High Throughput Calorimetry for Evaluating Enzymatic Reactions Generating Phosphate

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Abstract: A calorimetric assay is described for the high throughput screening of enzymes that produce inorganic phosphate. In the current example, cellobiose phosphorylase (EC 2.4.1.20) is tested for its ability to synthesize rare disaccharides. The generated phosphate is measured in a high throughput calorimeter by coupling the reaction to pyruvate oxidase and catalase. This procedure allows for the simultaneous analysis of 48 reactions in microtiter plate format and has been validated by comparison with a colorimetric phosphate assay. The proposed assay has a coefficient of variation of 3.14% and is useful for screening enzyme libraries for enhanced activity and substrate libraries for enzyme promiscuity.

Keywords: Calorimetry, high throughput screening, cellobiose phosphorylase, phosphate assay, pyruvate oxidase.

INTRODUCTION

Industrial biotechnology is constantly looking for enzymes that function under non-natural conditions, or enzymes that catalyze new reactions, or both. In the quest for these enzymes, several strategies are used. One approach is to screen genomic libraries from natural sources for enzymes with the desired characteristics. Another possibility is to create the enzymes in the laboratory by directed evolution. In both cases, success strongly depends upon a fast, convenient and reliable screening method [1, 2].

High throughput calorimetry is an attractive option: the universal character of the technique makes it applicable to all enzymatic reactions. Developments in microelectronic engineering allowed the design of high throughput calorimeters in microtiter plate format, typically containing 48 or 96 calorimetric units [3-5]. The difference in heat is measured between a test well where the biochemical reaction takes place, and a reference well that contains all but one essential reagent. Additions are done to both wells simultaneously thus minimizing common mode effects such as dilution heat upon injection. The open setup exposes both wells to the same ambient temperature changes, therefore avoiding extensive insulation as needed for traditional isothermal titration calorimeters (ITC). However, the miniaturization and accessibility to external influences is accompanied by a trade-off, and current micro-electronic technologies sacrifice some of the accuracy that is characteristic to the classical ITC instruments in favour of higher throughput. In a previous publication, a microtiter plate version of the open differential calorimeter [3] was used to establish a fast, low-cost assay for vitamin C quantification in food and pharmaceuticals as an alternative to HPLC analysis [6]. Vitamin C was oxidised with ascorbate oxidase and the area under the output signal versus time profile was linearly correlated to the vitamin C concentration.

In this work, we developed an assay to follow cellobiose phosphorylase (CBP) activity upon synthesis of cellobiose and phosphate from α-glucose-1-phosphate (G1P) and glucose. Since the calorimetric signal was too small to detect CBP activity in itself with the high throughput calorimeter (HTC), CBP was coupled to pyruvate oxidase (POX), an enzyme that converts phosphate and pyruvate into peroxide, CO_2 and acetyl phosphate. The peroxide was converted subsequently by catalase (CAT) into water and oxygen in a strong exothermic reaction. When pyruvate is depleted, the signal drops abruptly to baseline.

MATERIALS AND METHODS

High Throughput Calorimetry

The HTC and the micro-electronic wafer have been described before [3, 6]. A scheme of the calorimetric device is shown in Fig. (**1**). In brief, a silicon wafer consists of 96 wells that are interconnected pairwise by 64 underlying thermopiles resulting in 48 calorimetric units. As such, the difference in heat between one well of a calorimetric unit (the test well) and the connected well (the reference well) is generating a power of 20 mV/K. A disposable microtiter plate made out of cycloolefin copolymer (COC) is positioned on top of the wafer, the bottom of the wells in close contact with the wafer membrane. This setup allows reuse of the silicon wafer and prevents enzyme inactivation due to sticking to the wafer membrane. The wafer with the COC disposable on top is placed in a reader, an instrument that measures the voltage of all 48 calorimetric units simultaneously, and transfers the data to a computer. An

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eight-channel non-contact dispenser (Seyonic, Neuchatel, Switzerland) is mounted on a robot (Festo, Brussels, Belgium) that moves the dispenser from a microtiter plate to the reader, and to a washing station to clean the dispenser needles. The reader, the dispenser, the washing station and the station for microtiter plates are integrated in a single chamber that is humidified to a relative humidity of 96% to avoid evaporation.

