Biochemical Studies on L-Ascorbic Acid in Aquatic Animals—VIII

Purification and Properties of Dehydro-L-ascorbic Acid Reductase from Carp Hepatopancreas

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DAsA-reductase (EC 1.8.5.1) was purified from carp hepatopancreas by ammonium sulfate fractionation, Sephadex G-75 gel filtration and DEAE-Sephadex A-50 column chromatography, obtaining enzyme material enriched about 100-fold in specific activity with a 23% yield.

Some properties of the purified enzyme were studied with the following results. Without bovine serum albumin, the enzyme was relatively unstable. Optimal pH and temperature for the enzyme reaction were near 6.7 and 30°C, respectively. Co^{2+} , Zn^{2+} , Hg^{+} and Fe^{3+} strongly inhibited the enzyme activity, while Cu^{2+} , Mg^{2+} , and Mn^{2+} had no effect. The enzyme activity was only slightly affected if at all by EDTA, *o*-phenanthrolic acid, sodium azide, sodium arsenate and nucleotides, but was inhibited by sodium cyanide and CO gas. The K_m value was 5.7×10^{-4} M for DAsA and 1.5×10^{-3} M for GSH. The enzyme was specific for GSH as a hydrogen donor and less specific for DAsA as a hydrogen acceptor. Product inhibition was observed with excess GSSG but not with AsA.

In the previous paper¹⁾, the existence of Dehydro-L-ascorbic acid (DAsA)-reductase (EC 1.8.5.1) which catalyzes the transfer of hydrogen from reduced glutathione (GSH) to DAsA was shown in fishes. In spite of the accumulating knowledge of the enzyme in plant tissues²⁻⁷⁾, there are hardly any studies on the purification and properties of the enzyme in animal tissues except for the paper reported by HUGHES⁸⁾.

In the present paper, a method for the isolation and purification of DAsA-reductase from carp hepatopancreas and some properties of the purified enzyme are described.

Materials and Methods

Sample Culture carp, *Cyprinus carpio*, were obtained from commercial suppliers. All were living fish weighing about 400 g.

Materials Hemoglobin was prepared from carp as follows. The blood of carp was obtained by cutting off the posterior portion of the body. In order to prevent coagulation, sodium heparinate was used. Hemolysate of erythrocytes was collected from the blood by centrifugating at $1,000 \times g$ for 15 min, washing the cells 3 times with cold 0.9% NaCl, and finally hemolyzing with equal volume of water. The hemolysate was stirred vigorously with a third volume of ether for 10 min at a low temperature, and it was centrifuged at $10,000 \times g$ for 30 min to remove the stroma and ether. The hemoglobin layer was collected

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with a syringe and dialyzed overnight at 5°C against distilled water. The other reagents were obtained from commercial sources.

Assay of enzyme activity Measurement of DAsA-reductase activity was essentially the same as described in the previous paper¹⁾. The reaction mixture containing 22.8 μ moles of GSH, 11.4 μ moles of DAsA, 600 μ moles of phosphate buffer, pH 6.3, and enzyme solution in a total volume of 6 ml, was incubated at 30°C by bubbling nitrogen gas through the mixture. The reaction was stopped by the addition of 2 ml of 20% HPO_3 . After filtration through Toyo No. 5c paper, the amount of L-ascorbic acid (AsA) produced from DAsA was then determined by the 2,6-dichlorophenolindophenol method. Enzyme activity was expressed as μ moles of AsA formed enzymatically from DAsA per 10 min. When the effect of excess AsA on the enzyme reaction was studied, decrease of GSH was determined according to the method of GRUNERT⁹⁾ as an indication of the activity. HEPES buffer was used in studying the effect of metal ions on the activity in order to prevent their chelation.

Results and Discussion

Purification of the enzyme

1. *Pretreatment* Carp hepatopaneas was homogenized with 3 volumes of cold 0.9% NaCl in a POTTER-ELVEHJEM homogenizer. The homogenate was centrifuged at $28,000 \times g$ for 30 min.

