Analytical Biochemistry 389 (2009) 93–96

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Serum cholinesterase assay using a reagent-free micro pH-stat

Linus T.-H. Kao, Miklós Gratzl *

Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA

ARTICLE INFO

Article history:
Received 9 December 2008
Available online 27 March 2009

Keywords:
Cholinesterase assay
Electrochemical pH-stat
Serum

ABSTRACT

Enzyme activities in body fluids are often used as diagnostic markers for physiological conditions and diseases. Common enzyme assays use optical methods that often require the use of pseudosubstrates and associated dyes. Here we introduce a reagent-free micro pH-stat that can determine absolute enzyme activity without the need for exogenic reagents. This approach employs electrolysis for precise dosing of the requisite acid or base titrant to stat the pH of the sample. The micro pH-stat is based on the rotating sample system (RSS), a convection platform for microliter drops. Activities of serum cholinesterase in fetal bovine serum and human serum were analyzed with this approach. The performance of this system is comparable to that of standard techniques ($r^2 = 0.99$), yet it offers a broader range of detection. The reagent-free micro pH-stat has potential to be developed as a miniaturized device for point-of-care testing.

Enzyme activities in body fluids such as blood and urine are often used as diagnostic markers for physiological conditions and diseases [1]. To determine enzyme activity in a sample, the rate of conversion of the substrate to the product of the enzyme reaction needs to be assessed. Optical measurements using spectrophotometry and fluorimetry are the most common methods for enzyme assays. Other techniques such as radiometric, titrimetric, chromatographic, calorimetric, and chemiluminescence measurements have also been reported for particular enzyme assays [2]. Optical approaches depend on calibration with standard solutions and the use of pseudosubstrates and/or the addition of dyes and other reagents that may interfere with the reaction or constituents of the specimen. An alternative approach is based on pH-stating, a technique that allows the monitoring of the rate of reactions that induce a pH change in the solution. In a pH-stat, constant pH of the sample is maintained at a set level by the continuous addition of the required H^+ or OH^- that counterbalances the pH shift from the ongoing enzyme reaction. Therefore, the reaction rate can be readily obtained because it is equal to the rate of titrant addition at steady state. Previously, the use of water electrolysis as an alternative to titration with an actual acid or base reagent has been suggested for the electrolytic addition of H^+ or OH^- for enzyme assays in approximately 100-ml volumes of buffer [3,4].

Here we report on a novel approach for enzyme assays in microliter samples using electrochemical pH-stating. This approach takes advantage of the rotating sample system (RSS)[1] platform that has been introduced in our laboratory as a convective platform for optical and electrochemical analyses in small sample drops [5–9]. The electrochemical micro pH-stat that we propose here incorporates the main concepts of this platform, as shown schematically in Fig. 1. A hydrophobic ring with proper inner diameter holds the 20-μl sample in place, confining it into a hemispherical shape by virtue of high surface tension at the air–water interface. Vigorous rotation and mixing of the drop is generated by two antiparallel air jets tangential to the drop surface. Acid or base addition in our system is achieved by water electrolysis at a Pt mini-disc working electrode that is embedded in the stationary substrate. The magnitude of current is controlled by the actual difference between the real-time pH reading in the sample and the desired pH value set in the control system. We recently showed that in such a system, with precise current injection and efficient mixing, it is possible to maintain the desired pH within ±0.05 of the preset pH value in drops of artificial samples of red cell cholinesterase enzyme (red cell AChE, EC 3.1.1.7) prepared in physiological buffer [10].

The objective of this work was to bring the reagent-free micro pH-stat system a step closer to real-world applications by validating the approach for biological specimens, particularly serum. Here we analyzed serum cholinesterase (Serum ChE, EC 3.1.1.8) that often serves as a sensitive indicator of the synthetic capacity of the liver. A 30–50% drop in ChE activity is observed in acute and chronic hepatitis. A decrease of 50–70% occurs in advanced cirrhosis and carcinoma of the liver [11]. Furthermore, organic phosphorous compounds that are constituents of many insecticides inhibit activity of ChE. Hence, the decay in ChE activity in serum can be

* Corresponding author. Fax: +1 216 368 4969.
E-mail address: miklos.gratzl@case.edu (M. Gratzl).

[1] *Abbreviations used:* RSS, rotating sample system; AChE, red cell cholinesterase enzyme; ChE, Serum cholinesterase; DTNB (Ellman's reagent), 5,5′-dithio-bis-2-nitrobenzoate; FBS, fetal bovine serum; ATCh, acetylthiocholine iodide; PBS, phosphate-buffered saline.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.03.034