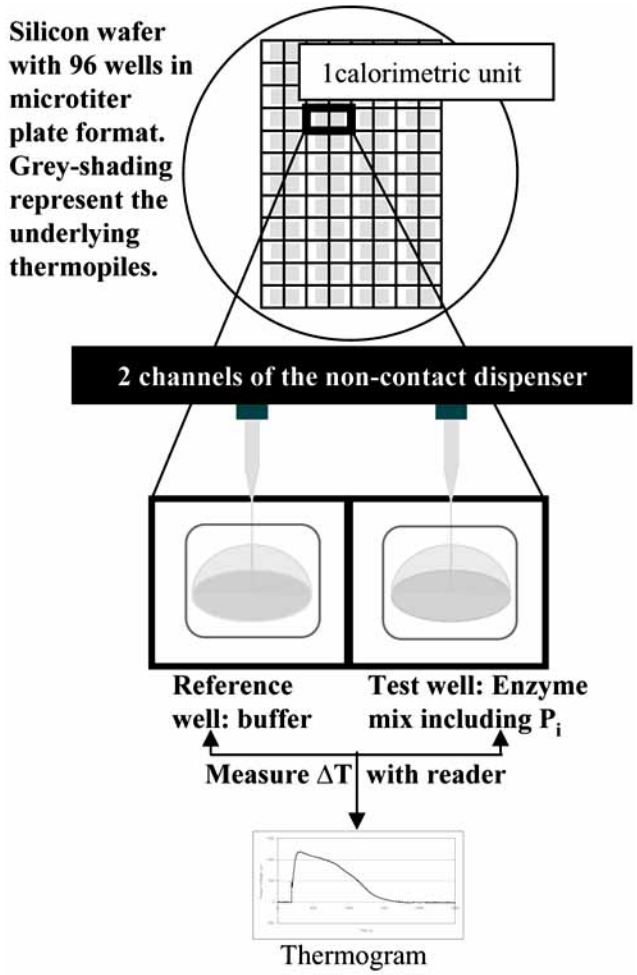

Fig. (1). Schematic overview of the micro-electronic wafer and measuring principle of the HTC. Each wafer consists of 96 wells corresponding to 48 calorimetric units. The reagents are put in the wells with a non-contact dispenser. The difference in temperature between both wells of the calorimetric unit creates a power that is measured with the reader. The data is transferred from the reader to a computer where it is shown graphically in a thermogram (a plot of output voltage versus time).

Reagents

Pyruvate oxidase (EC 1.2.3.3; Sigma P4591), catalase (EC 1.11.1.6), glucose, mannose, xylose, monobasic potassium phosphate (98% pure), sodium pyruvate (99% pure), thiamine pyrophosphate chloride, magnesium sulfate heptahydrate, Flavin adenine dinucleotide disodium salt hydrate, L-ascobic acid, sodium molybdate dihydrate and HCl, were purchased from Sigma (Bornem, Belgium). Galactose, dibasic potassium phosphate, and acetic acid were purchased from Acros (Geel, Belgium), sodium citrate tribasic dehydrate from Janssen Chimica (Geel, Belgium), ammonium molybdate tetrahydrate from Riedel de Haan (Seelze, Germany), and glucose-1-phosphate from Boehringer Mannheim (Mannheim, Germany). All reagents were used without further purification.

Enzyme Assays

All tests were performed at 25°C. POX and CAT were provided as lyophilized powder. POX contained flavin adenine dinucleotide (FAD) and sugar as a stabilizer. The enzyme powders were dissolved in 50 mM MES buffer pH 6.6 to obtain end concentrations of 0.5 U / well POX and 1 U / well CAT. No extra FAD or $MgSO_4$ were added since we could not observe a difference in reaction velocity (measured with HTC) upon supplementation. When thiamine pyrophosphoric acid (cocarboxylase or TPP) was added, a slight increase in reaction velocity was observed. Since the assay is meant for high throughput screening with crude cell extracts, we wanted to keep the setup as simple as possible and therefore decided not to supplement TPP during regular testing.

