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ISOLATION OF A PROTEIN WITH DIAPHORASE ACTIVITY FROM COTTON SEEDS AND A STUDY OF SOME OF ITS PROPERTIES

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The diaphorase activities of the proteins of different varieties of two species of cotton plant — *Gossypium hirsutum* L. and *G. barbadense* L. — have been studied. It has been shown that the proteins of the seeds of the cotton plant *G. barbadense* possess a low diaphorase activity in comparison with the proteins of the seeds of a *G. hirsutum* plant. On an electrophoretogram of the proteins, diaphorase activity was localized in two zones, with R_f 0.45 and 0.70. The diaphorase with R_f 0.45 has been isolated by electrophoresis in polyacrylamide gel (PAAG) and some of its properties have been studied. The diaphorase isolated oxidizes NADH and NADPH in the presence of various artificial electron Acceptors, and has two pH optima (at 7.20 and 8.70) and is characterized by relative thermal stability (at 80°C). In the case of the total extract, brief boiling does not lead to inactivation of the enzyme, which shows the presence in cotton seeds of a factor stabilizing this diaphorase. The molecular weight of the proteins isolated, according to gel filtration on Sephadex G-150, is 59,000, and from the results of SDS-PAAG electrophoresis it is 13,600, which shows a tetrameric structure of the enzyme.

It has been established previously that the seeds of cotton plants of the species *Gossypium hirsutum* L. have a higher level of diaphorase activity than the seeds of the species *G. barbadense* L. [1]. An electrophoretic investigation showed that diaphorase activity was possessed by electrophoretic bands with proteins having mobilities of 0.45 and 0.70, the band with a mobility of 0.45 oxidizing both NADH and NADPH in the presence of artificial electron acceptors [2]. In the present paper we consider the results of a further investigation of difference in the diaphorase activity of these two types of cotton plants and also a study of some properties of the protein isolated.

Diaphorase activity had previously been investigated for five varieties of *G. hirsutum*: S-427, 108-F, Tashkent-1, Tashkent-6, and Mexicanum, and four varieties of *G. barbadense*: S-6030, 8763-1, 9696-1, and 5904-1, at 20°C [1]. In the present stage, we have investigated the diaphorase activity of varieties 153-F, Tashkent-1, AN-chillyaki, AN-402, and Krasnolistnaya akala of the species *G. hirsutum* and S-6030, 5904-I, and 8704-I of the species *G. barbadense*, and this under standard conditions at 30°C.

The results obtained on the NAD(P)H–2,4-dichlorophenolindophenol (DCPIP) oxidoreductase activity of the soluble fraction of the proteins indicated that the level of diaphorase activity in the seeds of the cotton plant *G. hirsutum* was actually higher than in the seeds of *G. barbadense* (μmole/min per mg × 10^2) (see following page)

When these figures compared with those obtained previously [1], it is found that the interspecies difference in diaphorase activities is retained although, formerly [1], considerably lower results had been obtained, apparently because of the temperature factor.

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Variety of cotton plant	*NADH*	*NADPH*
G. hirsutum		
Tashkent-1	24,4±1,2	21,2±0,9
153-F	19,1±1,1	15,3±0,8
AN-chillyaki	27,0±1,2	22,9±1,0
AN-402	19,8±1,0	17,7±0,8
Krasnolistnaya akala	24,0±1,0	21,6±1,0
G. barbadense		
5904-I	15,1±0,7	12,8±0,8
8704-I	13,6±0,6	12,8±0,5
C-6030	14,2±0,8	12,0±0,6

To reveal proteins with diaphorase activity on the electrophoretograms, after electrophoresis the gels were incubated in DCPIP solution for 15 min and were then transferred to a solution of NAD(P)H. The zones corresponding to diaphorase activity appeared in the form of colorless bands on a blue background. Both in the case of the seeds of variety Tashkent-1 and in that of 5904-I (representatives of the two species) it was possible to detect two zones corresponding to diaphonase activity. The positions of the zones in the electrophoretograms were similar to those in the zymograms given in [2]. The first zone, with a mobility of 0.45, showed activity and after incubation for a few minutes in NAD(P)H solution was converted into a broad band, and a second zone with a mobility of 0.70 was converted into a narrow band. These zones were the main ones quantitatively on revelation by protein stains [3]. In the case of *G. barbadense*, the protein band with a mobility of 0.4 consisted of two closely adjacent components which it was possible to separate when the concentration of protein in the gel was low. In the case of the same fraction of the protein of *G. hirsutum* seeds, no such phenomenon was observed, and therefore the protein with a mobility of 0.45 was isolated from the seeds of the cotton plant of this species with the aid of electrophoresis in polyacrylamide gel. The protein isolated in this way was electrophoretically homogeneous (see Fig. 3) and its diaphorase activity had two pH optima: 7.20 and 8.70 (Fig. 1).

