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Monitoring carbohydrate enzymatic reactions by quantitative in vitro microdialysis

Swati J. Modi, William R. LaCourse *

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA

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Abstract

On-line in vitro microdialysis (MD) sampling followed by HPLC separation and UV absorbance detection (HPLC–UV) was used to monitor carbohydrate enzyme systems. Fundamental parameters (i.e., K_m and V_{max}) of hydrolysis reactions of 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-galactopyranoside, and 4-nitrophenyl-β-D-xylopyranoside were determined for a model enzyme, almond β-glucosidase. Accurate quantitation was achieved via internal standard methodology and compared to spectrophotometric data and literature K_m values, which were found to be 2.6 ± 0.5 mM (MD), 2.7 ± 0.4 mM (spec), and 2.5 mM (lit), for the substrate 4-nitrophenyl-β-D-glucopyranoside. A previously unpublished K_m value for the substrate salicin was also determined by this method. An application is shown for monitoring the glycoside salicin and its hydrolysis product saligenin in a commercially available willow bark product that is used for making tea. This versatile method has far-reaching applications to monitoring a variety of carbohydrates in enzymatic processes without complex sample preparation procedures and without volume loss.

Keywords: Salicin; Enzyme kinetics; β-Glucosidase; In vitro microdialysis; Carbohydrates; Nutraceuticals

1. Introduction

Enzymes are the reaction catalysts of biological systems. They have extraordinary catalytic power, often a high degree of substrate specificity and can greatly accelerate specific chemical reactions [1]. Michaelis–Menten enzyme kinetic parameters such as K_m and V_{max} offer a basis of comparison of different substrates for the same enzyme, or even comparison of different enzymes [2]. K_m, which is defined as substrate concentration at half maximum velocity (V_{max}), is an important parameter in enzyme studies as it is a measure of the apparent affinity of the substrate for the enzyme [1,2]. Thus, the substrate with the lowest K_m value has the highest apparent affinity for the enzyme [3].

Current methods for determining Michaelis–Menten enzyme kinetic parameters most often involve direct or indirect spectrophotometric methods [3,4]. In cases where spectra of substrate and product are very similar, direct spectrophotometric methods are difficult to carry out as the signals of both species are overlapping. Indirect methods couple a second enzyme or a reagent to act upon the product of the first enzymatic reaction in order to obtain an observable change in absorbance; the increased complexity of the analysis scheme results in a decrease in precision and accuracy [3]. If the substrate and product(s) are non-chromophoric and cannot be coupled to a secondary system, then an alternate detection approach is necessitated.

Microdialysis (MD) sampling circumvents these problems by allowing researchers to sample the reaction mixture continuously to obtain the complete kinetic profile [5,6]. Additionally, MD is easily coupled to HPLC and lends itself to the separation of substrate from product, thereby allowing kinetics of each to be observed individually by UV absorbance or even other forms of detection [7].

The main achievements of MD are the facilitation of continuous sampling, on-line sample clean-up, and the monitoring of small molecules of interest from complex matrices by employing a semi-permeable membrane with a specific molecular weight cut-off (MWCO) [8]. Small analytes able to pass through the membrane are collected by a flowing perfusion fluid and analyzed by various detection methods. This technique, commonly used in vivo, has opened up research in the area of pharmacokinetics and biological responses to various stimuli [9,10]. In the present study, in vitro microdialysis sampling was used to

* Corresponding author. Tel.: +1 410 455 2105; fax: +1 410 455 2608.
E-mail address: lacourse@umbc.edu (W.R. LaCourse).