In one set of assays, potassium phosphate was used as P_i source. A 100 mM potassium phosphate buffer pH 6.6 was diluted to get appropriate P_i concentrations, and the dilutions were mixed with the POX and CAT enzyme cocktail. The resulting mix was pipetted in the test well (10 µl / well). The reference well contained 10 µl of 50 mM MES buffer pH 6.6. To initiate the reaction, 1 or 2 µl of pyruvate dissolved in 50 mM MES-buffer pH 6.6 was added.

In another set of assays, P_i was generated by the enzyme cellobiose phosphorylase (EC 2.4.1.20). The CBP enzyme from *Cellulomonas uda* was expressed in *Escherichia coli* XL10-Gold (Stratagene, La Jolla, CA), and crude cell extract was prepared with Easylyse (Epicentre biotechnologies, Madison, WI, USA). The buffer composition of the resulting cell-lysate is 5 mM Tris-HCl pH 7.5, 0.5 % non-denaturing detergent, 2 mM $MgCl_2$ and easylyse enzyme mix. The detailed procedure is described in [7]. As a negative control (NC), *E. coli* XL10-Gold without recombinant CBP was prepared the same way as the CBP crude extract.

To assess the reproducibility over the whole wafer, the reference well contained 3 µl NC and 1 µl 0.3 M glucose in 0.2 M MES in a total volume of 10 µl. The test well contained 10 µl composed by 0.25 U POX, 0.5 U CAT, 3 µl CBP lysate and 1 µl 0.3 M glucose. The reaction was started by a single addition of 1 µl of 150 mM G1P and 150 mM pyruvate in all wells.

To determine the acceptor specificity of CBP, the reference well was filled with 3 µl NC in 0.2 M MES pH 6.6 in a total volume of 10 µl. The test well contained 3 µl CBP lysate, a mix of 0.25 U POX and 0.5 U CAT in 0.2 M MES pH 6.6. To all wells, 1 µl of 0.3 M acceptor was added. The reaction was initiated by adding 1 µl of a mix of 150 mM G1P and 150 mM pyruvate.

Colorimetric P_i Determination Method [8]

The initial rate of CBP when using different acceptors was assessed at 25°C in eppendorf tubes. Each tube

contained 285 µl of 30 mM G1P, 30 mM acceptor molecule and 0.2 M natriummolybdate in 50 mM MES pH 6.6. The reaction was initiated by adding 15 µl CBP lysate to each tube. At different time points, a 50 µl sample was removed and transferred to a microtiter plate previously filled with 150 µl detection reagent (a mix of two parts 0.12% (w/v) L-ascorbic acid in 1 N HCl and one part of 1% (w/v) ammonium molybdate tetrahydrate in distilled water). The low pH of this detection solution stopped the enzymatic reaction. After exactly 5 minutes, 150 µl stop reagent (2% sodium citrate tribasic dehydrate and 2% acetic acid in distilled water) was added to stop the colour development. The plates were allowed to equilibrate for at least 15 minutes before the absorbance at 655 nm was measured. For each time point, the corresponding blank was subtracted and the corrected absorbance was compared to a standard curve for phosphate to obtain the P_i concentration. By plotting the phosphate concentration for each time point against the time of sampling, the CBP velocity for each acceptor could be deducted.

RESULTS

Accuracy of the Pyruvate-Oxidase Based Phosphate Assay

Because pyruvate oxidase (POX) consumes inorganic phosphate (P_i) while converting pyruvate into acetyl phosphate, this enzyme can be used to measure phosphate concentration [9-12]. In bioanalytical calorimetry, it is common practice to add catalase (CAT) to oxidases because the coupled reduction of hydrogen peroxide generates a lot of heat, the deleterious effect of hydrogen peroxide is eliminated, and half the oxygen is regenerated [13]. A reaction scheme of POX and CAT is depicted in Fig. (**2**). To investigate the accuracy of a POX-based phosphate assay with our HTC, a standard curve was established for phosphate. All assays were performed at 25°C with 50 mM MES buffer pH 6.6. A typical HTC signal for phosphate, shown in Fig. (**3**), consists of a sharp peak followed by a tail that suddenly drops to baseline. With increasing phosphate concentrations, the initial peak becomes larger and the signal length decreases. Indeed, the pyruvate present in the assay mixture is consumed faster at high P_i concentrations and the reaction, therefore, is finished sooner. Interestingly, a calorimetric signal is also observed when no P_i is present. It has been reported, however, that POX can also convert pyruvate without P_i, albeit with different reaction products (Fig. **2**) [14, 15]. This slow reaction generates the longest signal and should be considered the background value in the assay.

