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ASPARAGUSATE DEHYDROGENASES AND LIPOYL DEHYDROGENASE FROM ASPARAGUS MITOCHONDRIA

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Summary

1. Lipoyl dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) and two asparagusate dehydrogenases from asparagus mitochondria were purified by a series of steps, freezing and thawing, sodium dodecylsulfate extraction, and chromatography on Sephadex G-200 and DEAE-cellulose.

2. Lipoyl dehydrogenase was highly specific for α-lipoic acid, which could not be replaced at all by asparagusic acid. Each of the asparagusate dehydrogenases was capable of reducing both asparagusic and α-lipoic acids by using NADH as hydrogen donor.

3. Reduction of α-lipoic acid with NADH by lipoyl dehydrogenase was activated by NAD^+, but that of asparagusic acid by asparagusate dehydrogenase was inactivated by NAD^+.

4. Lipoyl dehydrogenase and two asparagusate dehydrogenases differed in electrophoretic mobility on polyacrylamide gels.

Introduction

In previous papers, we elucidated the structure of asparagusic acid, dihydroasparagusic acid, *S*-acetyldihydroasparagusic acid, and asparagusic acid-*anti* and *syn*-*S*-oxides occurring in etiolated asparagus shoots [1,2]. As shown in Fig. 1, the structure of asparagusic acid resembles that of α-lipoic acid [3] in that the same dithiolane skeleton has a carboxylic acid. α-Lipoic acid, first discovered in bacteria [4,5], is a physiologically important cofactor involved in the dehydrogenation of pyruvate and α-ketoglutarate:

$$RCOCOOH + CoA \cdot SH + NAD^+ \rightarrow RCOS \cdot CoA + CO_2 + NADH + H^+$$

Abbreviations: $AsgS_2$, asparagusic acid; $LipS_2$, α-lipoic acid.

These facts led us to investigate the physiological role of asparagusic acid, and we found that asparagusic acids similarly stimulated pyruvate oxidation in *Streptococcus faecalis* 10C1 [7].

Lipoyl dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) was first discovered in bacteria [8] and shown to be a component of the α-lipoic acid-linked pyruvate and α-ketoglutarate dehydrogenation complexes [9,10]. Its physiological function is considered to be the oxidation of protein-bound dihydrolipoic acid with NAD^+ as electron acceptor:

$$Lip(SH)_2 + NAD^+ \rightleftharpoons LipS_2 + NADH + H^+$$

The enzyme has been purified and characterized from various kinds of animals [11,12] and micro-organisms [13,14]. Even less is known about the occurrence of lipoamide dehydrogenase in higher plants [15,16], and the nature of the enzyme has not been fully investigated. Since our preliminary experiments had shown that asparagusic acid stimulated pyruvate oxidation in asparagus mitochondria [17], further investigation was carried out to study the nature of the enzyme system for asparagusic acid. We wish to report here the solubilization and purification from etiolated asparagus mitochondrial fraction of two novel enzymes, tentatively named asparagusate dehydrogenases I and II, and also a lipoyl dehydrogenase. Evidence that the former two isolated enzymes and the latter enzyme are distinct in several characteristics is also presented.

Materials and Methods

Materials

The materials used were purchased from the following sources: sodium dodecylsulfate and amido black from Nakarai Chemicals, Ltd.; sodium deoxycholate from Merck Co.; Triton X-100, EDTA, acrylamide, *N,N'*-methylenebis-(acrylamide), and phenol reagent from Wako Pure Chemical Ind., Ltd.; *N,N,N',N'*-tetramethylethylenediamine from Canal Ind., Co.; nitro blue tetrazolium from Sigma Chemical Co.; NADH from Kyowa Hakko Kogyo Co., Ltd.; bovine serum albumin from Daiichi Pure Chemicals, Co., Ltd.; Sephadex G-200 from Pharmacia Fine Chemicals, DEAE-cellulose (DE-32) from Whatman Biochemicals, Ltd., respectively. Asparagusic acid used in the experiments was prepared according to our method [18]. (+)(−)-α-Lipoic acid and NAD^+ were obtained as described previously [17]. Other chemicals and organic solvents were of analytical grade.

