

### Short Communication

# Isolation of NADH Oxidation System from the Plasmalemma of Corn Root Protoplasts<sup>1</sup>

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### ABSTRACT

A plasmalemma-bound NADH oxidation system (Lin 1982 Proc Natl Acad Sci USA 79: 3773–3776) in corn root protoplasts was isolated by a mild treatment of intact protoplasts with trypsin. The majority of NADH stimulated O<sub>2</sub> consumption activity of the protoplasts could be recovered in the supernatant isolated from the intact protoplasts which have been treated with trypsin. The activation energy of NADH oxidation in the supernatant is similar to that of the intact protoplasts (8.7 versus 9.4 kilocalories per mole per degree). Unlike that of the intact protoplasts, an Arrhenius plot of the temperature response (from 5 to 25°C) of the activity in the supernatant shows no transition suggestive of a dissociation of the enzyme from the membrane. Trypsin treatment did not affect K<sup>+</sup> uptake into cell volume of the protoplast. However, the NADH-stimulated K<sup>+</sup> uptake and the increase of cell volume were greatly reduced. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of trichloroacetic acid-precipitated protein from the supernatant showed one extra peptide band with ~42 kilodalton molecular weight.

Recently, in the course of investigating the properties of corn root protoplasts (4), it was discovered that they oxidize exogenous NADH at a rapid rate, and that the additional O<sub>2</sub> consumption results in additional ion transport and hyperpolarization of the cells. To further characterize this NADH oxidation system, attempts were made to isolate the system from the plasmalemma through a mild treatment of intact protoplasts with trypsin. O<sub>2</sub> consumption, K<sup>+</sup> uptake, and packed protoplast volume were then measured in trypsin-treated protoplasts. SDS-polyacrylamide gel electrophoresis was conducted to examine the TCA-precipitated protein released into the supernatant after trypsin treatment.

### MATERIALS AND METHODS

Root protoplasts were isolated from 3-d-old etiolated corn (*Zea mays* L. Pioneer Hybrid 3320) seedlings as described (3). Methods for O<sub>2</sub> consumption, K<sup>+</sup> uptake, and packed cell volume measurements were the same as described previously (4). About 10 million protoplasts were incubated in 5 ml of 0.7 M mannitol, 0.2 mM CaCl<sub>2</sub>, and 1 mM Hepes buffer (pH 6.0) containing 20 μg trypsin at room temperature for 10 min. Supernatant and protoplasts were

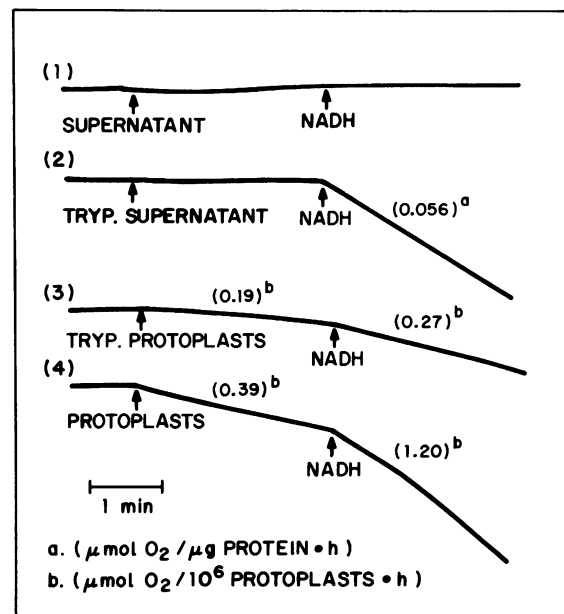


FIG. 1. Recorder traces of O<sub>2</sub> consumption. One hundred μl of (1) control supernatant and (2) trypsin-treated protoplast supernatant were used. About 1 × 10<sup>6</sup> of (3) trypsin-treated protoplasts and (4) control protoplasts were used. NADH concentration was 1.5 mM. (See text for detail).

separated by centrifuging the incubation mixture in microfuge tubes with 50 μl of a 1.0450 g/ml silicone oil pad in a microfuge (3). Trypsin-treated protoplasts used for O<sub>2</sub> consumption, K<sup>+</sup> uptake, and packed protoplast volume measurements were isolated by centrifuging the protoplast from the incubation mixture followed by three washes with 0.7 M mannitol, 0.2 mM CaCl<sub>2</sub>, and 1 mM Hepes buffer (pH 6.0). Proteins in the supernatant were precipitated with 10% (w/v) TCA. The proteins were then solubilized and heat dissociated with SDS. Electrophoresis was carried out on a 6% to 16% SDS-polyacrylamide gel (2, 5) and polypeptides were identified with Coomassie blue. Photographs were taken after the gels were dried. Mol wt standard proteins were purchased from Bio-Rad.

