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EFFECTS OF A *LEPIDIUM SATIVUM* ENZYME PREPARATION ON THE DEGRADATION OF GLUCOSINOLATES

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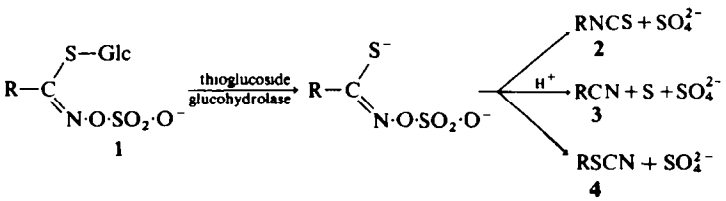
Key Word Index—*Lepidium sativum*; Cruciferae; glucosinolates; thioglucoside glucohydrolase; isothiocyanates; thiocyanates.

Abstract—The effects of a crude enzyme extract prepared from *Lepidium sativum* seeds, on the degradation of three pure glucosinolates (allyl-, benzyl- and 2-phenethyl-) were investigated in the presence of the known enzyme co-factor, ascorbic acid. Isothiocyanates and nitriles were obtained but no thiocyanates. For maximum isothiocyanate formation there was an optimum concentration of ascorbic acid which varied directly with the concentration of substrate but was independent of the particular glucosinolate. Formation of isothiocyanate from any glucosinolate was linear with time but enzymic production of 2-phenethyl isothiocyanate was activated by ascorbic acid to a greater extent than for the other two glucosinolates studied. Isothiocyanate was still the major product even at low pH although the thioglucosidase was only weakly active. Nitrile formation was always erratic in the presence of ascorbic acid. In the absence of ascorbic acid thioglucosidase was still active although to a much lesser extent, but in these circumstances benzyl thiocyanate was an additional product. There is thus a thiocyanate-forming factor in the extract of *L. sativum* seeds which is inactivated in the presence of ascorbic acid. This factor did not cause the formation of thiocyanate from 2-phenethylglucosinolate.

INTRODUCTION

Glucosinolates (**1**) are thioglucosides found characteristically in the Cruciferae. They undergo enzymic degradation as shown in Scheme 1 to give two main types of product, isothiocyanate (**2**) and nitrile (**3**), although thiocyanate (**4**) may also be formed. Certain other types of product are also possible from particular glucosinolates (epithiocyanoalkanes and oxazolidinethiones) and limited evidence has been gathered that alchols, aldehydes, thiols and methylthio compounds can also be formed, possibly as secondary products [1–3]. More than 70 glucosinolates are known to occur in nature, differing almost exclusively in the substituent R. It is, however, becoming increasingly obvious that the mechanism of enzymic degradation is not the same for all these glucosinolates and that current theories, as broadly outlined in Scheme 1, are inadequate to explain many recent observations. A few selected examples from some of our studies serve to illustrate these points.

First, there must be a major difference in the mechanism in that some Cruciferae are intrinsically isothiocyanate-producing whilst others naturally produce predominantly nitriles [4]. Furthermore, nitriles are sometimes formed in large amounts at pH levels greater than 7 [1], a reaction which should not occur according to the protonation mechanism indicated in Scheme 1. Indeed, the overall influence of pH on the mechanisms is not as straightforward as has been previously assumed [1]. The addition of a foreign enzyme to the plant system during analysis (a common approach presumably to ensure total substrate decomposition) can alter drastically the relative amounts of glucosinolate products [5]. Thus, the method of analysis (particularly sample preparation) which is adopted can affect the ratio of products [1,6] and temperature may be another important consideration [1,4]. The pathways of degradation also vary depending on the age and condition of the plant system [6–8]. Finally, the formation of the



Scheme 1. Enzymic degradation of glucosinolates.

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related alcohols, aldehydes, thiols, etc., as already mentioned, is not explained by current theories [1].