Preparation of mitochondrial fraction

Etiolated asparagus (*Asparagus officinalis* L.) shoots (about 14 cm length) were harvested from etiolated seedlings grown in the field of the northern part of Miyagi prefecture in Japan at the beginning of July (July 8, 1974) and cut up into short segments (0.5 cm) before blending in homogenizing buffer. Separation of the mitochondrial fraction from 2 kg of etiolated asparagus shoots was carried out as described previously [17]. The mitochondrial pellet was suspended in 67 mM phosphate buffer (pH 7.0) containing 10 mM EDTA to give a protein concentration of about 30 mg per ml.

Solubilization of lipoyl and asparagusate dehydrogenases

Enzyme activity was solubilized by several techniques, such as freezing and thawing, sonication, treatment with sodium dodecylsulfate, deoxycholate, Triton X-100, and ethanol. Freezing of the mitochondrial suspension at −50° C and thawing at 37° C was repeated at least twice over a period of 20 min. Sonication was carried out at 4° C for 5 min in an Artek Sonic Dismembrator under maximum power setting. The treatments of mitochondrial suspension with sodium dodecylsulfate, deoxycholate, Triton X-100, and ethanol were carried out at 37° C in a shaking water bath for 20 min. The solubilized enzyme was then separated by centrifugation at 36 000 × *g* for 10 min. The supernatant was dialyzed overnight against 67 mM phosphate buffer (pH 7.0) containing 10 mM EDTA; the small amount of precipitate formed during dialysis was removed by centrifugation at 36 000 × *g* for 60 min.

Enzyme assays

Asparagusate and lipoyl dehydrogenase activities were assayed at 25° C by measuring the decrease in absorbance at 340 nm due to NADH oxidation with a Gilford thermostated recording spectrophotometer, model 2400-2. Cuvettes of 1 cm light path were used. The reaction mixture contained the following components: 30 μmol phosphate buffer (pH 5.95), 0.38 μmol EDTA, 0.06 μmol NADH, 0.48 μmol asparagusic acid or (+)(−)-α-lipoic acid in a final volume of 0.3 ml; the reaction was initiated by the addition of an appropriate amount of enzyme. Each unit of asparagusate dehydrogenase and lipoyl dehydrogenase was defined as that amount of enzyme which oxidizes one nmol of NADH per minute under the conditions used and their specific activities were defined as units per mg of protein. Protein was determined by the method of Lowry et al. [19] with bovine serum albumin as standard, or by measuring the ultraviolet absorbance at 280 nm in a Zeiss PMQ II spectrophotometer.

Disc electrophoresis

Electrophoresis was carried out according to the procedure of Davis [20]. Activity staining of the enzyme was based on the method described by Cohn et al. [21] except that enzyme location buffer was substituted by 0.1 M phosphate buffer (pH 5.95); the electrophoresed enzymes on gel discs were placed in a solution containing 0.3 mg nitro blue tetrazolium and 0.6 mg NADH per ml 0.1 M phosphate buffer (pH 5.95) at 37° C for 60 min to detect diaphorase activity. The gel was also stained for protein with amido black.

Results

Solubilization of lipoyl and asparagusate dehydrogenases

The procedures attempted at solubilizing mitochondrial lipoyl and asparagusate dehydrogenases are summarized in Table I. The activity of lipoyl dehydrogenase could be released by freezing and thawing, sonication, and treatment with ethanol or Triton X-100. Particularly, the fraction possessing the highest specific activity was obtained when the mitochondrial suspension was treated by freezing and thawing. However, when the mitochondria were extracted with sodium dodecylsulfate or deoxycholate, the enzyme activity was

TABLE I

ATTEMPTS TO SOLUBILIZE LIPOYL AND ASPARAGUSATE DEHYDROGENASES FROM ASPARAGUS MITOCHONDRIA

Mitochondrial protein concentration prior to the treatment was about 32 mg/ml. Lipoyl and asparagusate dehydrogenase activities were determined as described in the text.