For the total aqueous extract of the cotton seed, diaphorase activity was shown over a wide pH range. The activity maxima at various pH values for NADH and for NADPH were similar in general outlines (Fig. 1). The thermal treatment of an extract did not appreciably affect the activity of the enzyme (Fig. 2). Even brief boiling (3 min) of the protein extract did not lead to inactivation of the enzyme.

The isolated protein with diaphorase activity also proved to be relatively heat-stable (Fig. 2): no appreciable fall in activity was observed up to 80°C. The results obtained give grounds for assuming the presence of a factor stabilizing the diaphorase in the cotton seeds. Also in favor of such a hypothesis are the results of other authors [4] who isolated from the cotton plant a factor of low molecular weight stabilizing certain enzymes.

In gel filtration of Sephadex G-150, the protein that had been isolated issued as a single fraction (Fig. 3) with a molecular weight of 59,000 D. The molecular weight of this protein according to the results of electrophoresis was 13,600 D and did not depend on the presence of 2-mercaptoethanol. The results of the molecular weight determinations by the two methods indicated a possible tetrameric structure of the enzyme. In the UV absorption spectrum, a single absorption maximum appeared at 275 nm.

The results of an investigation of the diaphorase activity in the NAD(P)H-DCPIP system are shown in Fig. 4. In this system, K_m was $3.63 \cdot 10^{-4}$ M for NADH and $7.19 \cdot 10^{-5}$ M for NADPH, while V_{max} for NADH was 0.78 and for NADPH 0.73. Similar values of K_m were obtained in the menadione–cytochrome system.

Better acceptors proved to be p-benzoquinone, vitamin K_3, and DCPIP. K_m for p-benzoquinone was $7.69 \cdot 10^{-5}$ M, and for vitamin K_3 it was $1.16 \cdot 10^{-5}$ M. The maximum rate for p-benzoquinone was higher for vitamin K_3.

p-Benzoquinone and vitamin K_3 are good mediators of the transfer of hydrogen from cotton-plant diaphorase to cytochrome. In the absence of mediators, cytochrome did not accept hydrogen.

2-Hydroxy-1,4-napthoquinone likewise possessed no acceptor activity. Similar results were obtained in the case of the DT-diaphorase from rat liver [5]. Quinones having no side chains in position 3 were more active, but with an increase in the length of the side chain their activity diminished. Quinones with a hydroxy group in the second or third positions were inactive as electron acceptors.

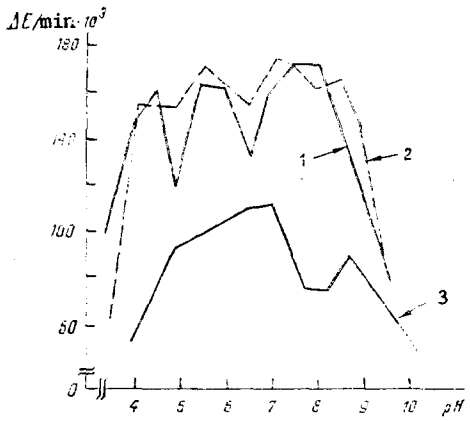

Fig. 1

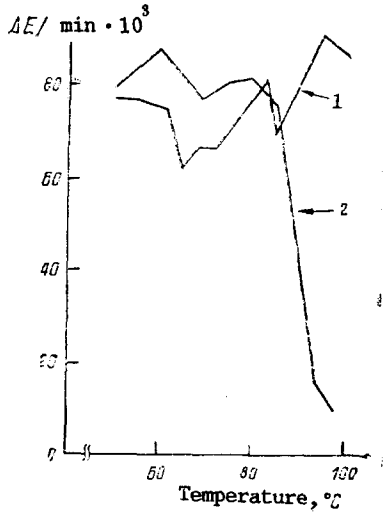

Fig. 2

Fig. 1. pH dependence of the NADH-DCPIP oxidoreductase (1) and the NADPH-DCPIP oxidoreductase (2) activities of the water-soluble fraction of the proteins of cotton seeds, and also the NADH-DCPIP oxidoreductase activity of the isolated diaphorase (3).