2. *Ammonium sulfate fractionation* The supernatant thus obtained was then fractionated with ammonium sulfate and the precipitates formed between 60 and 80% saturation were collected. The precipitates were dissolved in a small volume of 0.02 M sodium phosphate buffer, pH 7.0, and the insoluble matter was removed by centrifugation.

3. *Sephadex G-75 gel filtration* The supernatant was passed through a Sephadex

Fig. 1. Sephadex G-75 gel filtration of the $(\text{NH}_4)_2\text{SO}_4$ fraction of 0.9% NaCl extract of carp hepatopaneas. Column size: 2.5×40 cm, flow rate: 15 ml/hr, solvent: 0.02 M sodium phosphate buffer, pH 7.0. $\circ-\circ-$: E_{280} , $\bullet-\bullet-$: Enzyme activity.

G-75 column (2.5×40 cm) which had been previously equilibrated with the above phosphate buffer and eluted with the same buffer. A typical elution profile is shown in Fig. 1. Roughly, two peaks absorbing at 280 nm appeared and the enzymatically active protein was found in the second peak.

The enzyme preparation, partially purified by Sephadex G-75 gel filtration, assumed the characteristic red brown appearance of iron porphrin enzymes. As shown in Fig. 2,

Fig. 2. Absorption spectra of the enzyme partially purified with Sephadex G-75 gel filtration. The protein concentration was $0.86 \mu\text{g}$ per ml. The oxidized and reduced form were prepared by treating with sodium ferricyanide and sodium dithionite, respectively.

the spectrum of the enzyme is clearly that of a heme protein, since there are a large Soret peak at 415 nm and two smaller peaks in the spectral region between 500 and 600 nm. After treatment of the enzyme with potassium ferricyanide, the oxidized preparation displays peaks at 630, 500 and 400 nm. After treatment with sodium dithionite, the reduced preparation has peaks at 556 and 430 nm. The spectra are very similar to those of hemoglobin¹⁰. By comparing in more detail the spectral characteristics of the enzyme with those of hemoglobin prepared from blood of carp, it was found that this enzyme preparation contained a considerable amount of contaminating hemoglobin.

4. *DEAE-Sephadex A-50 column chromatography* The removal of hemoglobin from the enzyme preparation was carried out by chromatography on DEAE-Sephadex A-50. About 20 ml of the enzymatically active fractions was applied to a DEAE-Sephadex A-50 column (1.5×20 cm) pre-equilibrated with 0.02 M sodium phosphate buffer, pH 7.0. As shown in Fig. 3, elution was performed by changing buffer concentration from 0.02 to 0.2 M, stepwisely. The first eluted larger peak, having an absorbance at 415 nm, was found to contain a certain amount of hemoglobin by the spectroscopic analysis and

Fig. 3. Purification of DAsA-reductase from carp hepatopancreas by DEAE-Sephadex A-50 column chromatography. Column size: 1.5×20 cm, flow rate: 30 ml/hr.

had no activity of DAsA-reductase. The enzyme activity was found in the fractions eluted with 0.1 M buffer, and an absorbance at 415 nm was not observed in these fractions. Considering the results, it seems reasonable to conclude that the separation of hemoglobin from the enzyme preparation is almost complete.

Results of purification are summarized in Table 1. At the final step, the enzyme was

Table 1. Summary of purification of DAsA-reductase

Fraction	Total protein (mg)	Specific* activity	Yield (%)
Homogenate	3,432	0.028	100
Supernatant	1,672	0.051	87
Ammonium sulfate fractionation	440	0.158	71
Sephadex G-75 column treatment	155	0.271	44
DEAE-Sephadex A-50 column treatment	9	2.519	23

* AsA formed (μ moles)/10 min/mg protein.

purified about 100-fold with a yield of 23%. It has been reported that DAsA-reductase was purified about 10-fold in plant tissue⁶⁾ and several-fold in animal tissue⁶⁾. By comparing the degree of purification between these three preparations, DAsA-reductase here obtained from carp hepatopancreas is more homogeneous than the others.