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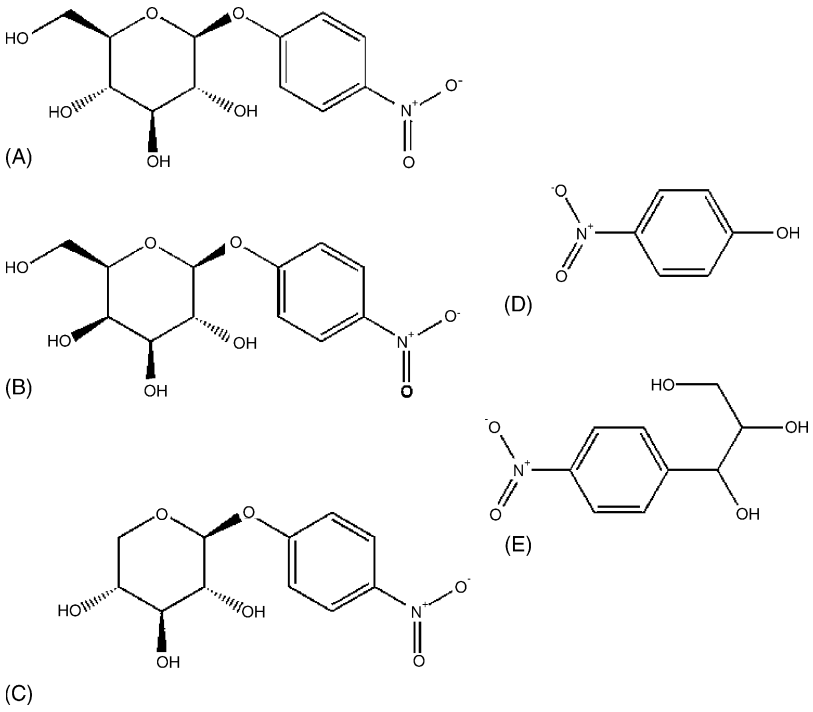

Fig. 1. Structure of 4-nitrophenyl-β-D-glucoside (A), 4-nitrophenyl-β-D-galactoside (B), 4-nitrophenyl-β-D-xyloside (C), 4-nitrophenol (D), and I.S. 1-(4-nitrophenyl)glycerol (E).

investigate the kinetics of hydrolysis for various substrates of the enzyme almond β-glucosidase [11,12].

As analytes must be recovered across the membrane, the percent recovery can vary greatly depending on the analytes, matrix, temperature, mass transport, perfusion flow rate, and membrane characteristics (i.e. material, diffusable geometry, and thickness) [13]. As such, the individual recovery value for each analyte may be difficult to obtain, especially in complex mixtures [14]. Therefore, many of the in vitro MD studies performed to date are qualitative, as opposed to providing quantitative results [15].

The defined model system employs selected constraints to explore the potential of accurate quantitative in vitro microdialysis. Microdialysis was used to monitor the enzymatic hydrolysis of carbohydrate substrates by β-glucosidase to obtain Michaelis–Menten enzyme constants. The enzyme catalyzes the hydrolysis of a broad array of substrates including the model nitrophenyl glycosides shown in Fig. 1A–C [12]. The reaction between β-glucosidase and these substrates generates 4-nitrophenol (4NP, Fig. 1D). The model system for studying enzyme kinetics involves coupling microdialysis to HPLC for separation and simple UV absorbance detection. An internal standard (I.S., Fig. 1E), 1-(4-nitrophenyl)glycerol, was employed for increased accuracy in quantitation, and the method was used for the *direct* determination of kinetic constants for these compounds [16].

The use of microdialysis in terms of on-line, sample clean-up for quantitative analyses has been reported previously by our group [8,17] and apparent rate constants have been estimated by microdialysis [17,18]. In this paper, HPLC following microdialysis sampling and sample clean-up is extended to the accurate determination of K_m values using chromophoric substrates, whose enzymatic parameters can be confirmed using standard spectrophotometric assays and literature values [12]. The analytical utility of microdialysis is further demonstrated by its ability to sample carbohydrates in complex matrices without perturbing the reaction under study. An application is shown for monitoring the glycoside salicin and its hydrolysis product saligenin in a commercially available willow bark product that is used for making tea [19–24]. In addition, the K_m value of salicin is reported for the first time by this novel application of in vitro MD–HPLC–UV.

2. Experimental

2.1. Chemicals

Methoxyacetic acid (MeOAc), disodium ethylenediaminetetraacetic acid (EDTA), [piperazine-1,4-bis(2-ethanesulfonic acid)] (PIPES), and sodium acetate were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) and sodium hydroxide (50/50, w/w) were obtained from Fisher Scientific (Springfield, NJ, USA). Mobile phases were filtered with 0.45 μm nylon-66 filters (Fisher Scientific) and a solvent filtration apparatus (Rainin, Woburn, MA, USA). Water was purified using an Elix-3 electrodeionization station coupled with a Milli-Q A10 water purification system (Millipore, Billerica, MA, USA).

The compounds 1-(4-nitrophenyl)glycerol, 4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-xylopyranoside, salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside], saligenin [2-hydroxybenzyl alcohol], and almond