Treatment	Lipoyl dehydrogenase		Asparagusate dehydrogenase	
	Total activity (units)	Specific activity (units/mg)	Total activity (units)	Specific activity (units/mg)
None	26.04	0.32	0.60	0.02
Freezing and thawing	71.76	2.81	2.28	0.07
Sonication	78.12	1.80	1.20	0.05
10% Ethanol	74.04	2.57	1.80	0.09
1% sodium dodecysulfate	13.86	0.12	21.60	0.21
1% Deoxycholate	6.96	0.06	4.68	0.04
1% Triton X-100	65.20	2.14	8.64	0.05

entirely lost. On the other hand, asparagusate dehydrogenase activity was hardly released by the treatment with freezing and thawing, sonication, and ethanol, whereas the enzyme activity could be extracted by the treatment with sodium dodecylsulfate, deoxycholate, and Triton X-100. The best result was achieved when the mitochondrial suspension was incubated with 1% sodium dodecylsulfate at 37° C. This result suggests that asparagusate dehydrogenase was located in the internal matrix of intact asparagus mitochondria. A detailed examination of the effectiveness of sodium dodecylsulphate in solubilizing asparagusate dehydrogenase from asparagus mitochondria was then carried out. Table II summarizes the influence of varying the sodium dodecylsulphate concentration and the time of exposure on the yield of the enzyme. Maximal solubilization was obtained when the mitochondrial suspension was treated with 0.5% sodium dodecylsulphate for 20 min at 37° C. This process gave 16-fold higher enzyme fraction in activity than without the sodium dodecylsulphate treatment. Increasing the concentration of sodium dodecylsulphate

TABLE II

EFFECT OF CONCENTRATION AND TIME OF EXPOSURE ON SOLUBILIZATION OF ASPARAGUS MITOCHONDRIAL ASPARAGUSATE DEHYDROGENASE

Mitochondrial protein concentration prior to the treatment was about 28 mg/ml. Asparagusate dehydrogenase activity was determined as described in the text.

Sodium dodecylsulfate concentration (%)	Exposure time (min)	Total activity (units)	Specific activity (units/mg)
0	20	0.75	0.03
0.1	20	11.60	0.33
0.3	20	17.36	0.37
0.5	10	20.10	0.43
0.5	20	27.36	0.48
0.5	30	21.50	0.38
0.7	20	19.68	0.32
1.0	20	18.50	0.27

gave poorer yields of the enzyme activity. Hence, the sodium dodecylsulphate concentration used in all subsequent solubilizations of asparagusate dehydrogenase was 0.5% in the ratio of 0.5 mg sodium dodecylsulphate per mg mitochondrial protein. Using freezing and thawing and sodium dodecylsulphate treatment it was possible to obtain from asparagus mitochondria, lipoyl dehydrogenase and asparagusate dehydrogenase, respectively. Thus we solubilized lipoyl dehydrogenase by applying the treatment of freezing and thawing and asparagusate dehydrogenase by the subsequent sodium dodecylsulphate treatment.

Purification of enzymes

All the steps were carried out at 4° C. The centrifugations and concentrations were performed in a Kubota KR-200A centrifuge and an Amicon PM-10 filter. It was found that the enzymes were more stable in phosphate buffer. Phosphate buffer was, therefore, used throughout the purification.

Lipoyl dehydrogenase

Step 1. Gel filtration on Sephadex G-200. A fraction of the enzyme from the freeze-thaw treatment was concentrated to 8 ml and filtered through a Sephadex G-200 column. The column was eluted with 67 mM phosphate buffer (pH 7.0). Some impurities were eluted in front of the enzyme and the active fractions in tubes 60—70 were combined (33 ml) and then concentrated to 5 ml. The enzyme was purified 3-fold by this procedure.