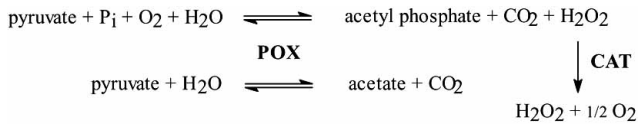

Fig. (2). The reactions catalysed by pyruvate oxidase (POX) and catalase (CAT).

A standard series of phosphate ranging from 0 to 8 mM was then analysed in three independent experiments. The signal lengths in function of P_i concentration is shown in Fig. (**4**). The reaction was initiated by adding pyruvate to a final concentration of 15 mM. Although the variation between the repeat measurements was sometimes relatively high (coefficient of variation (CV) ranging from 5.13% to 39.49%), a linear correlation between the P_i concentration and the signal length could be established. For concentrations ranging from 1 to 8 mM, an R^2-value of 0.91 was obtained. When including the 0 mM measurements, the R^2- value decreased to 0.89. However, since with 0 mM phosphate only the background signal is measured, this measurement point was ignored. Interestingly, the signal length obtained with 1 mM and 2 mM P_i are higher than the 0 mM P_i value, suggesting that small amounts of Pi inhibit the background reaction. Measuring higher P_i concentrations is possible, but this also requires higher pyruvate concentrations for a complete conversion of phosphate, which prolongs the assay time. A phosphate concentration series with 27 mM pyruvate is shown in Fig. (**5**). The R^2-value was 0.9758 and 0.9784 when including or excluding the 0 mM measurement respectively.

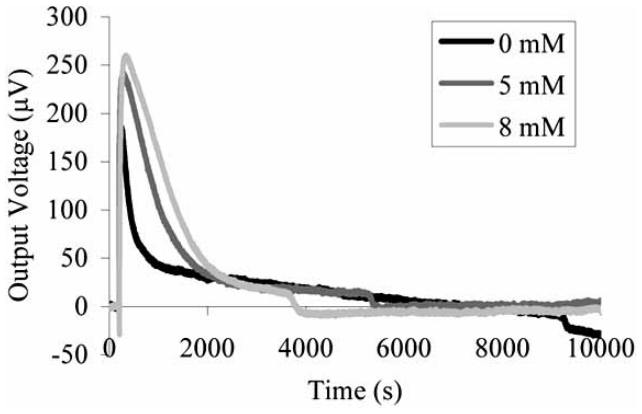

Fig. (3). Typical calorimetric signal for three different phosphate concentrations when potassium phosphate was used as P_i source. The reaction started when pyruvate was added (at 200 s) to obtain 15 mM pyruvate. Concentrations in the legend are the phosphate concentration in the total volume of the well.

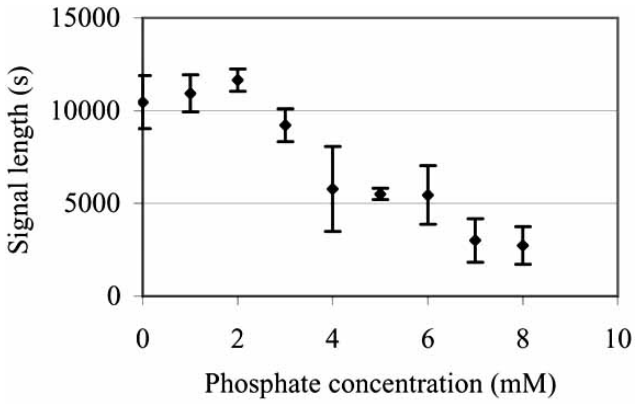

Fig. (4). Correlation between phosphate concentration and signal length with 15 mM pyruvate. All concentrations are expressed as end concentration in the total volume of the well. The values represent the mean ± SD of three experiments.