### RESULTS AND DISCUSSION

Previous results (4) suggested the existence of an energy-linked NADH oxidation system in the plasmalemma of corn root protoplasts. To further insure the plasmalemma localization of this system, intact protoplasts were briefly treated with trypsin (see

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“Materials and Methods”) to release the NADH oxidation system from the membrane. Supernatant obtained following trypsin treatment showed a high O<sub>2</sub> consumption upon the addition of 1.5 mM NADH (Fig 1, line 2), while equivalent amounts of supernatant from untreated protoplasts showed no activity (Fig. 1, line 1). The NADH-induced O<sub>2</sub> consumption in the supernatant was not caused by a breakage of protoplasts since no O<sub>2</sub> consumption activity could be detected in the supernatant alone. Furthermore, the number, K<sup>+</sup> uptake, and cell volume of protoplasts did not change with the trypsin treatment (will be discussed later). The effectiveness of NADH in stimulating O<sub>2</sub> consumption in trypsin-treated protoplasts was greatly reduced, *i.e.* from 3-fold stimulation down to 40% (Fig 1, lines 3 and 4). The lower control O<sub>2</sub> consumption rate in treated protoplasts might be due to the effect of trypsin on the plasmalemma arrangement.

In intact protoplasts, NADH stimulation of O<sub>2</sub> consumption is temperature dependent (4). The system interacts with the membrane and has an activation energy of 9.4 kcal/mol·degree above 9°C, and 35.7 below (4). Figure 2 shows an Arrhenius plot of temperature response (5–25°C) of NADH oxidation (measured the NADH induced O<sub>2</sub> consumption) in the supernatant. Unlike that of the intact protoplasts, there was no discontinuity in the plot in the supernatant, suggesting a dissociation of the enzyme

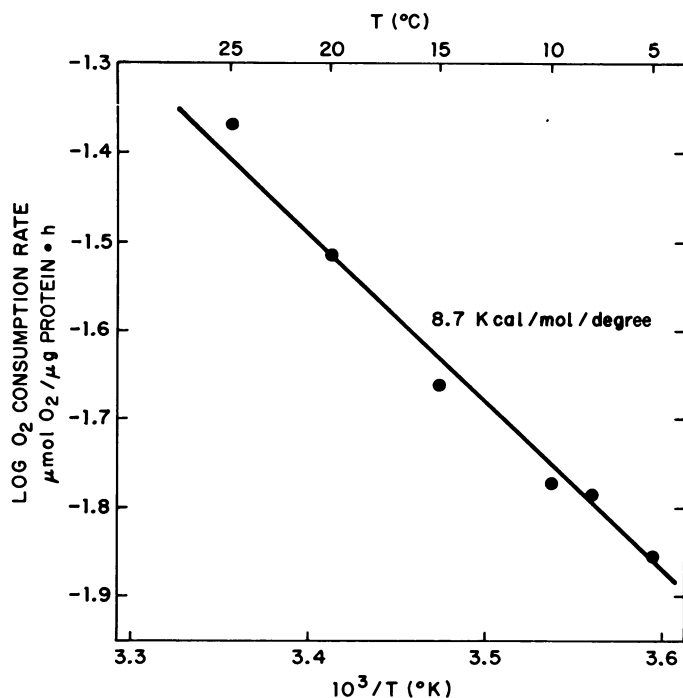


FIG. 2. Arrhenius plot of O<sub>2</sub> consumption of temperature response (5–25°C) of trypsin-treated supernatant. Activation energy, 8.7 kcal/mol·degree.