Properties of the purified enzyme

1. *Stability of the enzyme* As shown in Fig. 4, the enzyme stored at 5°C for 40 hr without bovine serum albumin lost 40% of its activity. Since the addition of bovine serum albumin prevented this loss, bovine serum albumin was added to the enzyme preparation

prior to enzyme assay.

2. *Time course and effect of enzyme concentration* As given in Fig. 5, the reduction of DAsA by this enzyme proceeds linearly with time up to 20 min. Figure 6 shows that the amount of AsA formed per 10 min increases in proportion to the protein concentration.

Fig. 4. Influence of added bovine serum albumin on the stability of DAsA-reductase. Bovine serum albumin (2.0 mg and 0.2 mg) were added to 1.0 ml of the enzyme solution (protein conc. 0.32 mg/ml) and the solutions were stored at 5°C for 20–40 hr; then the activities were assayed. Reaction mixture contained 0.32 mg of the enzyme protein, 22.8 μmoles of GSH, 11.4 μmoles of DAsA and 600 μmoles of phosphate buffer, pH 6.3, in a final volume of 6.0 ml. Incubation was carried out at 30°C by bubbling N₂ gas through the mixture.

Fig. 5. Time course of DAsA reduction. Assay conditions were the same as in Fig. 4.

Fig. 6. Effect of enzyme concentration on DAsA-reductase activity. Assay conditions were the same as in Fig. 4.

Fig. 7. Effect of pH on DAsA-reductase activity. Assay conditions were the same as in Fig. 4, except for the indicated pH.

3. *Effect of pH* The pH-activity curve of the enzyme is shown in Fig. 7. At 30°C for 10 min incubation, the optimum pH of the enzyme was near 6.7. This pH was slightly lower than the value reported for pea seeds⁶. In the pH range higher than 7.0, non-enzymatic reduction of DAsA by GSH was very rapid, so that exact activity measurements

were not possible.

4. *Effect of temperature* The effect of incubation temperature on enzyme activity is shown in Fig. 8. The optimum temperature appears to be near 30°C, under 5 min

Fig. 8. Effect of temperature on DASA-reductase activity. Assay conditions were the same as in Fig. 4, except for the indicated temperature. —●—: 10 min incubation, —○—: 5 min incubation.

or 10 min incubation. This temperature is somewhat lower than the value of 40°C reported for pea seeds⁶⁾. HUGHES⁸⁾ reported that the enzyme preparation from rat liver lost only 50% of its activity after heating at 98°C for 6 min. The lower optimum temperature of the enzyme from carp may be due to its lability to heat.

5. *Effect of metal ions* The effect of some metal ions on enzyme activity is shown in Table 2. Enzyme activity was markedly inhibited by Co^{2+} and Zn^{2+} at 5×10^{-3} M, by Hg^{2+} at 10^{-3} M and com-

Table 2. Effect of metal ions on DASA-reductase activity

Compound	Concentration (M)	Relative activity (%)
None	—	100
CoCl_2	5×10^{-3}	35
Hg_2Cl_2	1×10^{-3}	21
FeCl_2	1×10^{-3}	47
FeCl_3	5×10^{-3}	N.D.*
CuCl_2	5×10^{-3}	97
MgCl_2	5×10^{-3}	91
ZnCl_2	5×10^{-3}	12
MnCl_2	5×10^{-3}	91

* Not detected.

pletely destroyed by Fe^{3+} at 10^{-3} M. On the other hand, the presence of Cu^{2+} , Mg^{2+} and Mn^{2+} scarcely affect the activity at 5×10^{-3} M, although YAMAGUCHI and JOSLYN⁶⁾ have reported that Cu^{2+} brings about the greatest inhibition of the enzyme of pea seeds.

6. *Effect of chemicals* Some chemicals were added to the reductase system at a final concentration of 10^{-3} or 10^{-2} M. In the case of CO, CO gas was passed through the enzyme solution for 5 min before enzyme assay. As shown in Table 3, the metal chelating reagents, such as EDTA and *o*-phenanthrolic acid, and inhibitors to respiratory enzymes, such as sodium azide and sodium arsenate, scarcely affect the reaction rate under the experimental conditions. However, it was found that sodium cyanide at 10^{-2} M and CO are effective inhibitors. Nucleotide mono-, di- and triphosphate slightly stimulated the enzyme activity.

