Measuring the plankton CO_2 production throughout the water column by the isocitrate dehydrogenase (IDH) activity.

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ABSTRACT

Knowledge about Carbon flux in the ocean is limited by the complexity and expense of its measurement. Calculating it from plankton respiration provides an inexpensive alternative to sediment traps. Recently, the use of O_2-consumption-based respiration has been explored to calculate temporal and spatial variability in carbon flux in upwelling areas. Here, we explore the use of a new enzyme-based proxy for marine CO_2-production method based on the activity of different isocitrate dehydrogenase (IDH) isoenzymes, involved in the Krebs cycle. We evaluated the effects of different buffers, a range of pHs and temperatures, the IDH kinetic constants, the optimal concentrations of the main substrate (isocitric acid), and the cofactors ($NAD(P)^+$, Mg^{2+}) for different size fractions of the plankton community. As a result, we propose optimal substrates concentrations, pH and temperature in order to ensure the measurement of the maximum capacity of CO_2 production of the marine plankton community.

INTRODUCTION

Isocitrate dehydrogenase (IDH) is a key enzyme in the Krebs cycle, an essential biochemical pathway in carbohydrate catabolic metabolism. There, IDH is responsible for the production of one of the three CO_2 molecules released during this phase of cellular respiration.

Among the living organisms, different IDH isoenzymes play this special role [1]. In eukaryotes, a NAD^+-dependent IDH (NAD-IDH) at the mitochondrial matrix is involved in producing NADH for the electron transport system (ETS). In prokaryoyes, a $NADP^+$-dependent IDH (NADP-IDH) at the cytosol seems to be responsible of the same process. In addition to having NAD-IDH to feed the ETS, eukaryotes also have NADP-IDH activity outside their mitochondria in the cytosol. This cytosolic NADP-IDH activity is strong and has been reported for many animal tissues [2]. During this experimental study of a CO_2 production proxy, an assay based on measuring the NADP-IDH maximum Velocity (V_{max}) has been developed.

We have adapted existing IDH methodologies, drawing particularly on research of Berdalet *et al.* (1995) [3] using bacteria, and on research of Munilla-Moran and Stark (1989) [4] using fish liver, to develop an efficient kinetic NADP-IDH assay for the marine plankton community.

MATERIAL & METHODS

Zooplankton was collected from Sardina del Norte (Gáldar, Gran Canaria island) dragging a WP2 net horizontally in the upper euphotic zone (4m in a 12m water column). Samples were fractionated through 2000, 200, and 50µm nets. A fraction for the plankton between 0.7 to 50µm was collected at the surface with a bucket from Alcaravaneras Beach (100 m offshore, Las Palmas de Gran Canaria, Gran Canaria island), and passed through a 50µm net and 0.7µm glass fiber filter (GFF). All samples were stored in 2mL microcentrifuge tubes at -80ºC until analysis.

Kinetic Procedures: Samples were homogenated in 0.1M phosphate buffer. 50-200µm and 200-2000µm samples were sonicated for 45sec at 70% amplitude in a Vibracell VXC-130 Sonics® ultrasonic processor, whereas 0.7-50µm samples were homogenated by a Teflon® pestle PYREX® Potter-Elvehjem tissue grinder for 2 min. Crude homogenates were centrifuged (0-4ºC) at 4000rpm during 10 min. NADP-IDH activity was measured kinetically by following the production of NADPH at 340nm, at 18°C for 600 sec in a Cary 100 U-V visible spectrophotometer. In a final volume of 0.5ml of 0.1M phosphate buffer at pH 8.5, different concentrations of $MgCl_2$ (PANREAC 131396), DL-trisodium-isocitrate (SIGMA I1252) and β-$NADP^+$ (SIGMA N0505) were used, depending on the aim of each experiment. A molar extinction coefficient of 5.42 $mM^{-1}cm^{-1}$ was obtained for NADPH under these conditions.

Kinetic Properties Studies: The affinity between different substrates ($NADP^+$, Isocitric acid and $MgCl_2$) and the NADP-IDH was determined from Michaelis-Menten and Hanes-Woolf plots [5] at pH 8.5 and 18ºC, calculating their dissociation constants (*Km*) and the V_{max} of the enzyme. The substrate under study was used at different concentrations while the other reagents were maintained constant.

Buffer Studies: The response in the enzyme activity was analyzed from ten experiments with different combinations of buffer (0.025M MOPS, 0.1M TRIS and 0.1M phosphate buffer), cationic effectors (Mg^{2+} or Mn^{2+}) [4] and the presence of lysozyme in the homogenization buffer [3], at 18ºC and pH 8.5, 4mM DL-trisodium-Isocitrate, 0.08mM

$NADP^+$ and 2mM $MgCl_2$ with five different concentrations of purified NADP- IDH (SIGMA I2002).

pH Studies: The effect of pH on NADP- IDH activity was analyzed at 18ºC and eleven levels of pH that ranged from pH 5.5 to pH 11 with 0.2mM $NADP^+$, 2.1mM DL-trisodium-isocitrate and 2mM $MgCl_2$.