Table I. Effect of Trypsin Treatment on NADH-Stimulated K<sup>+</sup> Uptake and Packed Cell Volume Increase in Corn Root Protoplasts

	K <sup>+</sup> ( <sup>86</sup> Rb) Influx nmol/10 <sup>6</sup> protoplasts · h	Package Protoplast Volume μl/10 <sup>6</sup> protoplasts
Control	26.43 (100%)	30.72 ± 2.36 (100%)
NADH, 1.5 mM	80.13 (303%)	40.25 ± 2.50 (131%)
Trypsin treated*	27.75 (105%)	29.56 ± 1.89 (96%)
Trypsin + NADH*	38.32 (145%)	33.20 ± 3.10 (112%)

\* About 10<sup>7</sup> protoplasts were incubated with 20 μg trypsin at room temperature for 10 min prior to the measurement of K<sup>+</sup> uptake (see text for experimental details).

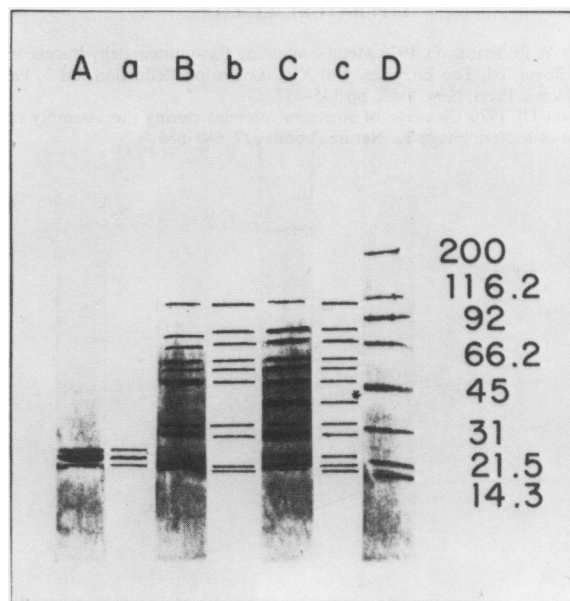


FIG. 3. SDS-polyacrylamide gel electrophoretogram of TCA-precipitated protein from supernatant separated from protoplasts. A, a, trypsin alone; B, b, control supernatant; C, c, trypsin-treated supernatant. D, mol wt standard proteins (*e.g.* myosin (200,000), β-galactosidase (116,200), phosphorylase B (92,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,300)). Star denotes the extra peptide band of the trypsin-treated supernatant. Twenty-five, 80, and 80 μg of protein were used in A, B, and C, respectively.

from the surrounding membrane. The 8.4 kcal/mol·degree activation energy is similar to that of the intact protoplasts (4).

Previously (4), it was shown that the stimulation of O<sub>2</sub> consumption by NADH in protoplasts is accompanied by a 2- to 3-fold increase in ion uptake and a 10% to 15% increase in cell volume. Table I shows that the treatment of protoplasts with trypsin greatly reduced the stimulating effect of NADH on K<sup>+</sup> influx, (from a 3-fold stimulation to 45%) and on cell volume, which decreased from 31% to 12%. However, trypsin treatment did not affect cell volume and K<sup>+</sup> uptake into the protoplasts in the absence of NADH. The data indicate that removal of the NADH oxidation system from the plasmalemma is responsible for the diminished NADH effect on ion uptake into the protoplasts.

To examine the protein profile of the supernatant, 10% TCA was used to precipitate protein from the supernatant. After the protein was solubilized with 100 μl SDS solution (2, 5), the mixture was electrophoresed on a 6% to 16% SDS-polyacrylamide gel and stained with Coomassie blue (see “Materials and Methods”). An extra peptide band, not associated with trypsin, with a mol wt of about 42 kd was observed in the trypsin-treated supernatant protein (Fig. 3). The size of this protein is close to that of reported NADH dehydrogenases (1). At present, it is not known whether this extra peptide has any NADH oxidation activity.

In conclusion, the present study further demonstrates the existence of an NADH-O<sub>2</sub> electron transport system associated with the plasmalemma. The *in vivo* function of this system is still unknown. This system can be isolated through a mild treatment of intact protoplasts with trypsin. Further study using nondenaturing gel electrophoresis is needed to characterize this NADH oxidation protein.

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