The extent and effects of the influence of ascorbic acid as an enzyme co-factor are also not clear [9–16]. Much of the confusion and oversimplification of glucosinolate degradation is possibly due to the original mechanisms having been deduced almost entirely from the limited data of *in vivo* experiments, one problem being the unknown effects on degradation caused by various amounts of numerous naturally-occurring co-extractives, such as ascorbic acid. In comparison relatively little, sound *in vitro* (model system) studies have been undertaken in support, although in 1965 Miller reported an excellent, albeit limited, survey of the decomposition of allylglucosinolate aglucone [17]. To resolve anomalies and to correct present misconceptions, the mechanisms of glucosinolate degradation must also be investigated in more detail in model systems, and the results compared with the wealth of data relating solely to natural systems. This paper describes some very preliminary work in this context using fairly simplistic model systems. Some results must necessarily be tentative at this stage since although pure glucosinolate substrates were employed, the enzyme preparation was relatively crude and could have contained some unknown interfering co-extractives. Work is presently in progress to purify this enzyme preparation.

Much of our previous *in vivo* studies have related to the glucosinolates of various types of cress, mainly *Lepidium sativum* (garden cress) [1,6] and *Nasturtium officinale* (watercress) [4,18], so an *L. sativum* enzyme preparation was employed in this project. The major glucosinolate of *L. sativum* is the benzyl compound, together with much lesser amounts of allyl- and traces of 2-phenethylglucosinolates [1,6]. The latter is also the major glucosinolate of *N. officinale* [4,18]. The overall objective of the present work was thus to investigate the effects of an *L. sativum* enzyme preparation on the degradation of these three glucosinolates. For this purpose it was necessary to obtain relatively large amounts of the pure substrates and although both allyl- and benzylglucosinolates are commercially available, the third is not. 2-Phenethylglucosinolate was therefore synthesized in 5 g quantity [19] by slight modification of the small-scale procedure devised by Benn [20].

One of the most puzzling aspects of glucosinolate degradation is that of the 70 known glucosinolates, only three have been shown to be capable of forming a thiocyanate, namely allyl-, 4-(methylthio)butyl- and benzyl-glucosinolates. One explanation for this is that a specific enzyme may be reponsible for this reaction, but many attempts to detect or isolate it have failed [21,22]. Another suggestion is that only glucosinolates possessing a specific common structural property, that is, the ability to form a particularly stable carbonium ion R^+ (see Scheme 1), are able to form thiocyanates via an ion pair mechanism. Considering the three glucosinolates which are the only ones proven to be capable of this reaction together with the bidentate nature of the SCN/NCS anion, this is a most attractive theory, but attempts to substantiate it have not been successful [23,24]. An additional complication, often ignored, is that apparently some plants which contain one or other of these three glucosinolates do not, in fact, yield thiocyanates (e.g. benzylglucosinolate of *Tropaeolum majus* [21, 22, 25], however, *L. sativum* seeds do produce benzyl thiocyanate from the endogeneous benzylglucosinolate [1,6]. Thus an important part of the present work was a further attempt to obtain an active extract from *L. sativum* seeds capable of converting synthetic benzylglucosinolate into thiocyanate and to test whether this extract had any action on synthetic 2-phenethylglucosinolate, which previously has not been shown to produce thiocyanate [5,18].

RESULTS AND DISCUSSION

An enzyme preparation was obtained from *L. sativum* seeds using Schwimmer's standard method developed for mustard [9], although it is claimed that superior methods have now been developed for thioglucosidases [12]. Preliminary assessment of this preparation by ultraviolet spectroscopy showed it to have no noticeable activity on allylglucosinolate until a few drops of l-ascorbic acid solution were added. Products formed, as determined by gas chromatography (GC), were allyl cyanide and allyl isothiocyanate, but no thiocyanate. It is very well known that thioglucoside glucohydrolases are activated by ascorbic acid and have been widely studied in this respect, e.g. [9–16], so this behaviour was no surprise and it was clear that all experiments should be carried out with the incorporation of ascorbate. Most previous work has studied thioglucosidase activation by ascorbic acid on the basis of the extent of substrate reaction, and ignored the nature or variation of the products of reaction. The latter clearly constituted the more important aspect of the present study.

