A simple and reliable method for determination of optimum pH in coupled enzyme assays

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ABSTRACT

Determination of the optimum pH in a coupled enzyme assay poses significant challenges because altering the pH of the reaction mixture can affect the performance of both enzymes. Here, we demonstrate a simple and reliable method to determine the pH optimum for pyruvate kinase using the pyruvate kinase/lactate dehydrogenase coupled enzyme assay. This simple and reliable method can be broadly adapted to determine the pH optimum for various enzymes that are assayed using a coupled enzyme assay.

METHOD SUMMARY

This article describes a simple method to determine the optimum pH for the activity of one enzyme in a coupled enzyme assay. Decoupling of the two-step reaction is achieved by heat denaturing the enzyme catalyzing the first reaction at different pHs followed by the addition of the second enzyme at a constant pH. The velocity of the second enzymatic reaction is determined by the concentration of the product produced in the first reaction, therefore, allowing the measurement of the optimum pH of the first enzyme by monitoring the rate of the second reaction.

The activity of many enzymes is

KEYWORDS

coupled enzyme assay • *Cryptosporidium parvum* • enzyme kinetics • lactate dehydrogenase • pH optimization • *Plasmodium falciparum* • pyruvate kinase

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strongly influenced by the pH of the reaction milieu. Although some enzymes exhibit activity in a broad range of pH [1–3], many show maximal activity at a narrow pH range [4–6], referred to as the pH optimum. The pH optimum is a key parameter that must be determined accurately as enzymatic activity, behavior and/or assembly can often be governed by key ionizable amino acid residues [7–9]. A classic example of how key residues and proximity of residues can influence an enzyme's pH activity optimum is the HIV type 1 (HIV-1) protease. HIV-1 protease, an aspartyl protease, has a pH optimum that is significantly higher than their eukaryotic counterparts [10,11]. One study attributes this increased pH activity range to the absence of a key serine residue, found in most eukaryotic aspartyl proteases, which can form a hydrogen bond with the carboxyl oxygen of the key aspartic acid residues within the active site [12].

To properly characterize enzymatic properties, it is especially important to perform kinetic analyses of enzymes at the pH optimum. Therefore, the pH optimum is typically determined prior to detailed kinetic analyses [13]. Determining this parameter is usually straightforward and involves measuring reaction velocities under different pH conditions while keeping other parameters constant. However, evaluating the direct kinetics of many enzymatic reactions can be technically challenging. In certain cases, this difficulty is overcome by coupling two enzymatic reactions together such that the product(s) of the first reaction is a substrate(s) for another reaction, catalyzed by a second enzyme, which can be easily monitored [14–16]. Considering that the activities of both enzymes depend on the pH of the reaction medium in coupled enzyme assays, the two enzymatic reactions must be decoupled to measure the pH optimum of the enzyme of interest.

Owing to the technical challenges inherent with coupled assays as mentioned earlier, the pH optimum is often not experimentally determined for the primary enzyme of interest. For instance, pyruvate kinase (PyK), an evolutionarily conserved enzyme that is essential in the regulation of cellular metabolism in both eukaryotes and prokaryotes, has been extensively studied for over 60 years [17–22]. Although the pH optimum of some mammalian PyKs have been experimentally determined, to our knowledge, measurement of the pH optimum of many prokaryotic and parasitic PyKs, such as *Cryptosporidium parvum* PyK (CpPyK), has not been reported [23–26]. PyK catalyzes the irreversible transfer of a phosphate ion from phosphoenolpyruvate to ADP yielding pyruvic acid (PA) and ATP (Figure 1) and serves as a major regulator of the glycolytic flux [27–29]. In a typical activity assay, the PyK reaction is coupled to lactate dehydrogenase (LDH, reaction 2 in Figure 1), which catalyzes the NADH-dependent reduction of PA to lactic acid. The LDH reaction velocity can be easily measured using UV spectroscopy by monitoring the decrease in absorbance at 340 nm attributed to the oxidation of NADH to NAD⁺ [22,30].

Ideally, in a coupled enzyme assay, the enzyme of interest should determine the overall rate of the reaction such that the measured kinetic parameters are relevant to the enzyme of interest rather than the coupled enzyme. At low pyruvate concentration, the initial velocity (v_0) for the LDH reaction is determined by the concentration of PA (Figure 1). Therefore, the rate of the LDH reaction can be used as a measure of the PyK reaction velocity. The pH dependence of the PyK reaction therefore cannot directly be evaluated in the coupled enzyme assay considering that the LDH reaction rate is also affected by pH [31,32]. Although previous studies have reported methodology for deter-

