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Short communication

A simple assay for formate dehydrogenase activity by gas chromatography–mass spectrometry ☆

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Abstract

A new method using GC–MS was devised for the convenient measurement of formate dehydrogenase (FDH) activity in crude tissue samples. FDH activity was detected by measuring headspace $^{13}CO_2$, which was enzymically converted from [^{13}C]formic acid. This method proved to be sensitive and simple for the estimation of FDH activity without complicated pretreatment.

Keywords: Formate dehydrogenase; Enzymes; Formic acid

1. Introduction

Several enzymatic reactions have been known which involve CO_2 emission. FDH (formate: NAD^+ oxidoreductase, EC 1.2.1.2) is one of the important enzymes which catalyzes the following reaction: $NAD^+ + HCO_2^- \rightarrow NADH + CO_2$. FDHs widely exist in bacteria, yeast, fungi, mammals, and plants [1–4]. Several types of FDHs have been reported with difference in cofactors, electron acceptors, substrates, and cellular localization [5,6]. Over the past several decades, FDHs have been extensively studied from the view-point of efficient industrial NADH regeneration due to the low cost for the substrate (formate) and easiness of removal of the emitted CO_2 [2].

A usual assay method for FDH activity is to measure the increasing rate of the absorbance at 340 nm, which is directly proportional to the increment of NADH in the test samples. However, this method has several limitations. First, opaque samples such as crude plant preparations are not detectable. Second, the FDH activity is not measured correctly in the samples which contain NADH oxidizing enzymes or any factors masking the UV absorption at 340 nm. Third, the linearity of the NADH increment of the test samples is only maintained for a short time [7], since the NADH oxidase consumes the newly produced NADH. Furthermore, tedious pretreatment is essential for removal of the factors, which cancel the original FDH activity or mask the net UV absorption.

In this paper, we describe a simple and convenient method with a ^{13}C-labeled compound to evaluate the FDH activity of crude tissue samples by measuring the emitted $^{13}CO_2$ using a GC–MS system.

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2. Experimental

2.1. Preparation of headspace gas sample

$NaH^{13}CO_3$(99 atom% ^{13}C) was purchased from Aldrich and used as a $^{13}CO_2$ source. $^{13}CO_2$ was produced in a 2 ml vial sealed with a rubber cap. Before sealing the vial with the cap, the test solution was sonicated for 5 min to degas. The solution (0.4 ml) contained 50 m*M* sodium phosphate buffer (SPB, pH 7) and $NaH^{13}CO_3$ (0–2.2 μmol/ml). The vial was maintained for 5 min at 25°C, and then the solution was acidified to pH 2 by the addition of 5 *M* HCl (100 μl) with a syringe and then mixed. After the successive 5 min incubation, an aliquot of the headspace gas sample (25 μl) was withdrawn through the rubber cap by a gas-tight syringe and then manually injected into the GC–MS.

2.2. FDH activity measurement

FDH reaction was performed in a 2 ml gas-tight vial. A reaction mixture (0.4 ml), contained 0.4 μmol NAD, 40 μmol SPB (pH 7), 20 μmol sodium [^{13}C]formate (99 atom% ^{13}C, purchased from Aldrich) and the enzyme solution. Commercially available FDH (derived from *Xylaria digitata*, Boehringer Mannheim) was used in the authentic FDH reaction. The reaction was initiated by addition of 0.5 *M* sodium [^{13}C]formate (40 μl). The reaction was carried out for 5 min at 25°C. The reaction was stopped by addition of 5 *M* HCl (100 μl) and the reaction mixture was incubated for 5 min. Preparation of the headspace gas sample was performed in the same manner mentioned above. The samples were subjected to GC–MS analysis which was carried out using QP-5000 (Shimadzu corporation), fitted with a CP-Silica porous-layer open tubular (PLOT) capillary column (30 m×0. 32 mm I. D.). GC–MS conditions: capillary column at 80°C; carrier gas, helium (He); injector at 200°C; Interface line at 250°C; flow-rate, 47 ml/min. Electron energy and electron current were set to 70 eV and 60 μA, respectively. Selected ion detection at 44 *m/z* for the $^{12}CO_2$ and 45 *m/z* for the $^{13}CO_2$ was carried out. One unit of the activity was defined as the amount of the enzyme which oxidizes 1 μmol of sodium formate per min at pH 7, 25°C.