It was found that different concentrations of ascorbic acid activated the *L. sativum* enzyme preparation to different extents, measured as the relative amounts of isothiocyanate produced at a set time from a certain concentration of glucosinolate. Summaries of some data obtained are given in Table 1; the complete figures have been published previously [26]. For each glucosinolate concentration there was an optimum ascorbic acid concentration and both weaker and stronger levels decreased isothiocyanate production. With weaker concentrations the activity decreased more rapidly. Plotting the full data (summarized in Table 1) gave a more accurate assessment of the optimum ascorbic acid concentration, which varied with the concentration of substrate. Thus for the two concentrations of allylglucosinolate studied in detail, 0.68×10^{-2} and 1.13×10^{-2} M, maximum activation of *L. sativum* enzyme preparation was effected at ascorbic acid concentrations of 2.8×10^{-3} and 4.6×10^{-3} M, respectively, the same concentration of enzyme preparation being used in all experiments. The ratio of these two pairs of values is virtually identical (1:1.66), suggesting a direct relationship between the concentration of ascorbic acid necessary for maximum enzyme activation and optimum allyl isothiocyanate formation (i.e. optimum substrate decomposition by this route). This does support the idea of a molecular relationship between ascorbic acid and glucosinolate during enzymic degradation to isothiocyanate. This deduction is based on only two sets of results but it is supported by the figures for the other two glucosinolates, where the optimum ascorbic acid concentration for activation is similar for equivalent concentrations of glucosinolate, and in the correct ratio, i.e. 5.1×10^{-3} M for 1.24×10^{-2} M benzylglucosinolate

Table 1. Effect of various concentrations of l-ascorbic acid as co-factor on the activity of *L. sativum* enzyme preparation on some different glucosinolates

l-Ascorbic acid conc (M)	Amount of isothiocyanate produced (mM) from glucosinolate after 60 mins			
	Allyl		Benzyl	2-Phenethyl
	0.68×10^{-2} M	1.13×10^{-2} M	1.24×10^{-2} M	1.10×10^{-2} M
5.0×10^{-2}	0.8	0.8	2.3	2.5
2.5×10^{-2}	2.2	1.7	2.8	5.1
5.0×10^{-3}	2.5	3.8	4.3	7.8
2.5×10^{-3}	5.2	3.2	3.1	7.0
5.0×10^{-4}	3.6	2.2	2.7	4.7
2.5×10^{-4}	0.6	0.9	2.0	3.6

Table 2. Formation (mM) of isothiocyanates and nitriles from glucosinolates by the action of an *L. sativum* enzyme preparation in the presence of the determined optimum concentration of l-ascorbic acid co-factor

Time (min)	Allyl				Benzyl		2-Phenethyl	
	0.68×10^{-2} M (ascorbic acid 2.8×10^{-3} M)		1.13×10^{-2} M (ascorbic acid 4.6×10^{-3} M)		1.24×10^{-2} M (ascorbic acid 5.1×10^{-3} M)		1.10×10^{-2} M (ascorbic acid 4.5×10^{-3} M)	
	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile
5	0.4	tr*	0.5	tr	0.4	tr	0.8	0.4
20	1.7	0.2	1.4	tr	1.4	0.2	3.2	0.4
35	3.3	0.7	2.6	0.3	2.6	0.4	4.7	1.5
55	4.8	tr	3.6	0.2	4.4	0.7	8.0	tr
70	6.0	0.4	4.5	tr	5.3	0.4	8.9	tr
90	6.5	tr	5.5	tr	6.3	0.2	10.4	tr

*tr = trace.

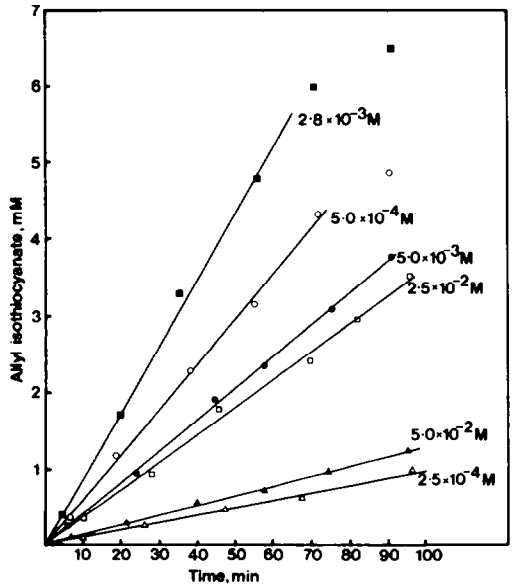

Fig. 1. Amounts of allyl isothiocyanate produced by the action of a *Lepidium sativum* enzyme preparation on allylglucosinolate (0.68×10^{-2} M) at various concentrations of ascorbic acid co-factor (▲ 5.0×10^{-2} M, □ 2.5×10^{-2} M), ● 5.0×10^{-3} M, ■ 2.8×10^{-3} M, ○ 5.0×10^{-4} M, △ 2.5×10^{-4} M).

and 4.5×10^{-3} M for 1.10×10^{-2} M 2-phenethylglucosinolate (compare also the two allylglucosinolate figures). It would appear, therefore, that ascorbic acid activation in this manner is independent of the particular glucosinolate substrate.

