Phytochemistry, Vol. 25, No. 5, pp. 1047–1051, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

ISOLATION AND EXAMINATION OF THIOGLUCOSIDE GLUCOHYDROLASE FROM SEEDS OF *BRASSICA NAPUS*

ALEXANDER J. MACLEOD and JOHN T. ROSSITER*

Department of Chemistry, King's College London, Strand, London WC2R 2LS, U.K.

(*Received* 27 *August* 1985)

Key Word Index—*Brassica napus*; Cruciferae; rape; thioglucoside glucohydrolase; myrosinase; glucosinolates; 2-hydroxybut-3-enylglucosinolate.

Abstract—Thioglucoside glucohydrolase (EC 3.2.3.1) was extracted from seeds of *Brassica napus* cv. panter (rape) and purified by a procedure including gel filtration and ion exchange chromatography. Its specific activity using allylglucosinolate as substrate was more than twice that when using 2-hydroxybut-3-enylglucosinolate, even though the latter glucosinolate is the natural substrate in *B. napus*. At least two isoenzymes were detected with pH optima of 4.4 and 8.0. 5-Vinyloxazolidine-2-thione was the sole product of enzymic degradation of 2-hydroxybut-3-enylglucosinolate above pH 5.4 reaching a maximum at pH 8.0, whilst 1-cyano-2-hydroxybut-3-ene was the main product at low pH reaching a maximum at 3.4.

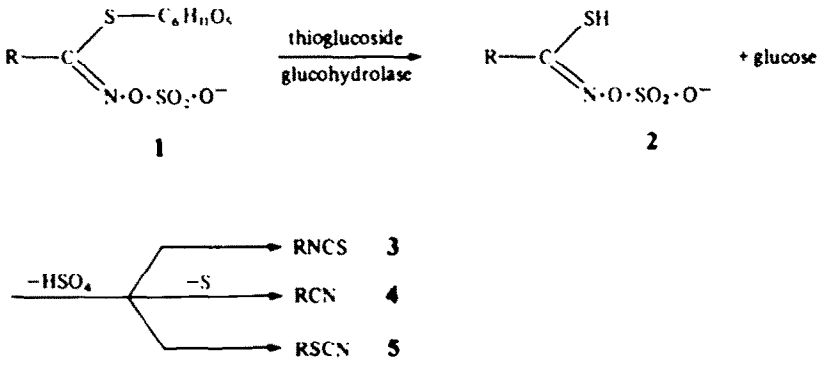
INTRODUCTION

To date, nearly 100 glucosinolates (**1**, Scheme 1) have been identified in nature, and mainly they differ only in the side-chain, R. They degrade enzymically, as shown in Scheme 1, to yield a variety of important products, including isothiocyanate (**3**), nitrile (**4**) and thiocyanate (**5**). Those glucosinolates possessing certain specific structural features in their side-chain are also capable of forming oxazolidinethiones and/or epithiocyanoalkanes.

The enzyme responsible for glucosinolate degradation is thioglucoside glucohydrolase (EC 3.2.3.1), sometimes referred to by the trivial name myrosinase. There has been some controversy concerning the nature of the enzyme, and especially regarding whether there is a dual functionality. Early workers suggested that myrosinase was both a sulphatase and a glucosidase, and Gaines and Goering claimed separation of the two fractions [1, 2]. Subsequently, however, it was shown that myrosinase cleaves only the thioglucosidic link [3, 4], the resultant aglucone (**2**) then spontaneously undergoing a Lossen rearrangement with concerted loss of sulphate to yield isothiocyanate as shown in Scheme 1. Nevertheless, thioglucoside glucohydrolase does occur in multiple forms in plants, but as isoenzymes. Many workers have characterized such isoenzymes in the Cruciferae [5–12]. Björkman and Janson separated three isoenzymes from *Sinapis alba* seed [5]. They referred to the predominant one as myrosinase C, and this was found to be a glycoprotein with M_r of *ca* 151 000, and consisting of two identical polypeptide subunits each with M_r of *ca* 62 000 together with the carbohydrate part [5]. Subsequently, Lönnerdal and Janson separated four isoenzymes in *Brassica napus* [6]. Henderson and McEwen have shown that variations in the proportions of the thioglucosidase isoenzymes have some taxonomic significance [9].

While a great deal of work has been carried out on characterizing thioglucosidase enzymes, relatively little

*Present address: Department of Chemistry, Nottingham University, Nottingham, U.K.