2.3. Preparation of plant samples

Rice seeds (*Oryza sativa* L. cv Hinohikari) were sterilized and planted in glass tubes (130 mm×40 mm I. D.), containing 1/2 MS salt and 0.1% Gellan Gum. The culture solution was adjusted to pH 6 with 1 *M* NaOH and then autoclaved. The plants were incubated at 25°C in a growth chamber with artificial light (100 $\mu E/m^2/s$) for a 16 h photoperiod. After 2 week incubation, the plants were harvested and washed with distilled water immediately before sample preparation. Plant materials were homogenized in a 100 m*M* SPB (pH 7), containing 0.1% Triton X-100, 0.02% 2-mercaptoethanol, with a pestle at 4°C. The homogenate was centrifuged at 12 000 *g* for 20 min at 4°C. The supernatant was dialyzed into the 50 m*M* SPB (pH 7) at 4°C. Protein concentrations were estimated by the Bio-Rad protein assay (Bio-Rad Labs).

3. Results and discussion

Test solutions containing different concentrations of $NaH^{13}CO_3$ were transferred into sealed tubes. Each solution was acidified by the addition of 5 *M* HCl and then allowed to stand at 25°C for 5 min. Gaseous $^{13}CO_2$ (25 μl) collected by a gas-tight syringe was subjected to GC–MS analysis. The selective ion chromatogram for *m/z* 44 and 45 are shown in Fig. 1. $^{13}CO_2$ content was determined by the peak area of the trace of monitoring selected ion. A linear correlation ($r=0.999$, $n=15$ and slope=7.25×10^4) between the amount of added $NaH^{13}CO_3$ and the corresponding chromatographic peak area was seen in the concentration range from 0.2 to 2.25 μmol/ml (Fig. 2). The intra-assay RSDs and inter-assay RSDs for the detection of $NaH^{13}CO_3$ by the GC–MS method were less than 1.54% ($n=24$) and 3.70% ($n=24$), respectively. This indicates that the headspace gas of this concentration range is quantitatively detectable.

Therefore, this method is applicable to the detection of FDH activity. When the different units of commercially available FDH were added to the

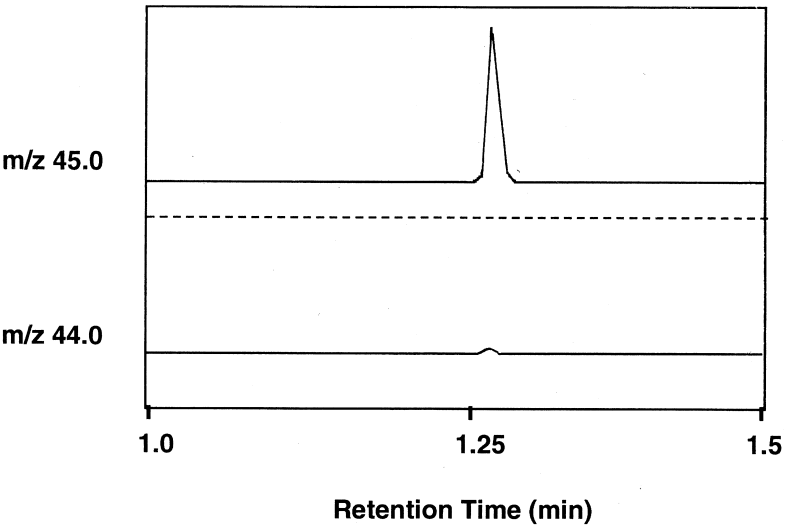

Fig. 1. Selective ion chromatograms for m/z 44 and 45 of headspace gas from the solutions (50 m*M* SPB, pH 7) containing $NaH^{13}CO_3$ (1.1 μmol/ml).

solution containing excess amounts of [^{13}C]formate and NAD the released $^{13}CO_2$ amount was directly proportional to the enzyme concentrations of 0.02–0.4 units/ml (r=0.998, n=18 and slope=0.787·10^{-3}) (Fig. 3). The intra-assay RSDs and inter-assay RSDs for the detection of $NaH^{13}CO_3$ by the GC–MS method were less than 1.85% (n=24) and 3.88% (n=24), respectively. In this enzyme system, the

