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Fast and accurate enzyme activity measurements using a chip-based microfluidic calorimeter

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ABSTRACT

Recent developments in microfluidic and nanofluidic technologies have resulted in development of new chip-based microfluidic calorimeters with potential use in different fields. One application would be the accurate high-throughput measurement of enzyme activity. Calorimetry is a generic way to measure activity of enzymes, but unlike conventional calorimeters, chip-based calorimeters can be easily automated and implemented in high-throughput screening platforms. However, application of chip-based microfluidic calorimeters to measure enzyme activity has been limited due to problems associated with miniaturization such as incomplete mixing and a decrease in volumetric heat generated. To address these problems we introduced a calibration method and devised a convenient protocol for using a chip-based microfluidic calorimeter. Using the new calibration method, the progress curve of alkaline phosphatase, which has product inhibition for phosphate, measured by the calorimeter was the same as that recorded by UV-visible spectroscopy. Our results may enable use of current chip-based microfluidic calorimeters in a simple manner as a tool for high-throughput screening of enzyme activity with potential applications in drug discovery and enzyme engineering.

Introduction

High-throughput measurement of enzyme activity is dependent on assays that enable time-resolved data collection to record activity of enzymes for a few seconds after addition of substrate. These assays generally require application of UV-visible or fluorescence spectroscopy in order to follow the color or the fluorescence of a substrate or a product [1]. Unfortunately, the natural substrates or products of many enzymes such as phosphatases, proteases, or kinases, do not have a color or are not fluorogenic, and thus, synthetically labeled substrates have to be used. Assays based on labeled synthetic substrates might not represent the true activity of enzymes, which might be misleading in identifying inhibitors as possible drug candidates or understanding the mechanism of the enzymatic reaction [2]. Furthermore, systematic approaches in enzyme engineering, using high-throughput screening for a desired activity, are difficult if the desired enzymatic reaction does not exhibit a color or fluorescence change. This can be solved by chemical or enzymatic coupled reactions, but this requires considerable development and optimization for every targeted enzyme reaction.

Therefore various label-free methods for enzyme activity measurements have been developed, based on different techniques, such as liquid chromatography-mass spectrometry (LC-MS) [3], capillary electrophoresis [4,5], a method based on macrocycle-fluorescent dye complex formation [6], and methods based on heat measurements with isothermal titration calorimetry (ITC) [7]. Recently, a method for time-resolved initial rate measurements using isothermal titration calorimetry (ITC) has been developed as a generic assay for the activity of enzymes. This method is based on a simple calibration reaction and enables accurate initial rate measurements by only recording a few early data points for the heat generated by an enzymatic reaction [8]. Although this method enables fundamental studies of many enzymes with their natural substrates and provides a tool for applying ITC instruments with high-throughput capability in measuring enzyme activity, still enzyme activity measurements using modern ITC instruments are significantly slower compared to those using UV-visible or fluorescence spectroscopy. This is because the ITC instruments, which have a measurement cell of 200–1400 µl, require a thermal equilibration time of 10–20 min to establish isothermal conditions necessary for sensitive measurements.

To address this problem and enable fast equilibration the sample volume required for measurements needs to be decreased. This has been achieved by applying microfluidic technology. New chip-based

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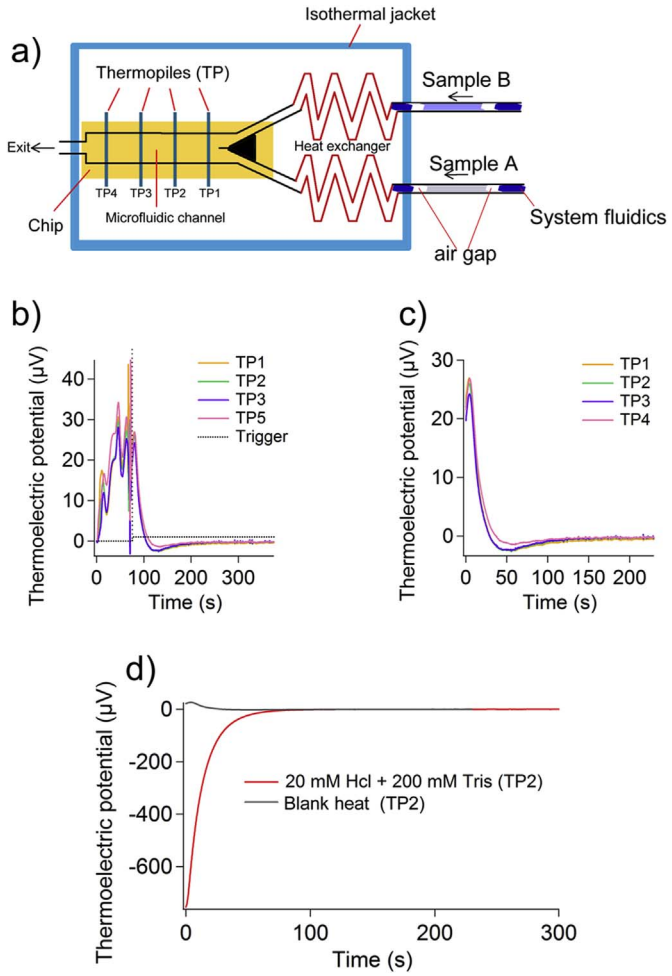