Figure 1. The reaction schematic of the coupled pyruvate kinase and lactate dehydrogenase reaction to monitor pyruvate kinase kinetics. In the first reaction, pyruvate kinase (colored in red) irreversibly converts phosphoenolpyruvate to pyruvic acid (or pyruvate) while also converting ADP to ATP. In the second reaction, pyruvic acid is then reversibly converted to lactic acid using lactate dehydrogenase. During this step, NADH is oxidized to form NAD⁺ at the same rate as pyruvic acid production, allowing the analyst to follow the reaction rate of pyruvate kinase using UV-Vis spectroscopy by measuring the decrease in absorbance at 340 nm from NADH being consumed in reaction two ($\epsilon = 6300 \text{ cm}^{-1}\text{M}^{-1}$).

mining the pH optimum of PyK within this coupled assay [23,24,26], we report an alternative, reliable and simple method for decoupling a coupled enzyme assay to determine the pH optimum of PyK using recombinant versions of *CpPyK* [33] and *Plasmodium falciparum* LDH (*PfLDH*) [34]. The described method could potentially be adapted to determine the pH optimum of other PyKs and could be broadly applied to determine pH optima in other coupled enzyme assays.

MATERIALS & METHODS

Recombinant *CpPyK* was expressed and purified from *Escherichia coli* as detailed in Cook *et al.* [33]. Recombinant *PfLDH* was purified from *E. coli* using ammonium sulfate precipitation, affinity chromatography on Blue Sepharose resin (GE Healthcare), followed by size exclusion chromatography on Superdex 200 column (GE Healthcare; IL, USA.) [34]. Reagents used were from Sigma-Aldrich (MO, USA.).

The outline for a typical coupled assay to measure PyK activity is shown in Figure 1 in which PyK catalyzes the first reaction (1, conversion of phosphoenolpyruvate [PEP] to PA) and LDH catalyzes the second reaction (2, conversion of PA to lactic acid). Standard coupled assays are performed in 1.0 ml of total reaction mixture containing 50–100 mM Tris buffer (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 1 mM PEP, 1 mM ADP, 0.1 mM NADH and 1 μg of *PfLDH*. The reaction is initiated by adding 1 μg *CpPyK* and absorbance at 340 nm is monitored in a temperature-controlled spectrophotometer (HP 8452A Diode Array; Hewlett Packard, CA, USA) at 25 or 30°C. The optimum pH for *PfLDH* activity is in the range of 7.0–8.0 [34].

To analyze the effect of pH on PyK activity, however, the two reactions must be decoupled. We decoupled the reactions by heat inactivating PyK at the end of reaction 1 and then used the heat-inactivated PyK reaction mixture as a source of PA to drive reaction 2 followed by the addition of NADH and *PfLDH*. In control experiments, we determined *CpPyK* enzyme is completely and irreversibly inactivated by incubation at 70°C for 5 min (data not shown). We also noted that the *CpPyK* reaction was not affected by buffer concentration in the range of 10–100 mM. To ensure that the addition of the mixture after reaction 1 does not affect the pH of the *PfLDH* reaction, *CpPyK* assays were performed in 10 mM buffer. The pH of each mixture for reaction 1 was adjusted after addition of all reagents before adding *CpPyK*. Two different buffer systems were used: sodium acetate (pH range: 4.0–6.5) and Tris (pH range 7.0–9.0).

The PyK reaction was carried out in 10 mM buffer of various pHs containing 100 mM KCl, 5 mM MgCl₂, 1 mM ADP and 1 mM PEP in a total volume of 1 ml in Eppendorf tubes. Reaction 1 was initiated by adding 2.5 μg of *CpPyK* to the tube and placed in 30°C water bath for 1 min. For heat inactivation of *CpPyK*, the tubes were then immediately placed into a 70°C water bath for 5 min, followed by cooling on ice for 2 min. The reaction mixtures were then subjected to centrifugation at 4500 $\times g$ for 5 min. From each tube, 800 μl of the supernatant were transferred to a 1-ml quartz cuvette containing 200 μl of 1 M Tris buffer at pH 7.5. The second reaction was initiated by adding NADH (final concentration 0.1 mM) and 1 μg of *PfLDH*. The LDH reaction was then monitored for 1 min by following the

absorbance at 340 nm at 25°C. These experiments were repeated at least four times. To ensure that reaction 1 is the rate determining step of the coupled reaction, the amount of *PfLDH* to add in reaction 2 was determined by incrementally titrating increasing amounts of *PfLDH* in control experiments until the v_o of reaction 2 no longer increased. The amount of *PfLDH* found within those experiments was then doubled to ensure that *PfLDH* would remain in excess under all of the experimental conditions (data not shown).