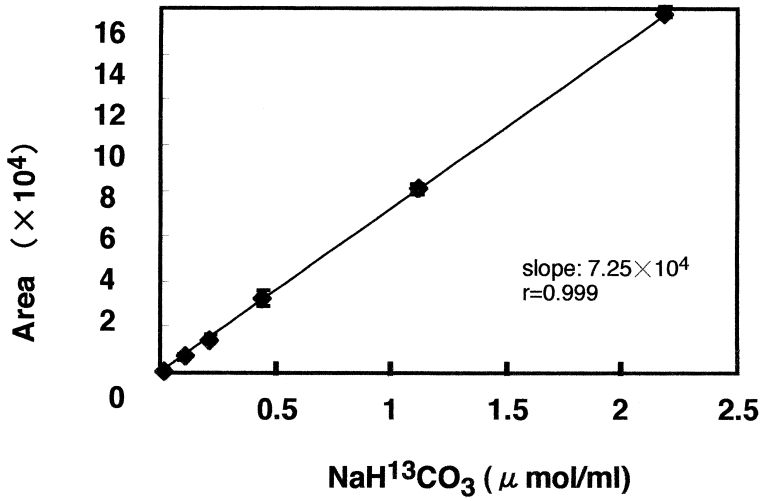

Fig. 2. Effect of amount of $NaH^{13}CCO_3$ on the CO_2 concentration in the headspace gas. Each concentrations of $NaH^{13}CO_3$ was added to the reaction solution and incubated at 25°C for 5 min. One hundred μl of 5 *M* HCl was added and a further 5 min incubation was carried out. Twenty five μl of headspace gas was analyzed by GC–MS. Each data point represents the mean ±SD of three experiments. Intersection was forced with control of 0 m*M* $NaH^{13}CO_3$. In those cases where no SD values (bars) are given, the bar size did not exceed the symbol size.

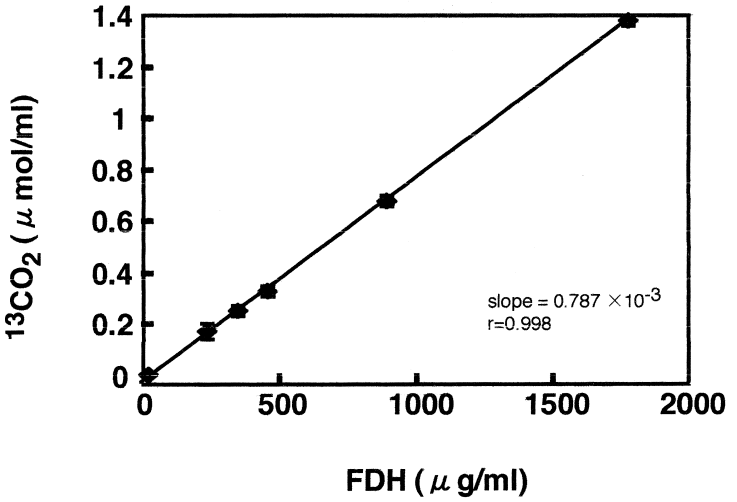

Fig. 3. Effect of the amount of FDH on CO_2 production in the head space gas. Sodium [^{13}C]formate (20 μmol) and NAD (0.4 μmol) were incubated with commercial available FDH (derived from *Xylaria digitata*). After 5 min incubation at 25°C, the reaction was stopped by the addition of 5 *M* HCl (100 μl). After a further 5 min incubation, 25 μl of headspace gas was analyzed by GC–MS. Each data point represents the mean ±SD of experiments. Intersection was forced with control of 0 μg FDH. In those cases where no SD values (bars) are given, the bar size did not exceeded the symbol size.

reaction time (5 min) for the FDH activity is adequate to evaluate the CO_2 accumulated in the sealed vial.

Test samples from rice seedlings were diluted with SPB to adjust its concentration to the reliably measurable range described above. FDH activity of the aerial part or root from rice plant showed 11.9 or 21.7 mU/mg.

The activity of the crude samples could not be detected with a routine colorimetric method.

These results indicate that the headspace gas analysis with GC–MS is quite effective for the detection of the FDH activity of a crude sample. Our improved method is expected to be used as a sensitive and convenient detection tool for the evaluation of FDH activity, since this method is free from complicated pretreatment and interference factors which mask the net UV absorption. Also, this method is simply manipulated and easy to use due to the fact that there is no radioactivity, as compared to other methods [8–10].

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References

[1] C.M. Chow, U.L. Rajbhandray, J. Bacteriol. 175 (1993) 3703.
[2] S.J. Allen, J.J. Holbrook, Gene 162 (1995) 99.
[3] G. Sawers, Antonie Van Leeuwenhoek 66 (1994) 57.
[4] E.A. Cossins, S.K. Sinha, Can. J. Biochem. 43 (1965) 685.
[5] G. Ferry, FEMS Microbiol. Rev. 87 (1990) 377.
[6] V.O. Popov, V.S. Lamzin, Biochem J. 301 (1994) 625.
[7] J.R. Quayle, Methods Enzymnol. 9 (1966) 360.
[8] Z. Lin, R. Sparling, Can. J. Microbiol. 41 (1995) 1048.
[9] T.S. Haynes, D.J. Klemm, J.J. Ruocco, L.L. Barton, Anaerobe 1 (1995) 175.
[10] E. Tschrsin, W.R. Wolf, D. Lacroix, C. Veillon, K.Y. Patterson, Appl. Environ. Microbiol. 60 (1994) 4310.