Accuracy of the CBP Coupled Assay

The assay was intended for screening directed evolution libraries of recombinant cellobiose phosphorylase (CBP)

from *Cellulomonas uda* for increased activity on unnatural substrates. In nature, CBP synthesises cellobiose with the concomitant production of phosphate from the donor substrate α-glucose-1-phosphate (G1P) and acceptor substrate glucose in a reversible reaction. The reaction scheme is depicted in Fig. (**6**). Therefore, the synthesis reaction of CBP can be coupled to the pyruvate-oxidase based phosphate assay to probe CBP activity.

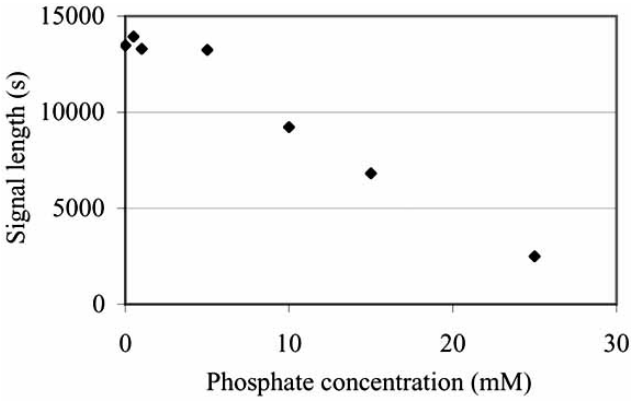

Fig. (5). Correlation between phosphate concentration and signal length with 27 mM pyruvate.

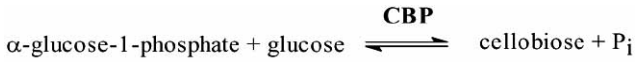

Fig. (6). The reaction catalysed by cellobiose phosphorylase (CBP).

The CBP gene from *Cellulomonas uda* was expressed in *E. coli* XL10 Gold, and cell-lysate was used as recombinant enzyme source. Different conditions were tested that varied the amount of CBP lysate, and POX and CAT concentration. The test wells were filled with a mix containing the three enzymes, the reference wells contained negative control lysate (NC), obtained from an *E. coli* strain without the recombinant CBP. The condition where the test well contained 3 µl CBP cell extract, 0.25 U POX and 0.5 U CAT, and the reference well was filled with 3 µl NC in a total volume of 10 µl turned out to work well, and it was used for further experiments. Glucose, G1P and pyruvate were added to a final concentration of 25 mM, 12.5 mM and 12.5 mM respectively. The reproducibility of this coupled CBP assay was measured by performing the same experiment in all 48 calorimetric units of the wafer simultaneously. The length of each calorimetric signal was measured and the CV was determined to be 3.14% (Table **1**), which makes this assay suitable for use in high throughput screening.

Finally, other carbohydrates than glucose were tested for their efficacy to function as acceptor substrate in the synthesis reaction of CBP. In Fig. (**7**), it is obvious that glucose is the preferred acceptor thus generating cellobiose. However, xylose and mannose are converted as well in this order of preference. Galactose is not an acceptor, as the coupled reaction stops at about the same time as the blank reaction, where no acceptor was added. The obtained ranking coincides with results obtained with a colorimetric phosphate assay where the initial rate of enzymatic reaction with the different acceptors was measured. A comparison of both methods is shown in Table **2**. Although the ranking is the same, the relative values for both assays are not identical. Therefore the HTC assay should be regarded as a semi-quantitative screening assay.

Table 1. Assay Reproducibility Over an Entire Plate[a]