Step 2. Second gel filtration on Sephadex G-200. The concentrated enzyme fraction was refiltered through a Sephadex G-200 column which was eluted with 67 mM phosphate buffer (pH 7.00). The enzyme was eluted in a single peak. This step afforded a further 8-fold purification of the solubilized enzyme.

Step 3. Chromatography on DEAE-cellulose. The enzyme from the previous step was concentrated and adsorbed onto a DEAE-cellulose (DE-32) column which had been equilibrated with 10 mM phosphate buffer (pH 7.0). The column was washed with 10 mM phosphate buffer (pH 7.0) to remove unad-

TABLE III

SUMMARY OF THE PURIFICATION OF LIPOYL DEHYDROGENASE OF ASPARAGUS MITOCHONDRIA

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
1. Extract by freezing and thawing	50	244	96.40	0.40	100	1
2. Sephadex G-200	33	72	75.10	1.05	78	3
3. Sephadex G-200	14	7.4	68.50	9.25	71	23
4. DEAE--cellulose	65	0.32	64.50	201.00	67	502

sorbed proteins and a linear phosphate gradient from 10—200 mM was then applied to the column. The fractions containing lipoyl dehydrogenase activity were eluted with 120 mM buffer in a single peak. These fractions were pooled and concentrated. The purity of the preparation thus obtained was tested on polyacrylamide-disc gel electrophoresis by staining with amido black and the enzyme preparation was observed to be homogeneous. In addition, as shown in Fig. 3, lipoyl dehydrogenase activity was located in the same band by activity staining. The purification scheme employed is summarized in Table III.

Some characteristics of lipoyl dehydrogenase

Stability of enzyme. Aqueous solution of enzyme preparation in 67 mM phosphate buffer (pH 7.0) was stable for at least two months at −80° C but was less stable at 4° C. EDTA and phosphate ion stabilized the dehydrogenase activity during storage.

Substrate specificity. As shown in Table V, although the enzyme exhibited a strong activity toward α-lipoic acid, no activity of the enzyme was detected at all with asparagusic acid.

Activation by NAD^+. Table V also shows the effect of NAD^+ on lipoyl dehydrogenase activity. The reduction of α-lipoic acid with NADH catalyzed by lipoyl dehydrogenase was strongly activated by the addition of NAD^+.

Asparagusate dehydrogenases

Step 1. Gel filtration on Sephadex G-200. A fraction of the enzymes from the treatment of sodium dodecylsulfate was concentrated to 4 ml and then applied to a Sephadex G-200 column. The column was eluted with 67 mM phosphate buffer (pH 7.0). The fractions were analyzed for protein and two dehydrogenase activities. Asparagusate and lipoyl dehydrogenase activities were eluted at the same fraction in tubes 50—65. This step resulted in 4 and 3-fold purification for asparagusate and lipoyl dehydrogenase activities, respectively (Table IV).

Step 2. Second gel filtration on Sephadex G-200. Fractions from the preceding step were combined and concentrated to 3 ml. The resulting solution was filtered through a Sephadex G-200 column. The column was eluted with 67 mM phosphate buffer (pH 7.0). Asparagusate and lipoyl dehydrogenase activities were eluted in the same fraction in tubes 18—30.