Scheme 1. Enzymic degradation of glucosinolates.

attention has been devoted to their substrate specificity. However, Björkman and Lönnerdal have shown the activity of myrosinase C from *Sinapis alba* to vary with the type of glucosinolate used as substrate [7]. The specific activities of thioglucosidases can also vary, and quite markedly, depending on the source. Thus, the enzyme activity in *Sinapis alba* has been shown to be ten times greater than in *Brassica napus* and *B. campestris*, even though the total thioglucosidase in *S. alba* is fifteen times that in *B. napus* [6].

Elaborate methods have been used to separate and purify thioglucosidases. Originally, Schwimmer simply used acetone fractionation to purify the enzyme from commercial mustard powder (mainly *Brassica juncea*) [13]. Vose extended this type of procedure and separated two thioglucosidases from *Sinapis alba* using polyacrylamide gel electrophoresis [10]. However, possibly the most successful methods to date have been devised by Björkman and co-workers, and have utilized such techniques as gel filtration, ion exchange, isoelectric focusing, gel electrophoresis and ultracentrifugation [5, 6, 14].

Whilst it is the thioglucosidases of the higher plants which have been best studied, some workers, in particular Ohtsuru *et al.* [15–17], have investigated other sources of the enzyme, and found it to be present in the fungus *Aspergillus sydowi* and the bacterium *Enterobacter cloacae* [16]. Recently, thioglucosidase has even been located in certain muscles of the thorax and head of the aphid *Brevicoryne brassicae* [18], and it would be interesting to discover whether this metabolizes glucosinolates in the same way as plant thioglucosidases.

The thioglucosidase isoenzymes have been found to have different properties to one another, particularly with regard to their pH and temperature optima, and in their responses to ascorbate [7–10]. The quoted pH optima for various enzyme preparations from various sources cover a surprisingly wide range from 4.0 to 9.0 [7, 15, 17–23], although the diverse ionic strengths and buffers used by different workers complicate any direct comparison of results. However, the pH optima determined by Björkman and Lönnerdal for the isoenzymes of *Sinapis alba* (4.0–5.8) and of *Brassica napus* (4.5–5.2) [7] are much lower than those obtained by most other workers for thioglucosidases, which are usually between 6.5 and 9.0 [15, 17, 19, 20, 22], although a value of 5.0 has been reported for the thioglucosidases of *Brevicoryne brassicae* [18] and *Lepidium sativum* [23].

The pH at which glucosinolate degradation actually occurs has an important effect on the distribution of the products. Thus, it has been found in many cases that enzymic hydrolysis at acidic pH leads predominantly to the nitrile (**4**, Scheme 1), while hydrolysis at neutral or near-neutral pH leads predominantly to the isothiocyanate (**3**). The mechanism of cyanide formation is believed to be proton-dependent, with the proton acting on the thioglucosidase-produced aglucone (**2**), and there is much evidence in favour of this mechanism [23–27], although Virtanen suggested that the process was enzymic rather than non-enzymic [28].

Most enzymic degradative work using model systems has been carried out on extracted glucosinolates, although some has utilized a pure glucosinolate [29]. Since such extracted substrates would be expected to contain impurities, it is quite possible that these might have interfered and given misleading findings. To obtain most meaningful results, it is therefore obviously necessary to work with pure, synthetic glucosinolates and purified enzymes. Partly for this reason, recently we synthesized 2-hydroxybut-3-enylglucosinolate (progoitrin) as the pure substrate [30]. This paper therefore describes our preparation and examination of purified thioglucoside glucohydrolase as the other component of the basic model system. In particular, we studied the effect of pH on the products of reaction.

RESULTS AND DISCUSSION

2-Hydroxybut-3-enylglucosinolate is the main glucosinolate of *Brassica napus* (rape), and therefore this species (cv. panter) was a reasonable choice as the source of thioglucoside glucohydrolase to study this glucosinolate's enzymic degradation. The procedure for extracting and purifying the enzyme was broadly based on previous methods, and especially those of Björkman [14], but it included a number of modifications and improvements.