Fig. 2. Diaphorase activity of the water-soluble fraction (1) and of the diaphorase (2) of cotton seeds after treatment at various temperatures.

It is known that, for many quinone reductases, p-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol in a concentration of 10^{-4} M are powerful inhibitors [6]. We studied the inhibiting action of solutions of various nitrophenol derivatives on the activity of the cotton-plant diaphorase, the solutions of the preparations being used in concentrations of from 10^{-4} to 10^{-3} M:

Inhibitor	Concentration	Inhibition, %
2,4-Dinitrophenol	5×10^{-4} M	12,5
	$7{,}5\times10^{-4}$ M	20,7
2,6-Dinitrophenol	5×10^{-4} M	6,2
	$7{,}5\times10^{-4}$ M	16,5
2,5-Dinitrophenol	5×10^{-4} M	7,5
	$7{,}5\times10^{-4}$ M	17,1
2-Nitrophenol	5×10^{-4} M	11,5
	$7{,}5\times10^{-4}$ M	21,2
4-Nitrophenol	5×10^{-4} M	10,3
	10^{-3} M	13,4
	6×10^{-3} M	27,9

Among the inhibitors studied no competent ones were detected: the activity of the enzyme was inhibited only by 10-25%, while inhibition by nitrophenyl derivatives of other enzymes amounted to from 75 to 95% [6].

One of the enzymes possessing diaphorase activity is nitrate reductase. The enzyme contains a diaphorase component and possesses NADH—cytochrome c reductase activity [7, 8]. The pH optima of the NADH nitrate reductase from the leaves of eight-day wheat shoots are located at 6.6 and 8.9. Three forms of nitrate reductase using as electron donors NADH, NADPH, or NADH and NADPH simultaneously have been isolated from the rootlets of five-day maize shoots [9]. The cotton-plant diaphorase did not possess NAD(P)H—cytochrome reductase activity.

The main part of the p-benzoquinone reductase activity of a homogenate of pea leaves was detected in the soluble fraction after the precipitation of the chloroplasts, mitochondria, and peroxysomes [10]. It is assumed that the qunione reductase activity is connected with processes of respiration.

In an investigation of the soluble proteins of the leaves of seven-day barley shoots by electrophoresis in 7.5% polyacrylamide gel, seven bands with diaphorase activity were detected

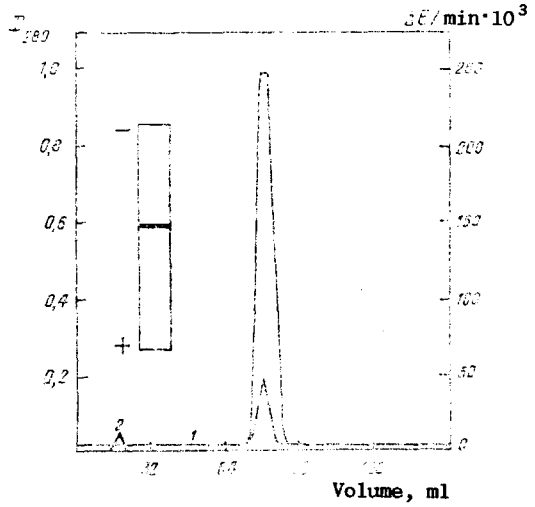

Fig. 3

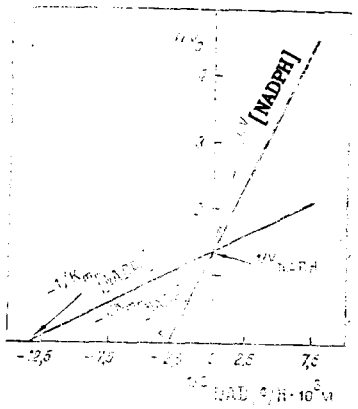

Fig. 4

Fig. 3. Gel filtration of the cotton-plant diaphorase on Sephadex G-150 [1) optical density; 2) activity]. A sketch of an electrophoretogram of the isolated cotton-plant diaphorase is shown on the left.