Row[b]	Column AB (s)	Column CD (s)	Column EF (s)	Column GH (s)	Average (s)	SD (s)	CV
1	2842.4[c]	2863.9	2831.8	2917.4	2863.9	38.1	1.33%
2	2815.7	2718.1	2844.3	2917.5	2823.9	82.5	2.92%
3	2798.5	2676.5	2807.4	2880.0	2790.6	84.4	3.02%
4	2815.1	2787.8	2775.8	2922.9	2825.4	67.0	2.37%
5	2916.9	2780.6	2740.7	2922.3	2840.1	93.2	3.28%
6	2774.7	2725.9	2717.0	2875.9	2773.4	72.9	2.63%
7	2809.2	2816.4	2683.0	2897.9	2801.6	88.7	3.17%
8	2716.4	2713.4	2711.6	2937.3	2769.7	111.8	4.04%
9	2643.9	2693.3	2684.9	2914.1	2734.1	122.0	4.46%
10	2743.9	2695.6	2637.3	2861.1	2734.5	95.0	3.47%
11	2730.8	2704.6	2715.3	2716.9	2716.9	10.8	0.40%
12	2811.8	2725.4	2663.5	2908.1	2777.2	106.4	3.83%
Average (s)	2784.9	2741.8	2734.4	2889.3		**Average[d] (s)**	2787.6
SD (s)	69.7	57.3	67.0	58.7		**SD (s)**	87.5
CV	2.50%	2.09%	2.45%	2.03%		**CV**	3.14%

[a]The length (in seconds) measured on all 48 signals from an entire wafer. After each row and under each column, the average, SD and CV of that row or column is given.
[b]As in a microtiter plate, the rows are numbered from 1 to 12.
[c]The columns are labelled AB, CD, EF, and GH as each cell represents one calorimetric unit on the wafer.
[d]The average, SD and CV for all 48 signals.

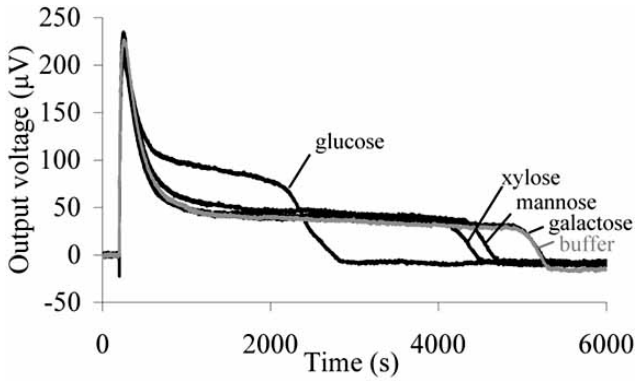

Fig. (7). Calorimetric signals for the CBP assay with different acceptors (glucose, mannose, xylose, galactose). As a negative control, buffer was added instead of acceptor. The higher the reaction rate at which CBP is converting the acceptor molecule together with G1P into a rare dissaccharide, the shorter the signal. Galactose is not an acceptor for CBP and the signal with galactose lasts as long as the negative control.

Table 2. Comparison of the HTC Assay with the Spectrophotometric Phosphate Assay [15] for Determining the Acceptor Specificity of CBP

Acceptor	Spectrophotometry[a]		HTC[b]	
	µM P_i/min	Relative	Length (s)	Relative
glucose	3.45	100%	2868.55	100%
xylose	2.37	69%	4533.46	32%
mannose	0.53	16%	4714.6	25%
galactose	0	0%	5333	-1%
buffer	0	0%	5315	0%

[a]The initial rate of the enzymatic reaction was determined by measuring the phosphate concentration at different time points.

[b]The length of each reaction was measured. To calculate CBP velocity and the signal length relative to glucose, the figures found for each acceptor were corrected by subtracting the blank (buffer) measurement.

CONCLUSION

The classical method to determine P_i quantifies molybdenium blue formed by the reduction of phosphomolybdate under acidic conditions [16]. In this report, we present an alternative assay based on calorimetry. The assay is applicable to enzymatic reactions that generate phosphate. In that respect, it can be used to explore the substrate specificity of phosphate generating enzymes. Vice versa, a library of mutated enzymes can be screened for improved activity on a certain (new) substrate. The calorimetric method was used to probe the reaction rate of CBP from *Cellulomonas uda* on different substrates. The developed assay has a CV of 3.14%.

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ABBREVIATIONS

CAT = Catalase

COC = Cylcoolefin copolymer

CBP = Cellobiose phosphorylase

CV = Coefficient of variation

FAD = Flavin adenine dinucleotide

G1P = α-glucose-1-phosphate

HTC = High throughput calorimeter

ITC = Isothermal titration calorimetry

NC = Negative control cell extract

P_i = Inorganic phosphate

POX = Pyruvate oxidase

SD = Standard deviation

TPP = Thiamine pyrophosphoric acid

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