Step 3. Chromatography on DEAE-cellulose. Fractions possessing asparagusate and lipoyl dehydrogenase activities from the preceding step were combined and concentrated to 8 ml. The concentrate was then applied to a DEAE-cellulose column that had been equilibrated with 10 mM phosphate buffer (pH 7.2). After the sample had been adsorbed on the top of the column, a few ml of 10 mM phosphate buffer (pH 7.20) were added and elution was begun with 600 ml of a linear phosphate gradient in a concentration range from 10 to 500 mM phosphate buffer (pH 6.95). The fractions were analyzed for protein and the two dehydrogenase activities. Asparagusate dehydrogenase activities were eluted at phosphate concentrations of 150—160 mM (Fraction I) and 165—190 mM (Fraction II), respectively and furthermore, two lipoyl dehydrogenase activities were also eluted at the same phosphate concentration. Thus, each protein peak contained both asparagusate and lipoyl dehydrogenase activities.

TABLE IV

SUMMARY OF THE PURIFICATION OF ASPARAGUSATE DEHYDROGENASES OF ASPARAGUS MITOCHONDRIA

Purification step	Volume (ml)	Total protein (mg)	Asparagusate dehydrogenase				Lipoyl dehydrogenase			
			Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
1. 0.5 % sodium dodecylsulfate extract	50	315	58.80	0.19	100	1	85.40	0.27	100	1
2. Sephadex G-200	135	129	98.40	0.76	167*	4	117.15	0.91	137*	3
3. Sephadex G-200	60	50	70.30	1.95	119*	10	339.52	4.73	397*	18
4. DEAE--cellulose	I 24	0.282	12.45	44.12	21	236	31.98	113.40	37	418
	II 30	0.308	18.54	60.23	32	322	47.16	153.22	55	565
5. DEAE--cellulose	I 21	0.255	12.20	63.97	21	342	30.05	163.12	35	602
	II 24	0.285	17.25	83.23	29	445	46.50	213.01	54	786

* Anomalous excess yield of the activity in these steps are probably due to removal of sodium dodecylsulfate.

TABLE V

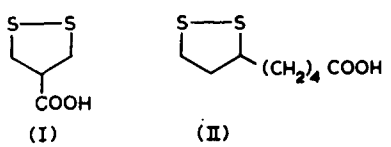
SUBSTRATE SPECIFICITIES AND NAD^+ ACTIVATION OF LIPOYL AND ASPARAGUSATE DEHYDROGENASES

Substrate	NAD^+ (mM)	Specific activity (units/mg)		
		Lipoyl dehydrogenase	Asparagusate dehydrogenase I	Asparagusate dehydrogenase II
$LipS_2$	0	175.30	112.50	152.62
$LipS_2$	0.2	422.91	208.82	307.18
$AspS_2$	0	0	44.15	60.00
$AspS_2$	0.2	0	36.38	46.80
$AspS_2$	0.4	0	26.17	35.53

Step 4. Second chromatography on DEAE-cellulose. Two fractions, Fractions I and II from the preceding step were combined, dialyzed, and concentrated to 10 ml. The resulting solution was adsorbed on a DEAE-cellulose column and the enzymes were further purified by a gradient of phosphate buffer. The phosphate concentration varied linearly from 10—500 mM. The elution pattern and activity of the enzyme is shown in Fig. 2. The first (Fraction I) and second (Fraction II) protein peaks both coincided with the asparagusate dehydrogenase activity and furthermore, each protein peak also possessed lipoyl dehydrogenase. The purity of the Fraction I and Fraction II thus obtained was checked on polyacrylamide gel electrophoresis by staining with amido black and both fractions were observed to be homogeneous. In addition, as shown in Fig. 3, both fractions showed a single band by activity staining with NADH-nitro blue tetrazolium. Results obtained after each step of the preparation leading to the two fractions are summarized in Table IV. In the final purification step, Fraction I revealed specific activities of 64.0 and 163.1 units per mg toward asparagusic acid and α-lipoic acid and Fraction II showed specific activities of 83.2 and 213.0 units per mg toward both the acids, respectively. In both Fractions I and II, the ratio of specific activity toward asparagusic acid to that toward α-lipoic acid was 2.55 to 1. Fractions I and II thus isolated were tentatively named asparagusate dehydrogenases I and II, respectively, based on the assumption that asparagusic acid may be the natural substrate of the enzymes. In view of specificity, it seems that these two enzymes are novel enzymes.