Temperature Studies: The effect of temperature (T) on NADP-IDH activity was analyzed at pH 8.5 and nine temperatures ranged from 5 to 60ºC with 0.2mM $NADP^+$, 2.1mM DL-sodium-isocitrate and 2mM $MgCl_2$.

RESULTS & DISCUSSION

Kinetic properties: The apparent *Km* values, calculated by Hanes-Woolf plots [5] for the main substrate and cofactor, were 0.246mM for isocitric acid (Fig.1) and 0.024mM for $NADP^+$. In the case of $MgCl_2$, an allosteric relationship led to a *Km* of 2.632mM. The optimal values to measure the potential activity of NADP-IDH in this community were 2mM for isocitric acid, 0.3mM for $NADP^+$ and 10mM for $MgCl_2$.

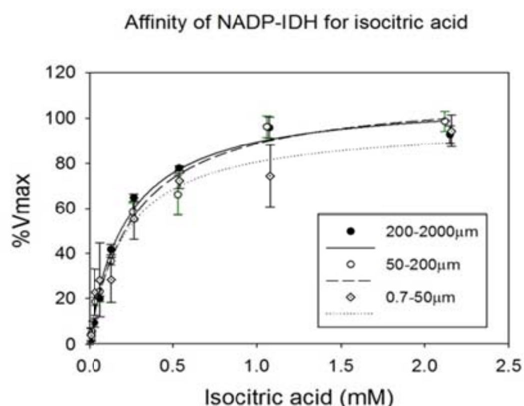

Fig. 1. Michaelis-Menten plot to determine the NADP-IDH kinetic constants for isocitrate. Equivalent experiments were also developed for $NADP^+$ and $MgCl_2$.

T effects: The Arrhenius relationship led to an optimum T at 30ºC for the NADP-IDH activity. The energy of activation (Ea) in the zooplankton community is 58790.42 J mol¯1 and the frequency factor (A) 6.14 $x10^9$ µmol NADPH h^{-1} mg $protein^{-1}$. The Q_{10}, measured from 5 to 30ºC, is 2.3.

pH effects: The peak distribution of the activity with the pH showed a maximum value at pH 8.2 (Fig.2). A measurement below pH 7.62 and above pH 8.75 will report activities lower than 50% of its V_{max}.

Buffer studies: Mg^{2+} and 0.1M phosphate buffer seemed to be the most appropriate combination in yielding high reaction velocities under low pure NADP-IDH solution. No results for Mn^{2+}-phosphate buffer were obtained because the chemical reaction between the reagents prevented the correct spectrophotometer readings.

From these experiments, the new IDH assay was developed and successfully applied to different fractions of coastal plankton community, as well as to euphotic-zone sediment traps samples during KOSMOS GC2014 campaign. The application of the IDH assay will improve our knowledge about the metabolic state of plankton communities in oligotrophic, eutrophic, the open ocean and coastal waters.

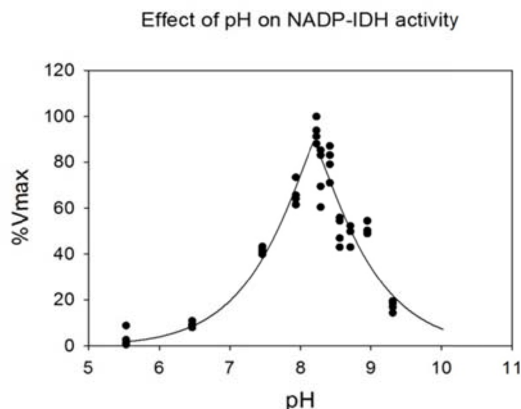

Fig. 2. pH effects on NADP-IDH activity.

Combining these enzyme assays with physiology measurements for CO_2 emission may also improve the spatial and temporal resolution of carbon metabolism studies on oceanographic surveys, as well as the Carbon flux research with data from deep-sea samples.

ACKNOWLEDGMENTS

We thank D. Ortega, G. Muntaner, R. Pérez, A. Marrero, R.Triay-Portella, M. Grau, D.R. Bondyale, A. Garran, A. Štindlová, E. Bru and V. Romero-Kutzner for their help with the living samples. Thanks also to our collaborators in the BIOACID Project (KOSMOS GC 1.0 and 2.0 experiments). This study was supported by project BIOMBA (CTM2012-2729-MAR). University of Las Palmas de Gran Canaria (PIFULPGC-2013-CIENCIAS-1) supports MTE, and CIE (Canary Islands) Tricontinental Atlantic Campus (CEI10/00018) funded TTP.

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