Table 3. Effect of chemicals on DAsA-reductase activity. The enzyme was treated with the chemicals for 5 min prior to addition of substrates.

Reagent	Concentration (M)	Relative activity (%)
None	—	100
EDTA	1×10^{-2}	139
EDTA	1×10^{-3}	115
<i>o</i> -Phenanthrolic acid	1×10^{-2}	91
Sodium azide	1×10^{-2}	96
Sodium azide	1×10^{-3}	107
Sodium arsenate	1×10^{-2}	111
Sodium arsenate	1×10^{-3}	87
Sodium cyanide	1×10^{-2}	7
Sodium cyanide	1×10^{-3}	83
Carbon monoxide		46
AMP	1×10^{-2}	96
AMP	1×10^{-3}	122
ADP	1×10^{-2}	98
ADP	1×10^{-3}	117
ATP	1×10^{-2}	81
ATP	1×10^{-3}	124

7. *MICHAELIS constant* Figure 9 and 10 show the relationship between the reaction velocity and the concentration of DAsA and GSH, respectively. The enzyme was saturated at about 1.0×10^{-3} M of DAsA and 2.0×10^{-3} M of GSH. The apparent MICHAELIS constant (K_m) was 5.7×10^{-4} M for DAsA and 1.5×10^{-3} M for GSH.

Fig. 9. Effect of DAsA concentration on DAsA-reductase activity. Assay conditions were the same as in Fig. 4.

Fig. 10. Effect of GSH concentration on DAsA-reductase activity. Assay conditions were the same as in Fig. 4.

8. *Substrate specificity* The specificity of the enzyme with regard to hydrogen acceptor was examined. Dehydro-D-arabo ascorbic acid and dehydro-L-glucoscorbic acid were prepared by oxidation of their reduced form with bromine. The comparison of these hydrogen acceptors is given in Table 4. Dehydro-D-arabo ascorbic acid, an optical isomer of DAsA, was able to accept hydrogen from GSH as effectively as DAsA, although dehydro-L-glucoscorbic acid did so only to the extent of 30% of DAsA. As

Table 4. Comparison of hydrogen acceptors. Assay conditions were the same as in Fig. 4, except for the indicated hydrogen acceptor.

Substrate	Relative activity (%)
Dehydro-L-ascorbic acid	100
Dehydro-D-arabo ascorbic acid	111
Dehydro-L-glucos ascorbic acid	30

Table 5. Comparison of hydrogen donors. Assay conditions were the same as in Fig. 4, except for the indicated hydrogen donor.

Substrate	Relative activity (%)
GSH	100
L-Cysteine	N.D.*
Succinate	N.D.
iso-Citrate	N.D.
G-6-P	N.D.
NADH	N.D.
NADPH	N.D.

* Not detected.

shown in Table 5, among the hydrogen donors tested, others than GSH are not able to transfer hydrogen to DAsA at all. These results indicate that the enzyme is specific for GSH as hydrogen donor.

Fig. 11. Effect of GSSG on DAsA-reductase activity. Assay conditions were the same as in Fig. 4, except for the addition of GSSG.

enzyme was moderately inhibited by GSSG. Considering these results, DAsA-reductase system *in vivo* may be controlled with GSH. Unless some mechanism for the reconversion of GSSG to GSH is available, it is considered that the reduction of DAsA may be insufficient owing to a lack of hydrogen donor and to product inhibition. As a result DAsA undergoes further change to products having no vitamin C activity. Therefore, the DAsA-reducing enzyme system may be regarded as an AsA-preserving mechanism in carp.

9. Effect of reaction product As shown in Fig. 11, 40% of enzyme activity is lost by the addition of oxidized glutathione (GSSG) above a concentration of 1.2×10^{-3} M. On the other hand, inhibition was not observed by AsA up to a concentration of 2.0×10^{-3} M.

The DAsA-reductase from carp hepatopancreas appears to be more specific for GSH as hydrogen donor than for DAsA as hydrogen acceptor. In addition, the

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