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HPLC-based kinetics assay facilitates analysis of systems with multiple reaction products and thermal enzyme denaturation☆

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ABSTRACT

Glucosinolates are plant secondary metabolites abundant in *Brassica* vegetables that are substrates for the enzyme myrosinase, a thioglucoside hydrolase. Enzyme-mediated hydrolysis of glucosinolates forms several organic products, including isothiocyanates (ITCs) that have been explored for their beneficial effects in humans. Myrosinase has been shown to be tolerant of non-natural glucosinolates, such as 2,2-diphenylethyl glucosinolate, and can facilitate their conversion to non-natural ITCs, some of which are leads for drug development. An HPLC-based method capable of analyzing this transformation for non-natural systems has been described. This current study describes (1) the Michaelis–Menten characterization of 2,2-diphenyethyl glucosinolate and (2) a parallel evaluation of this analogue and the natural analogue glucotropaeolin to evaluate effects of pH and temperature on rates of hydrolysis and product(s) formed. Methods described in this study provide the ability to simultaneously and independently analyze the kinetics of multiple reaction components. An unintended outcome of this work was the development of a modified Lambert *W*(x) which includes a parameter to account for the thermal denaturation of enzyme. The results of this study demonstrate that the action of *Sinapis alba* myrosinase on natural and non-natural glucosinolates is consistent under the explored range of experimental conditions and in relation to previous accounts.

1. Introduction

Organic isothiocyanates (ITCs, **2**, Scheme 1) are a well-studied class of compounds with an array of documented biological applications, including anti-inflammatory [1–5], antioxidant [6–9], and antitumor effects [10–14]. Of note, consumption of many *Brassica* vegetables (e.g. broccoli, cauliflower, kale, cabbage) confers anti-cancer properties in part due to their high natural concentration of glucosinolates (**1**), β-thioglucoside-*N*-hydroxysulfate precursors of ITCs. Over 200 naturally-occurring variants of **1** have been discovered which vary in the aglycone sidechain (R) [15]. The enzyme myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) catalyzes hydrolysis of the thioglucosidic bond in **1** in a mechanism that follows Michaelis–Menten kinetics [16]. While ITCs are the principle hydrolysis product at physiological pH and temperature, nitriles (**3**) have been observed to form at acidic pH [17,18], and amines (**4**) can result when the electrophilic ITC is hydrolyzed by water to a carbamic acid that rapidly undergoes decarboxylation [17].

Although natural glucosinolates and their ITCs have been relatively well-described, fewer accounts have been made concerning non-natural analogues, those compounds bearing sidechains structures not observed in natural sources [19]. Screening efforts have identified non-natural ITC leads with promising activity against human disease [20–23]. These results have been aided by a key 2008 study which demonstrated that non-natural glucosinolates are substrates for myrosinase, and that these precursors can

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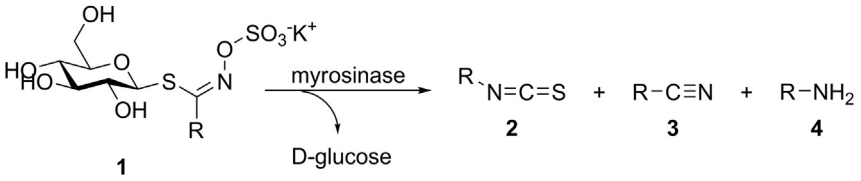

Scheme 1. Myrosinase-catalyzed hydrolysis of glucosinolates and possible resultant organic products.

be quantitatively converted to non-natural ITCs by the enzyme [23]. However, the broader structural diversity of non-natural analogues challenged the traditional ultraviolet–visible (UV–Vis) spectrophotometric methods of monitoring this enzymatic method of unmasking bioactive ITCs [16]. In 2014, a novel HPLC approach for analyzing reaction kinetics was described and validated against the UV–Vis method; key advantages of the HPLC approach were the ability to chromatographically separate and simultaneously track analytes with diverse polarity/solubility profiles, such as **1** and **2** [19]. This methodology has more recently been expanded to LC–MS systems to aid in the evaluation of samples from complex mixtures and has broader implications as an analytical approach [24].