After extraction of *B. napus* thioglucosidase with buffer, the extract was dialysed for a few hours to remove small peptides and to allow formation of a precipitate, which was removed by centrifugation. This process was carried out to avoid subsequent protein precipitation on the Sephadex G-50 column. Initial purification was achieved using the Sephadex G-50 gel, which allowed fast separation of the thioglucosidase. At this stage, the elution profile of *B. napus* thioglucosidase indicated at least two isoenzymes. Active fractions were combined, and concentrated by ultracentrifugation. Further purification was then achieved using a column of Whatman DE-52 ion exchanger with a linear salt gradient, and again the elution profile indicated at least two isoenzymes. This step provided a purification factor of 11.3 over the Sephadex G-50 separation, and yielded a final specific activity for the enzyme of 0.92 μmol/min/mg protein, using allylglucosinolate as substrate. In earlier work, a specific activity of only 0.164 μmol/min/mg protein was obtained by us for the thioglucosidase of commercial mustard powder, but extracted and purified using the less sophisticated original procedures of Schwimmer [13].

Using *Sinapis alba* thioglucosidase, Björkman and Janson found allylglucosinolate to be hydrolysed at about twice the rate of 2-hydroxybut-3-enylglucosinolate [5]. It is interesting that we observed exactly the same behaviour with our thioglucosidase preparation. Thus, when 2-hydroxybut-3-enylglucosinolate was used as substrate, the thioglucosidase exhibited a specific activity of only 0.426 μmol/min/mg protein, less than half that obtained when using allylglucosinolate as substrate. In many respects this is surprising, since the former glucosinolate is undoubtedly the natural substrate for the thioglucosidase of *Brassica napus* cv. panter, but since allylglucosinolate was hydrolysed at a faster rate, then the enzyme cannot be specific for 2-hydroxybut-3-enylglucosinolate. In that case, it must be the nature of the glucosinolate side-chain which determines the rate of enzyme hydrolysis. Taking into account previous studies on substrate specificity [5, 7] together with the results reported here, it would seem that those glucosinolates with more hydrophilic side-chains, such as those with a hydroxyl group present, are hydrolysed at a slower rate than those with less hydrophilic side-chains. It may be that non-specific hydrogen-bonding from such glucosinolates to amino or amido groups of the enzyme might hinder the seating of the glucosinolate into the active site of the enzyme.

Considering now the effects of pH on the enzymic degradation of the synthetic 2-hydroxybut-3-enylglucosinolate, initial studies on its reaction with mustard thioglucosidase in an unbuffered medium showed that the pH decreased with time as glucosinolate degradation progressed. Data are shown in Table 1, and the only product was 5-vinyloxazolidine-2-thione (goitrin), since the pH did not fall sufficiently for the proton-dependent formation of 1-cyano-2-hydroxybut-3-ene. This oxazolidinethione is formed in this case by spontaneous cyclization of the unstable isothiocyanate. Similar results have previously been reported for allyl- and 2-phenethyl-glucosinolates with the same enzyme preparation, and an explanation given for this type of behaviour. However, in both these other cases the decrease in pH during the same time period was more severe, so that eventually nitrile was also produced [27]. With 2-hydroxybut-3-enylglucosinolate, it can be seen (Table 1) that both the decrease in pH and the production of oxazolidinethione were beginning to level off after about 40 min as the amount of substrate available became the limiting factor.

The effects of controlled pH on the degradation of synthetic 2-hydroxybut-3-enylglucosinolate by the purified *Brassica napus* thioglucosidase were evaluated in two ways. First, a limited hydrolysis was carried out (for 50 min) to obtain pH optima for this particular system, and then prolonged hydrolysis (for 4 hr) was also performed in order to determine the relative amounts of glucosinolate products at different pH. Results of the first set of experiments are given in Table 2, and the optimum pH (for maximum glucosinolate degradation) for this thioglucosidase is at about 4.4. This agrees very well with the value of 4.5 previously obtained by Björkman and Lönnerdal for myrosinase C, the most abundant isoenzyme, of *Brassica napus* [7]. However, these workers also detected two other isoenzymes with pH optima at 5.2 [7], which we did not. As stated earlier, most other workers have reported higher pH optima for thioglucosidases, usually between about 6.5 and 9.0 [15, 17, 19, 20, 22]. It is interesting, therefore, to note that our results (Table 2) also show a second, although smaller, peak of glucosinolate degradation at a pH of about 8.0, more in line with these other observations. Clearly this must indicate another isoenzyme in our system, but not found in previous studies of *B. napus* thioglucosidase [7].

A seeming peculiarity of the results in Table 2 is that at best only about 32% glucosinolate degradation was obtained in 50 min under these circumstances, whereas in unbuffered medium very nearly 100% degradation occurred within 40 min (Table 1). The reason for this is that a much larger quantity of thioglucosidase (of different origin) was used in the preliminary assay in order to enable direct comparison with similar earlier studies on other glucosinolates [27]. However, when prolonged enzymic hydrolysis (for 4 hr) was carried out, near quantitative degradation was again observed, as can be seen from the results of these experiments, given in Table 3.