In all instances ascorbate-activated enzymic degradation of glucosinolate to isothiocyanate was linear with time up to at least 60 min and often to *ca* 100 min (when in some cases most of the substrate had been consumed and a plateau developed). Fig. 1 illustrates this for one set of data (0.68×10^{-2} M allylglucosinolate) but similar straight line graphs were obtained in all other cases [26]. Table 2 gives some detailed values for isothiocyanate formation, at ascorbate-activated maximum, from the four concentrations of glucosinolates studied. Also quoted in this table are the figures for nitrile production which are somewhat erratic, as was the case with all the other experiments in this series (i.e. at different concentrations of ascorbic acid). It follows, therefore, that the mechanism of enzymic formation of nitriles must be basically different from that for isothiocyanates, and certainly it is independent of ascorbic acid as an enzyme co-factor. This may partly explain some of the observed anomalies of glucosinolate degradation in natural systems [4], but more detailed correlation is necessary.

Although the optimum concentration of ascorbic acid for *L. sativum* enzyme activation was constant for similar

Table 3. Extent of activation of *L. sativum* enzyme preparation by the same concentration of l-ascorbic acid co-factor (4.5×10^{-3} M) for the degradation of the same concentration of different glucosinolates (1.10×10^{-2} M)

Time (min)	Amount of isothiocyanate produced (mM) from glucosinolate		
	Allyl	Benzyl	2-Phenethyl
8	0.6	0.7	1.1
23	1.5	1.6	3.5
38	2.7	2.7	5.2
60	3.9	4.3	8.3

concentrations of different glucosinolates, the extent of activation varied. Table 3 compares appropriate data for equal concentrations of glucosinolates at the same level of ascorbic acid. These data are selected arbitrarily; the same effect was shown at other concentrations. The rate of enzymic formation of isothiocyanate is about the same for allyl- and benzyl-glucosinolates but is much faster for 2-phenethylglucosinolate. In addition, the amount of product formed by the latter within any set time period is much greater (about double) than for the other two glucosinolates. It is difficult to explain this result since the main substrate for an *L. sativum* thioglucosidase was expected to be benzylglucosinolate [1, 6]. However, Bjorkman and Lonnerdal have observed similar variations in the extent of ascorbate activation using *Brassica napus* enzymes, and they suggested that enzymes from different sources are not specifically adapted to a particular set of glucosinolates [14]. Nevertheless, this does not explain why one glucosinolate should be activated to a greater extent than another but, again, this could account for some of the observed natural variation in glucosinolate behaviour. It is possible that the differing levels and availability of ascorbic acid in different plants, different parts of plants, plants of different ages and history, etc. contribute to natural inherent variations in isothiocyanate formation, but without necessarily influencing nitrile formation.

One of the other uncertainties is the effect of pH on glucosinolate degradation. All previous experiments were carried out at pH 6.75 but Table 4 gives data for allylglucosinolate (0.68×10^{-2} M) degradation by ascorbate-activated (2.8×10^{-3} M) *L. sativum* enzyme over a narrow range of acid pH levels. At pH 2.85 the enzyme was almost inactive but between pH 3.15 and 3.75 small amounts of both isothiocyanate and nitrile were formed. The enzyme was less active at these relatively high acidities than at a pH near neutrality (compare data in Table 2) and its activity appeared to diminish after about 1 hr. The surprising feature of the results in Table 4 is that, without exception, the major product of degradation at any time was the isothiocyanate, when on all present theories it should have been the nitrile via the protonation mechanism (see Scheme 1). The influence of pH on enzymic glucosinolate decomposition is clearly not as simple as presently supposed (see also ref. [1]).