2,2-Diphenylethyl ITC (**8**, Fig. 1) is one example of a non-natural analogue that demonstrates promise as an anticancer agent. Mutations to p53 are estimated to be present in about half of human cancers, and limit the ability of p53 to exert its normal function as a tumor suppressor [25]. Mutant p53 has also been implicated in promoting tumorigenesis through a gain of function mechanism [26]. A 2011 study identified **8** as a potent and selective inhibitor of mutant p53 [22]. This same ITC was also one of the three lead compounds identified in a 2008 evaluation of non-natural ITCs, where the authors noted its consistent, potent antiproliferative effects against an array of human cancer cell lines [23]. Importantly, this study demonstrated that the synthetic non-natural glucosinolates **5** was a substrate for myrosinase and, upon exposure to the enzyme, was converted to its analogous ITC **8** [23,27]. More recent efforts further demonstrate that non-natural ITCs can be generated *in situ* via the reaction of non-natural glucosinolates and myrosinase [19]. Together, this combined work supports the premise that non-natural ITCs and their glucosinolate prodrugs are viable targets for the rational design of novel anticancer agents, a field that has not been extensively explored.

conditions demonstrated both time-dependence of increasing acidity and preferential formation of the nitrile product (**3**) at low pH [18]. Studies describing the pH-dependence of myrosinase catalytic activity on natural substrates have noted a maximum efficiency at pH 6.5 for enzyme isolated from *Sinapis alba* [29] and pH 6.5–8.5 for *Carica papaya* [30]. Similarly, thioglucohydrolases demonstrate an Arrhenius-type increase in the rate of catalysis of natural glucosinolates between 0 and 40 °C, an optimum temperature range between 40 and 55 °C, and complete thermal inactivation by 80 °C [30–32]. If the catalytic mechanism of myrosinase on non-natural glucosinolate substrates parallels the mechanism for natural substrates, it would be expected that similar dependence patterns on pH, temperature, and their effects on the ratio of organic product distribution (e.g. **2**–**4**) would be observed.

The underlying motivation for this study was to test the hypothesis that the myrosinase-catalyzed hydrolysis of non-natural glucosinolate **5** would mirror the parameters observed for natural glucosinolates. The validated HPLC approach is ideally suited for these enzymological studies, as it offers the possibility of simultaneously and independently evaluating glucosinolate and any resultant organic product(s), a feature which has broader methodological considerations and applications [19]. Initial rates (V_0) for glucosinolate hydrolysis and product formation would be obtained from nonlinear reaction progress curves fit with the Lambert $W(x)$ [19,33]; these elucidated V_0 would provide explicit rate information, an advantage over many previous accounts which solely reported relative rates [31,32].

To test this hypothesis, two glucosinolates were selected as representative examples of natural and non-natural analogues. 2,2-Diphenylethyl glucosinolate (**5**) was a non-natural glucosinolate that has not yet been subject to complete kinetic characterization, whose resultant ITC (**8**) has potential utility as an anticancer agent

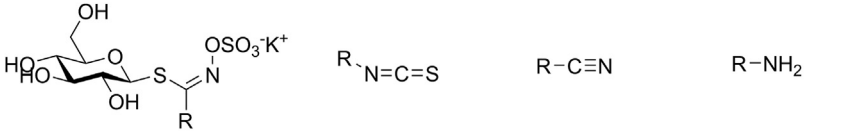

Fig. 1. Glucosinolates, isothiocyanates, nitriles, and amines.

Although non-natural glucosinolates are known substrates for myrosinase, the kinetic studies conducted to date have been focused on the goals of proof-of-principle [23] and validation of the general HPLC methodology [19]. A comprehensive evaluation of myrosinase toward non-natural substrates has not been conducted to demonstrate that these analogues mirror the outcome(s), tolerance, and mechanism exhibited by natural glucosinolates. Since myrosinase does not require any additional cofactors for complete function [28], the major parameters that affect its role as a catalyst are pH and temperature. Kinetic evaluation of **7** in unbuffered [23]. Its inclusion was secondarily intended to provide greater understanding of the enzyme-catalyzed conversion of **5** to **8** toward future cellular studies directed at the use of **5** as a prodrug for **8**. Glucotropaeolin (**6**) was a natural glucosinolate whose Michaelis–Menten and specific activity characterization has been previously described [19]; its inclusion would allow parallel evaluation and direct comparison of both natural and non-natural glucosinolate substrates in pH and temperature experiments. Through the implementation of reaction progress curves tracking each analyte, these studies would provide detailed information on the optimal