Fig. 1. Measurement of heat using a chip-based microfluidic calorimeter. Data for thermopile 2 are shown and are corrected for the blank heat. Measurements were performed at 37 °C. (a) Schematic representation of a chip-based microfluidic calorimeter. This schematic view is based on the original design by Lerchner et al. [12,13]. The calorimeter consists of a flow cell that is located on a chip with four thermopiles. Each inlet of the microfluidic cell is connected to a heat exchanger. All components are placed in an isothermal jacket. (b) The sample in each inlet to the flow cell is separated from the system fluid (Milli-Q water) using two air gaps, one in front and one in the back. (c) The raw data for measurement of generated thermoelectric potential due to mixing two samples of exactly the same solution of MOPS buffer. The initial data before flow stops are disturbed due to the passage of the air gap and flow over the thermopile. The trigger signal that changes from zero to one, shows the time at which the flow stops and measurements begin. (d) Time zero is set at the trigger signal. (e) The thermoelectric potential generated due to the mixing of 20 mM HCl with 200 mM Tris.

microfluidic calorimeters have been developed [9–11] that have a sample volume of less than 20 µl and a thermal equilibration time of a few seconds. Fig. 1a is a schematic overview of an isothermal chip-based microfluidic calorimeter, which was initially designed by Lerchner et al. [12,13]. In this calorimeter two laminar flows reach the microfluidic cell where heat measurements are made with a set of four thermopiles located in a row directly under the measurement cell. These thermopiles measure the heat based on the heat conduction principle [14]. However, use of this microfluidic calorimeter or other types of chip-based microfluidic calorimeters for accurate measurement of enzyme activity has remained a challenge, due to three inter-related problems: 1) the decrease in volume of the measuring cell and thus the volumetric heat generated compared to conventional ITC instruments, 2) the lack of complete mixing in a sufficiently short time [15], and 3) the lack of calibration methods to translate heat measurements into enzyme activity.

To address the above problems, we have applied a chip-based microfluidic calorimeter (Fig. 1a) for enzyme activity measurements. We developed a new calibration method which enabled the use of simple calculations to accurately determine the activity of enzymes. Practical application of this calibration method was demonstrated for alkaline phosphatase, which catalyses the dephosphorylation of different substrates [16] and is a target for drug discovery [17].

Methods

Chemicals

3-morpholinopropane-1-sulfonic acid (MOPS), *para*-nitrophenyl phosphate (PNPP) and HCl were obtained from Sigma-Aldrich. KCl, and Tris were obtained from Merck. NaCl, NaOH, KH_2PO_4 and $MgCl_2$ were obtained from J.T. Baker. Alkaline phosphatase from bovine intestinal mucosa was purchased from Sigma-Aldrich.

Microfluidic calorimeter experiments

A chip-based microfluidic calorimeter (ChipCAL) was used (TTP Labtech). For each experiment the solutions were filtered on 0.45 µm Whatman filters (GE Healthcare) and were degassed prior to injection into the microfluidic cell in order to prevent formation of air bubbles. After each experiment the microfluidic cell was washed with Milli-Q water. At the end of a set of experiments, before turning the instrument off, the microfluidic cell was cleaned by further washing with Milli-Q water. Of the four thermopiles registering the heat changes only the data of thermopile 2 were used. Thermopile 1 and 4 were not considered in view of occasional disturbance of the measurement due to air bubbles at the end or beginning of the flow cell. Thermopile 3 gave similar results as thermopile 2.

Isothermal titration calorimetry

A VP-ITC from Microcal (Malvern) with a cell of 1.4 ml was used to perform isothermal titration calorimetry (ITC) experiments. The instrument settings were: high feedback mode, stirring speed 502 rpm, reference power 63 µJ/s and a filtering time of 2 s. The instrument was calibrated electrically using the procedure provided by the maker. This calibration procedure involved the administration of a defined power to a resistive heater located next to the measurement cell. Samples were degassed and heated up to reaction temperature before the experiment. At the beginning of each experiment 2 µl of the sample was injected into the cell. The data point resulting from this injection was discarded in view of inaccuracy in the volume and sample concentration in the first injection. Subsequently 3 µl of the sample was injected twice to obtain two measurements for the reaction enthalpy. For the Tris-HCl calibration experiments the sample in the syringe contained a 1 mM HCl solution in Milli Q, while the cell contained a 200 mM Tris solution at pH 10.6. For the phosphate calibration experiments the sample in the syringe contained a 10 mM KPi solution in a 200 mM MOPS, 20 mM NaCl buffer at pH 5, while the cell contained a 200 mM MOPS, 20 mM NaCl solution at pH 8. The experiment was designed such that the heat developed after the injection had dissipated and the signal returned to the baseline before the next injection. Samples were degassed and heated to the experimental temperature prior to all experiments. The dilution enthalpy of the sample was determined and subtracted from the reaction enthalpy as a blank. The integral of the obtained curve was taken as the reaction enthalpy.

Calibration sample preparation

To test the suitability of several calibration methods, the following solutions were injected into the ChipCAL: 1) 200 mM Tris solution, pH 10.6 versus 5–20 mM HCl in Milli Q water; 2) 200 mM MOPS 20 mM NaCl pH 8.0 versus 5–20 mM KH_2PO_4 in 200 mM MOPS 20 mM NaCl