ascorbic acid clinically (16-17). On the other hand, *E. coli* (3-4) and *St. albus* (2) possess a high activity of reducing dehydroascorbic acid, protecting the decomposition of ascorbic acid. In animals, dehydroascorbic acid is enzymatically delactonized to 2,3-diketogulonic acid, which cannot become dehydroascorbic acid by the reverse reaction (11-12). Therefore, 2,3-diketogulonic acid lacks the antiscorbutic activity, whereas dehydroascorbic acid possesses the activity, and what is eliminated in the urine following excessive administration is not dehydroascorbic acid, but ascorbic acid (18). No dehydroascorbic acid reductase has ever been found in animals and bacteria. The present paper clarified the presence of dehydroascorbic acid reductase in bacteria. The properties of the enzyme were

similar to that obtained from the plant (19).

As it is known that, *E. coli* reduces dehydroascorbic acid to ascorbic acid in the presence of glucose (4), and the bacteria have glutathione reductase (20), dehydroascorbic acid is assumed to be reduced into ascorbic acid by the following reaction sequence in the bacterial cells.

The presence of dehydroascorbic acid reductase in bacterial cells would serve the regeneration of ascorbic acid, protecting its decomposition, because, once dehydroascorbic acid is delactonized, it is easily decomposed under oxidation. The delactonization of dehydroascorbic acid proceeds by an enzymatic reaction in animal tissues (10-11), and dehydroascorbic acid delactonizing enzyme was reported to be identical to lactonase I (aldonolactonase, gluconolactonase) (11-12). The gluconolactonase was found by Brodie and Lipmann (14) in bacteria. Thus, dehydroascorbic acid will be easily delactonized enzymatically in the bacterial cells, when it is not readily reduced to ascorbic acid. Therefore, the presence of the reductase might be desirable to the host, although it is not quite clear what role the enzyme plays physiologically in the bacterial cells.

SUMMARY

1. The enzymatic reduction of dehydro-L-ascorbic acid in *Escherichia coli* was reported at the physiological pH range with the assay condition without loss of resulting ascorbic acid.

2. The enzyme, dehydroascorbic acid reductase, was partially purified, and some properties were observed. Hydrogen donors, hydrogen acceptors, and cofactor requirement were studied.

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