A major objective of this project was to obtain an extract of *L. sativum* seeds capable of generating thiocyanate from benzylglucosinolate. However, in none of the experiments so far discussed was any detectable amount of thiocyanate formed. No peak was produced on GC at the known relative retention time of the thiocyanate and since no other component eluted at this point the sensitivity of the analysis was high. However, it was observed that although the *L. sativum* enzyme preparation appeared inactive in the absence of ascorbic acid, when assessed by ultraviolet spectroscopy, small amounts of degradation products were observed on high sensitivity GC analysis. Careful examination of these products by comparison of their relative retention times with those of authentic standards suggested that benzylglucosinolate gave benzyl thiocyanate. This particular sample was carefully concentrated *in vacuo* and analysed by high sensitivity GC/MS. Although the mass spectra of benzyl isothiocyanate and thiocyanate are similar [6], this was irrelevant in that two clearly resolved peaks were obtained on GC/MS of the sample, both of which showed this same basic spectrum, so both isothiocyanate and thiocyanate were present. Retrospective single ion monitoring using the data system following GC/MS confirmed beyond doubt the presence of the two resolved peaks with virtually identical spectra.

Since the *L. sativum* enzyme preparation showed only weak activity in the absence of ascorbic acid, relatively low yields of all products were obtained on degradation of

Table 4. Effect of pH on the products of degradation of allylglucosinolate (0.68×10^{-2} M) by the action of *L. sativum* enzyme preparation and ascorbic acid (2.8×10^{-3} M) co-factor

Time (min)	Amounts of products formed (mM) at various pH							
	2.85		3.15		3.45		3.75	
	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile	Isothiocyanate	Nitrile
10	tr*	tr	tr	tr	0.5	0.4	0.7	tr
20	tr	tr	0.5	tr	0.7	0.4	0.9	tr
30	tr	tr	0.5	tr	1.2	0.5	1.1	tr
40	tr	tr	0.6	tr	1.5	0.5	1.1	tr
50	tr	tr	0.7	0.3	1.6	0.5	1.3	0.2
60	tr	tr	0.9	0.4	1.8	0.5	1.3	0.2
90	tr	tr	1.0	0.5	1.8	0.7	1.4	0.2
120	tr	tr	1.0	0.7	1.8	0.8	1.2	0.4

* tr = trace.

Table 5. Products of degradation of benzylglucosinolate (2.8×10^{-2} M) and 2-phenethylglucosinolate (2.5×10^{-2} M) obtained by the action of *L. sativum* enzyme preparation in the absence of ascorbic acid

	Amounts of products formed (mM)					
	Benzyl			2-Phenethyl		
Time (min)	Isothio-cyanate	Nitrile	Thio-cyanate	Isothio-cyanate	Nitrile	Thio-cyanate
10	0.2	tr*	tr	0.2	tr	—
30	0.2	tr	tr	0.3	0.2	—
50	0.3	0.1	0.1	0.4	0.2	—
140	1.2	0.1	0.4	0.7	0.4	—
18 hr	2.0	0.4		1.4	0.9	—

* tr = trace.

benzylglucosinolate. Some data are given in Table 5, and can be compared with those in Table 2, noting the higher concentrations of glucosinolates used in these ascorbate-free experiments. The extent of enzyme activation for the production of isothiocyanate by ascorbic acid is clearly seen in comparing these tables. However, there is no doubt that the thioglucosidase does still function in the absence of ascorbic acid. The nitrile production in this series was no longer erratic, thus supporting the proposal that ascorbic acid is not necessarily involved in promoting enzymic degradation to nitrile. As benzyl thiocyanate production appeared also to increase with time, samples were allowed to incubate overnight. This proved counterproductive since thiocyanate was no longer detected, and it is possible that during the longer incubation it isomerised to the thermodynamically more stable isothiocyanate. However, production of benzyl thiocyanate in the model system from synthetic benzylglucosinolate by the action of an extract of *L. sativum* seeds is the first positive indication of the existence of a thiocyanate-forming enzyme or 'biological' factor. Although ascorbic acid was an important co-factor for the thioglucosidase in the efficient formation of isothiocyanate, its presence totally prevented glucosinolate degradation to thiocyanate. Presumably the ascorbic acid blocked the active site of the thiocyanate-producing factor. Previous efforts to identify or isolate such a factor may well have failed simply due to the use of ascorbate buffers—a very common procedure—but in addition it has been widely assumed that ascorbic acid is essential for any enzymic degradation of glucosinolates. It is intriguing that one compound should seem to have opposing effects on a substrate degradation, but this may be a method of biological control over the relative amounts of the two important secondary metabolites produced. It is interesting, for example, that benzyl thiocyanate is obtained from the seeds but not from the leaves of *L. sativum* [6].