Table 3 also shows that oxazolidinethione was not produced at pH levels below *ca* 3.4, whilst nitrile was not formed at pH values greater than *ca* 5.4. This latter limit is further extended beyond that found in Table 2, i.e. pH 4.7, presumably due to the longer period of the hydrolysis allowing for trace amounts of nitrile to increase beyond

Table 1. Enzymic* degradation of 2-hydroxybut-3-enylglucosinolate (1.35 μmol) in an unbuffered medium

Time, min	5-Vinyloxazolidine-2-thione, μmol	pH
0	—	6.94
4	—	6.68
8	trace	6.47
12	trace	6.33
16	0.14	6.25
22	0.62	6.10
30	1.11	6.03
38	1.33	6.03

*Using mustard thioglucosidase.

Table 2. Enzymic* degradation of 2-hydroxybut-3-enylglucosinolate (0.5 μmol for 50 min) at controlled pH

pH	1-Cyano-2-hydroxybut-3-ene, μmol	5-Vinyloxazolidine-2-thione, μmol	Total products, μmol
2.6	0.073	—	0.073
3.4	0.079	0.017	0.096
4.1	0.042	0.108	0.150
4.4	0.034	0.126	0.160
4.7	0.022	0.123	0.145
5.4	—	0.120	0.120
6.4	—	0.077	0.077
7.0	—	0.075	0.075
7.7	—	0.085	0.085
8.0	—	0.092	0.092
8.3	—	0.082	0.082
8.8	—	0.065	0.065
9.7	—	0.065	0.065

*Using purified *Brassica napus* thioglucosidase.

Table 3. Enzymic* degradation of 2-hydroxybut-3-enylglucosinolate (0.6 μmol for 4 hr) at controlled pH

pH	1-Cyano-2-hydroxybut-3-ene, μmol (% theoretical)	5-Vinyloxazolidine-2-thione, μmol (% theoretical)	Total products, μmol
2.6	0.164 (27.3)	—	0.164
3.0	0.218 (36.3)	—	0.218
3.4	0.300 (50.0)	0.085 (14.2)	0.385
3.8	0.256 (42.7)	0.249 (41.5)	0.505
4.4	0.115 (19.2)	0.410 (68.3)	0.525
5.0	0.044 (7.3)	0.470 (78.3)	0.514
5.4	0.022 (3.7)	0.490 (81.7)	0.512
5.8	—	0.520 (86.7)	0.520
6.4	—	0.524 (87.3)	0.524
7.0	—	0.530 (88.3)	0.530
8.0	—	0.535 (89.2)	0.535
8.8	—	0.426 (71.0)	0.426
9.7	—	0.360 (60.0)	0.360

*Using purified *Brassica napus* thioglucosidase.

detection limits. The decreasing yields of nitrile below pH 3.4 could be due either to its instability at such low pH levels or to enzyme inhibition. In the same way that nitrile formation did not eventually become quantitative at lower pH, neither did oxazolidinethione at higher pH. It reached a maximum at *ca* 8.0 but then also declined rapidly with more extreme pH. This may also be due to chemical degradation or reaction, but this pH does, of course, coincide exactly with the pH optimum of one of the *Brassica napus* thioglucosidase isoenzymes.

Although the effects of prolonged hydrolysis support current theories on the main proton-dependent mechanism of nitrile formation, it should be emphasized that in natural systems it has been observed on a number of occasions that nitrile can be the dominant product of glucosinolate degradation at neutral, or even basic pH [23, 31–34]. Attention has previously been drawn to this difference between the model system and natural autolysis [27], and clearly in the natural system some extra feature must be present which directs glucosinolate degradation to nitrile under these conditions. This could be as simple as the presence of Fe^{2+} [26, 35], but further proper model system experiments are necessary to evaluate the possibility.

EXPERIMENTAL

Synthesis of substrate and standards. 2-Hydroxybut-3-enylglucosinolate was prepared by a multi-stage synthesis from propenal and α-D-glucose [30]. 5-Vinyloxazolidine-2-thione was prepared from 1,2-epoxybut-3-ene in three stages by the method of ref. [36]. 1-Cyano-2-hydroxybut-3-ene was prepared in two steps from buta-1,3-diene and calcium hypochlorite using standard procedures [37].