Some properties of aspargusate dehydrogenase I and II

Stability of the enzyme. Preparations of both enzymes in 67 mM phosphate buffer (pH 7.0) were very unstable at 4°C and their activities were completely lost in two weeks. However, the activities of the two enzymes were



(I) (II)

Fig. 1. Chemical structures of asparagusic acid (I) and α-lipoic acid (II).

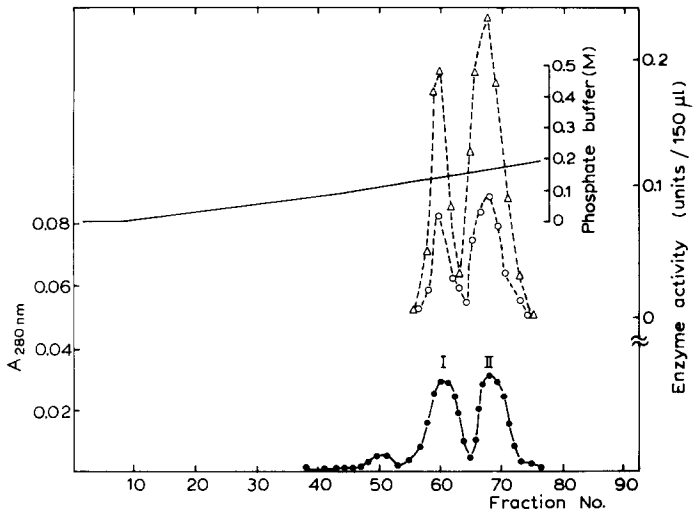


Fig. 2. DEAE-cellulose column chromatography of asparagusate dehydrogenase. An enzyme solution containing Fractions I and II from the preceding step (10 ml, asparagusate dehydrogenase activity 3.10 units/ml, A_{280nm} = 0.060) was applied to a DEAE-cellulose column (1.4 cm X 37). The column was eluted with 600 ml of a linear phosphate gradient. The enzyme was collected in 3-ml fractions. ●——●, absorbance at 280 nm; ○------○ asparagusate dehydrogenase activity; △------△, lipoyl dehydrogenase activity.