Having obtained the thiocyanate-forming factor in crude form, its activity on a supposedly non-thiocyanate forming glucosinolate was investigated. From the data given in Table 5 it can be seen that no 2-phenethyl thiocyanate was detected although in other respects the enzyme preparation functioned as with benzylglucosinolate. Very careful and detailed examination was undertaken of samples for the presence of 2-phenethyl thiocyanate, and again the sensitivity and selectivity of the searches were high. None of this thiocyanate was detected during many replicate analyses.

In conclusion, it would appear, therefore, that a combined theory for the mechanism of thiocyanate production by glucosinolates is most feasible in which an enzyme or some other biological factor is necessary, but which functions only on glucosinolates which possess particular structural properties. Attempts are now in progress to purify and characterize the thiocyanate-producing factor of *L. sativum* seeds for further study.

EXPERIMENTAL

Enzyme preparation. Authenticated *L. sativum* seeds (100 g) were crushed to a fine powder in a coffee grinder. The remaining steps were carried out in a cold room at ~0°. The powder was defatted × 10 with 300 ml portions of cold, dry Me_2CO and dried *in vacuo*. *Ca* 800 ml of cold distilled H_2O were added and the mixture stirred for 2 hr. After centrifugation (30 min) the supernatant was discarded and a further 200 ml of cold distilled H_2O added and the mixture stirred for a further 2 hr. Further centrifugation gave *ca* 200 ml of supernatant which was diluted with 100 ml cold Me_2CO and the ppt. removed by centrifugation. Cold Me_2CO (400 ml) was carefully added with stirring to the clear supernatant liquid. Centrifugation gave a white starchy mass which was suspended in *ca* 150 ml distilled H_2O, dialysed in H_2O for 40 hr and lyophilized to a white powder, yield 0.5 g.

Assessment of enzyme activity by UV spectroscopy. An aq. soln of allylglucosinolate (0.68×10^{-2} M) showed UV λ_{max} at 227 nm. Addition of an aq. suspension of enzyme preparation (25 mg in 1.5 ml) did not cause any change in glucosinolate absorption after 80 min, but incorporation of aq. l-ascorbic acid soln (2.5×10^{-2} M) brought about a rapid decrease in absorption.

Determination of products of enzymic degradation of glucosinolates by GC. Ascorbic acid buffers of the required concn and pH were prepared by mixing various amts of an aq. soln of 0.1 M l-ascorbic acid with the appropriate vol. of an 0.2 M aq. soln of $Na_2H\ PO_4$. It was sometimes necessary to adjust the pH finally by the additionof a small amt of NaOH. The reaction mixture was prepared by mixing 0.25 ml of the ascorbic acid soln with 0.1 ml of an aq. suspension of enzyme preparation (25 mg in 1.5 ml) and 0.1 ml of an aq. soln of the glucosinolate of the appropriate concn. An aliquot (1 μl) of this mixture was directly examined by GC at various time intervals. In some analyses ascorbic acid was not incorporated, but otherwise the reaction mixture was prepared in the same manner. GC analysis was accomplished using a Pye-Unicam model 104 equipped with

heated FID. For allylglucosinolate products analysis a 1.5 m × 4 mm i.d. glass column was used packed with 10% PEG 20M coated on 100–120 BSS mesh acid-washed Diatomite C. It was operated isothermally at 100° with a N_2 carrier gas flow rate of 60 ml/min. For products of the other glucosinolates an 0.3 m column containing 3% PEG 20M was used, at a temp. of 110° and a flow rate of 60 ml/min. Identification of degradation products was made by comparison of GC RR_t with authentic standards. Quantification of results was accomplished by manual peak area measurements and comparison with data obtained on GC of known amounts of pure standards of all glucosinolate products.

Identification of products of enzymic degradation of glucosinolates by GC/MS. Identification of products was confirmed by GC/MS, particularly in the case of benzyl thiocyanate using an AEI model MS30 equipped with a DS 50 data processing system. A Pye-Unicam 104 is linked on-line via a silicone membrane separator. The same GC conditions were employed as above (but using He as carrier gas). Significant MS parameters were: ionization potential, 70 eV; ionization current, 300 μA; source temp., 230°; resolution, 1500; scan speed, 3 sec/decade (repetitive throughout run). The background subtraction facility and in particular the retrospective single ion monitoring facility of the data system were extensively employed in this project.

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