Separation and purification of thioglucoside glucohydrolase (EC 3.2.3.1). Ground seeds of *Brassica napus* cv. panter (15 g) were defatted with hexane (10 × 100 ml) and the glucosinolates removed by extraction with ice-cold 70% Me_2CO. After filtration the seed powder was extracted with NaOAc buffer (0.01 M, pH 5.9, 50 ml) for 10 min at 4°. The extract was then centrifuged at 13000 *g* for 15 min at 4°, and the supernatant decanted and dialysed at 4° for 2 hr against 0.01 M NaOAc buffer. The extract was then centrifuged again at 13000 *g* for 15 min before applying it to a 30 × 4.4 cm Wright column packed with Sephadex G-50. Proteins were eluted with NaOAc buffer (0.01 M, pH 5.9) at a flow rate of 66 ml/hr. The elution of protein was followed by measuring the *A* at 280 nm of 15 ml fractions. Thioglucosidase activity of each fraction was assessed by adding 1 ml to 1 mg of pure allylglucosinolate, and incubating the mixture at 40° for 1 hr. Products were extracted with CH_2Cl_2 (2 × 5 ml), the extracts dried (Na_2SO_4) and concd under vacuum at room temp. The residue was taken up in 0.5 ml CH_2Cl_2 and the allyl isothiocyanate present determined by GC. Thioglucosidase activity was represented by the peak area due to allyl isothiocyanate for each fraction. Fractions with thioglucosidase activity were then combined and concd to 20 ml using an Amicon filtration apparatus (*M*, cut-off, 15000) at 30 psi. The concd extract was then applied to a 10 × 1.5 cm Wright column packed with Whatman DE-52 ion exchange resin (equilibrated overnight with 0.01 M NaOAc buffer, pH 5.9). Protein was eluted with a linear salt gradient (0–0.2 M NaCl, 0.01 M NaOAc, pH 5.9) at a flow rate of 30 ml/hr. Fractions (10 ml) were collected and assayed for protein and thioglucosidase activity as before. Fractions with thioglucosidase activity were combined and concd as before.

Protein determinations, for assay of specific activities, were achieved by the Folin Ciocalteau method with a calibration curve obtained using bovine serum albumin.

Enzymic degradation of 2-hydroxybut-3-enylglucosinolate in an unbuffered medium. 2-Hydroxybut-3-enylglucosinolate (1.45 μmol) in 1 ml of deionized H_2O was mixed with 10 mg of mustard thioglucosidase prepared by the method of ref. [13]. The mixture was incubated at 40°, and the pH measured at certain time intervals. At these same times the concn of 5-vinyloxazolidine-2-thione was determined in the mixture by extraction with CH_2Cl_2 (600 μl) followed by FID-GC using a 1.5 m × 5 mm i.d. glass column packed with 5% Apiezon L coated on Celite, column temp. 130°, injection point temp. 150°, detector temp. 250°, N_2 flow rate 30 ml/min. Quantification was by comparison with standards of synthetic oxazolidinethione chromatographed under the same conditions.

Enzymic degradation of 2-hydroxybut-3-enylglucosinolate at controlled pH. Buffers from pH 2.8 to 8.0 were prepared from 0.2 M Na_2HPO_4 and 0.1 M citric acid; buffers above pH 8 were prepared from 8.5% NaCl, 0.5 M NaOAc, 0.143 M Na barbitone and 0.1 M HCl. 2-Hydroxybut-3-enylglucosinolate (0.5 or 0.6 μmol) in 50 μl of appropriate buffer soln was mixed with 100 μl of *Brassica napus* thioglucosidase preparation and the mixture incubated at 40° for 50 min or 4 hr. Products were extracted with CH_2Cl_2 (600 μl) and 5-vinyloxazolidine-2-thione assayed as described above. 1-Cyano-2-hydroxybut-3-ene was also determined in the extracts by FID-GC, but using a 1.5 m × 4 mm i.d. glass column packed with 10% neopentyl glycol succinate (NPGS) coated on Celite, column temp. 150°, injection point temp. 150°, detector temp. 250°, N_2 flow rate 30 ml/min. Quantification was again accomplished by injecting known quantities of the synthetic standard under the same GC conditions.

Acknowledgements—We thank the Agricultural Research Council for support and Karlshamns AB, Karlshamn, Sweden for provision of authenticated seeds of *Brassica napus*.

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