The pH optimum of *CpPyK* was determined by analyzing the rate of decrease in absorbance at 340 nm due to the consumption of NADH at each pH condition. Therefore, the v_o of reaction 2 is calculated as molarity of NAD⁺ formed over time (or NADH consumed) using the extinction coefficient of NADH ($\epsilon = 6300 \text{ cm}^{-1}\text{M}^{-1}$). The linear behavior of the v_o of reaction 2 for longer than 1 min at each pH demonstrated that NADH was not limiting during reaction 2 (data not shown). Because the rate of the LDH reaction (2) is dependent on the concentration of PA produced in reaction 1, the pH of reaction 1, which yielded highest LDH activity represents optimum pH for *CpPyK* activity.

An online step-by-step protocol of the assay described above with suggestions, reagents and equipment lists can be found at [41].

RESULTS & DISCUSSION

In a set of control experiments, we determined that *CpPyK* was completely and irreversibly inactivated by heating at 70°C for 3 min (data not shown). We also verified that the concentration of the buffer in the range of 10–100 mM did not affect *CpPyK* activity (data not shown). Moreover, addition of 10 mM acetate or Tris buffer of different pH values to 1 M Tris HCl, pH 7.5, in a ratio of 4:1 did not alter the pH of the latter buffer. Therefore, 800 μl of the reaction mixture from *CpPyK* reactions performed in 10 mM acetate or in 10 mM Tris HCl buffer after heat inactivation of the *CpPyK* enzyme were used to assay LDH activity at a constant pH.

Because the v_o of the LDH reaction (2) is dependent on the concentration of PA, the rate of reaction 2 should be proportional to the v_o of reaction 1 (PyK). Our analysis demonstrates that *CpPyK* is enzymatically active in a broad range of pHs from 5.5 to ►

8.0, with maximal activity at pH 7.0 (Figure 2). CpPyK showed little activity at pH 4.0–5.0 and increased more than threefold at pH 5.5. The activity decreased at pH 8.5 and higher. These data demonstrate that the CpPyK is active over a broad pH range; however, the enzyme exhibits minimal activity at highly acidic conditions (pH 5.0 or below). Although many enzymes prefer a narrow range of pH for activity, others exhibit activity over a broad range of pHs. Among the latter group are some parasitic enzymes [35], a number of small molecule kinases [36,37] and PyKs from rabbit muscle PyK [23] and cyanobacterium *Synechococcus* PyK [38].

The determination of the optimum pH for an enzyme is important for the characterization of enzymatic properties and is often challenging to determine for enzymes within a coupled reaction [13]. Knowing whether the enzyme of interest has a narrow or broad range of pH activity is not only an important consideration for kinetic studies but could also be used to determine the potential chemical ruggedness or pH stability of an enzyme [39,40]. The method described here could potentially be used to accurately determine the pH optimum of other coupled enzyme assays or be modified to determine optimum parameters for the enzyme catalyzing the second reaction. One limitation of the proposed method is that any heat sensitive biochemical or chemical entity that may be necessary for the successful performance of the second reaction may be inactivated or damaged during the heat denaturation of the first enzyme. This method could also be expanded to determine and optimize other important chemical and physical properties of an enzyme within a coupled assay such as temperature, salt type and concentrations and/or other allosteric effectors.

We report a simple, cost-effective and reliable method for determining the pH optimum of an enzyme within a coupled assay by decoupling the two-step reaction with heat inactivation of the first enzyme. Additionally, the potential expansion of this method to include other parameters, such as optimum temperature, salts and allosteric effectors, could also be very beneficial. Accurate characterization of all enzymes within coupled assays is essential in the development of future coupled assays or

downstream applications that use coupled assays.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0126

AUTHOR CONTRIBUTIONS

L Bowman, R Motamed and P Lee made equal and significant experiment contributions. K Aleem and AS Berawala also contributed to the experiments. KL Hayden, DJ Bzik and D Chattopadhyay made significant contributions to the preparation of the manuscript and guidance on the experiments. L Bowman also contributed to the preparation of the manuscript

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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Figure 2. The v_0 of *Plasmodium falciparum* lactate dehydrogenase as a response to pyruvate production by *Cryptosporidium parvum* pyruvate kinase at varying pH conditions. The data shown are the average initial reaction velocities (in $\mu\text{M}/\text{min}$ of NAD^+) for the *Plasmodium falciparum* lactate dehydrogenase (PfLDH) reaction (2). *Cryptosporidium parvum* pyruvate kinase (CpPyK) reactions (1) were performed at various pH conditions ranging from 4.00 to 9.00 for 1 min, heat-inactivated and 800 μl of reaction mixture was used for the PfLDH reaction (total volume 1 ml) at pH 7.5. Error bars indicate standard error with a sample size of $n = 4$ for each pH condition. v_0 : Initial velocity.

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