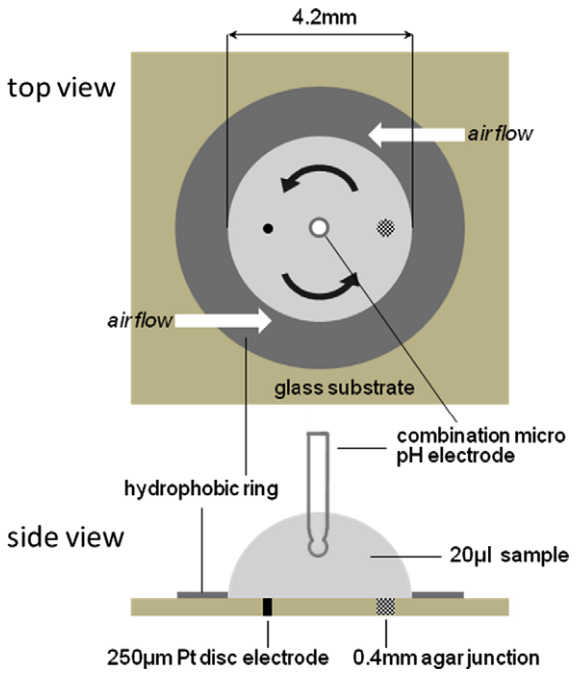

Fig. 1. Schematic of the electrochemical micro pH-stat built on the RSS platform. A 20-µl sample drop is surrounded and confined by a hydrophobic ring on a glass substrate containing an embedded Pt disc electrode and a junction. The dark arrows represent flow patterns generated in the drop by two tangential antiparallel air jets. A combination micro pH electrode is immersed to a depth of 1 mm from the top into the sample. The schematics are drawn approximately to scale.

used as an early diagnostic indication of possible insecticide poisoning [11,12]. A variety of alternative ChE substrates for optical assays have been reported [13–16]. Although optical assays are fairly automated, the lack of standards makes it difficult to compare results between laboratories [12]. Also, the use of artificial substrate and additional color-changing reagents may interfere with the reaction of interest. For instance, Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoate [DTNB]) can react with sulfhydryl groups [13].

The activity of ChE can be measured directly by pH-stating because acetic acid is produced as a side product. Here we propose an electrochemical pH-stating technique that requires only the actual substrate to initiate the reaction and, therefore, is independent of other exogenic reagents. Given that OH^- is continuously delivered by electrical current and nearly 100% current efficiency is achieved in serum, the method provides an absolute measurement and, thus, calibration with standard solutions is not necessary. Our results indicate that the accuracy and precision of the electrochemical pH-stat assay is comparable to that of standard techniques ($r^2 = 0.99$). Minimal sample consumption and the fact that the requisite instrument can be miniaturized render the electrochemical micro pH-stat a simple, cost-effective, and potentially portable system for enzyme assays.

We note that electrochemistry in specimens of biomedical origin is generally challenging due to the complex composition of the sample that can lead to contamination of the electrode surface and/or competing oxidation or reduction of constituent molecules, especially at the high overpotentials needed for water electrolysis. In addition, changes in viscosity and surface tension in serum may influence the hydrodynamics of the RSS, and the strong buffer capacity of serum may reduce the sensitivity of pH-stating. Despite these technical challenges, the quality of the data obtained in this work is similar to that obtained in artificial buffer seen in our previous publication [10]. This is an important argument for using galvanostatic methods in analyses of biological fluids.

Materials and methods

Materials

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA). All aqueous solutions and subsequent dilutions were prepared with 18.2 MΩ-cm deionized water (Milli-QUV Plus, Millipore, Billerica, MA, USA). Substrate solutions were prepared freshly before the experiments. Serum samples stored in a refrigerator are stable for 7 days. Native and heat-inactivated fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT, USA).

Apparatus

The fabrication of the micro pH-stat cell (Fig. 1) was described in our previous work [10]. A 250-µm-diameter Pt mini-disc electrode (Alfa Aesar, Ward Hill, MA, USA) was employed as working electrode. Agarose (1% [w/w] type I) was prepared in heated 0.1 M KNO_3 solution and filled into the junction hole embedded in the glass substrate. A hydrophobic ring of 4.2 mm inner diameter for sample positioning and confining it to a hemispherical shape (Fig. 1) was made of painted silicon elastomer (Dow Corning, Midland, MI, USA). A micro pH-electrode (MI 4154, Microelectrodes, Bedford, NH, USA) with a 1-mm-diameter tip was immersed at the vertical axis of the sampled drop to a depth of 1 mm for real-time pH monitoring.

Procedures

Determination of current efficiency in the electrochemical micro pH-stat

We measured current efficiency by adding 5 µl of HNO_3 (2–8 mM) to lower the pH of 15 µl of FBS. A constant current was subsequently applied to neutralize the acid and bring the pH of the sample back to its original value. Once the original pH was restored, the duration of current injection was recorded and then current efficiency was obtained by using Faraday's law. We define current efficiency as the percentage of moles of acid added to the sample divided by the added charge necessary to restore the initial pH.

ChE assay in FBS with electrochemical pH-stating

ChE (0.9–2.4 U/ml) from equine serum was prepared in heat-inactivated FBS. Intralipid (0.15%, Baxter Healthcare, Deerfield, IL, USA) was added to the enzyme solution to ensure effective mixing [17]. The substrate acetylthiocholine iodide (ATCh) was prepared in 0.1 M NaCl and adjusted to pH 8.0.

The ChE assays were performed at room temperature. Substrate solution (15 µl) was placed on top of the RSS cell. Once the pH reading was stabilized at 8.0, 5 µl of 10- to 100-fold diluted ChE solution was added to the substrate solution. The protons generated by the enzyme reaction were neutralized by OH^- titration generated electrolytically by the micro pH-stat system. In each pH-stating experiment, the sample pH was maintained within 0.1 pH unit of the preset pH for 1 min. The enzyme activity was obtained by averaging the current over 1 min of steady-state pH-stating and applying Faraday's law to convert current into molar amount.

Spectrophotometric control assay

The ChE assay using Ellman's procedure was adapted as control [13]. All of the solutions except the enzyme solution were prepared with 0.1 M phosphate-buffered saline (PBS) with the pH adjusted