Fig. 4. Lineweaver-Burk plot for the cotton-plant diaphorase in the NAD(P)H-DCPIP system.

[11]. It was found that all these bands were different molecular forms of ferredoxin NAD^+ reductase.

Thus, the results of the investigations showed that the protein with diaphorase activity from cotton seeds exhibits in artificial systems properties close to the diaphorases from other sources, but the absence of inhibition of its activity makes it difficult to assign this enzyme to a definite class. Further investigations are necessary to establish the functional role of the protein isolated.

EXPERIMENTAL

Seeds of Tashkent-1, 153-F, AN-chillyaki, AN-402, and Krasnolistnaya akala cotton plants, of the species *G. hirsutum*, and 5904-I, 8704-I, and S-6030 plants, of the species *G. barbadense*, were used. The seeds were prepared for the extraction of the proteins by a known method [12]. The defatted seeds were extracted with distilled water in a ratio of fluor to water of 1:250. The extract was clarified by centrifugation at 10,000g for 15 min. The amount of protein in the extract was determined by Lowry's method [13]. The diaphorase activity of the extracts obtained was monitored from the decrease in the optical density of a solution of DCPIP at 30°C on a SF-18 recording spectrophotometer. The reaction system consisted of 3 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.08% Triton X-100, 0.05 mM NADH or NADPH, and 0.04 mM DCPIP. The reaction was initiated by the addition of the protein solution as has been described in a number of publications [5]. The diaphorase activity was expressed in micromoles of DCPIP reduced per 1 mg of protein in 1 min.

The electrophoretic separation of the proteins was carried out in 7.5% polyacrylamide gel by Davis' method [14], and in the presence of sodium dodecyl sulfate by the method of Laemmli and Favre [15].

To isolate the protein with diaphorase activity, the gels after electrophoresis were incubated successively in solutions 0.5 mM in DCPIP (10-15 min) and 2.5 mM in NADH or NADPH. Diaphorase activity was also revealed with the aid of Nitrotetrazolium Blue [11, 16]. The zones corresponding to diaphorase activity were cut out from unstained electrophoretograms and the protein was extracted with distilled water. The protein extract was passed through a column of Sephadex G-25, and the protein eluate was lyophylized.

The diaphorase activity of the protein obtained was studied in the presence of various electron acceptors. The change in the optical density of the solutions was recorded on a Unicam-1800 spectrophotometer (United Kingdom). The oxidation of NADH and NADPH by benzoquinones and naphthoquinones was evaluated from the decrease in the optical density of the

NADH and NADPH at 340 and 366 nm, respectively. In a study of the diaphorase activity in the menadione–cytochrome or the p-benzoquinone–cytochrome system, the course of the reaction was monitored from the increase in optical density at 580 nm. The following extinction coefficients (liter/mole•cm) were used: $6.22 \cdot 10^3$ at 340 nm for NADH and NADPH; $2.1 \cdot 10^4$ at 600 nm for DCPIP; and $1.85 \cdot 10^4$ at 550 nm for reduced minus oxidized cytochrome [5].

The gel filtration of the protein solution was performed on columns (1.5 × 96 cm) with Sephadex G-25 and G-50 fine (Sweden). As standard proteins in the determination of the apparent molecular weight of the cotton-plant diaphorase we used: cytochrome c — 12,300 D; myoglobin — 17,800 D; chymotrypsinogen — 25,000 D; ovalbumin — 45,000 D; bovine serum albumin - 67,000 D (FRG); carboxypeptidase — 34,000 D; and soybean trypsin inhibitor — 21,000 D (Hungary).

SUMMARY

1. It has been shown that the seed proteins of the cotton plant *Gossypium hirsutum* possess a higher level of diaphorase activity than the proteins of *G. barbadense*.

2. A protein with diaphorase activity from the seeds of the cotton plant *G. hirsutum* is relatively heat-stable and has a molecular weight of 59.000 D. It consists of four polypeptide chains identical in molecular weight (13,600 D).

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