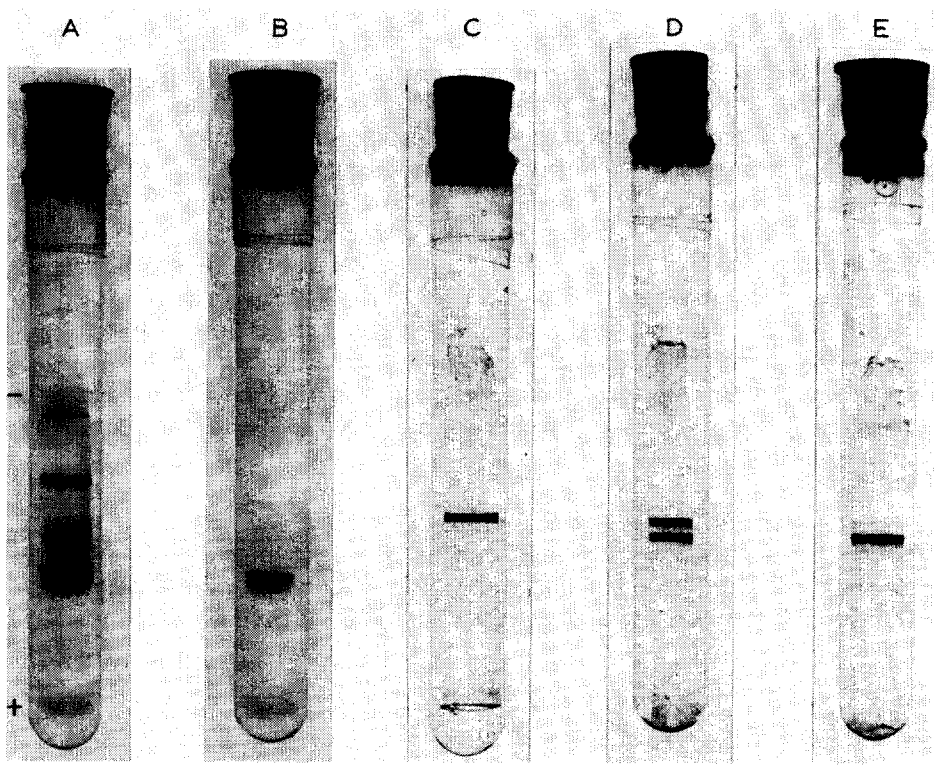


Fig. 3. Polyacrylamide gel electrophoresis of lipoyl dehydrogenase, asparagusate dehydrogenase I, and asparagusate dehydrogenase II. Electrophoresis was carried out in 7.5% polyacrylamide gel (0.5 cm X 5) at 4° C in Tris/glycine buffer (pH 8.3) for 40 min with 5 mA/tube. Activity was stained with nitro blue tetrazolium and NADH as described in the text. Gel A, crude extract of asparagus mitochondria after freezing and thawing; Gel B, lipoyl dehydrogenase; Gel C, asparagusate dehydrogenase I; Gel D, a mixture of asparagusate dehydrogenases I and II; Gel E, asparagusate dehydrogenase II.

stable for at least one month at −80° C. EDTA and phosphate ion stabilized both the enzymes.

Substrate specificity. As shown in Table V both enzymes were capable of reducing asparagusic and α-lipoic acids. It was found that they had a higher activity toward α-lipoic acid than toward asparagusic acid.

Effect of NAD^+. Table V also shows the effect of NAD^+ on asparagusate dehydrogenase activity. The reduction of α-lipoic acid catalyzed with the asparagusate dehydrogenase I and II was activated by NAD^+, whereas that of asparagusic acid catalyzed with the enzymes was inhibited by NAD^+.

Discussion

Lipoyl dehydrogenase and asparagusate dehydrogenases I and II have been purified 502, 342 and 445-fold from the crude extract of asparagus mitochondria, respectively. Asparagusate dehydrogenases showed a characteristic behavior apparently different from lipoyl dehydrogenase; lipoyl dehydrogenase exhibited a strong activity for α-lipoic acid but did not show any activity for asparagusic acid. On the other hand, asparagusate dehydrogenase I and II were capable of reducing both asparagusic and α-lipoic acids using NADH as hydrogen donor. The fact is quite unique, because many lipoyl dehydrogenases have been isolated up to now from various kinds of animals and microorganisms but asparagusate dehydrogenase has never been isolated. Hence, it seems reasonable to assume that both asparagusate dehydrogenases are novel enzymes.

The reduction of α-lipoic acid with NADH catalyzed by lipoyl and two asparagusate dehydrogenases was activated by NAD^+. In this respect, the enzymes behave like those of pig heart [22] and yeast [23]. Massey and Veeger concluded that NAD^+ prevents the complete reduction of the enzyme by NADH and thus protects the NADH-lipoamide reductase activity from inactivation [24]. However, on the contrary, the reduction of asparagusic acid catalyzed by both asparagusate dehydrogenases was inhibited by the addition of NAD^+. EDTA and phosphate ion stabilized the activities of the three enzymes. Moreover, it was found that asparagusic acid strongly inhibited the activity of lipoyl dehydrogenase isolated from asparagus mitochondria (Yanagawa, H., unpublished). Our previous report has also provided evidence that asparagusic acid inhibits the growth of various kinds of higher plants [25]. Hence, these two observations may suggest that asparagusic acid participates in enzymatic pyruvate and α-ketoglutarate dehydrogenation in asparagus [17] and probably in other higher plants.

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