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BENZYLGLUCOSINOLATE DEGRADATION IN *LEPIDIUM SATIVUM*: EFFECTS OF PLANT AGE AND TIME OF AUTOLYSIS

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Key Word Index—*Lepidium sativum*; Cruciferae; garden cress; glucosinolates; benzyl thiocyanate; autolysis.

Abstract—Benzylglucosinolate degradation products were analysed in extracts of the seedlings of *Lepidium sativum* ('curled cress' and 'plain cress'). Benzyl thiocyanate was positively identified in extracts of both types of cress, but it could not be detected after the onset of development of the true leaves. The relative percentages of benzylglucosinolate degradation products varied appreciably with age of the seedlings and with the length of time the shredded plant material was allowed to autolyse. Both types of cress behaved similarly on autolysis but differences were observed with seedling age.

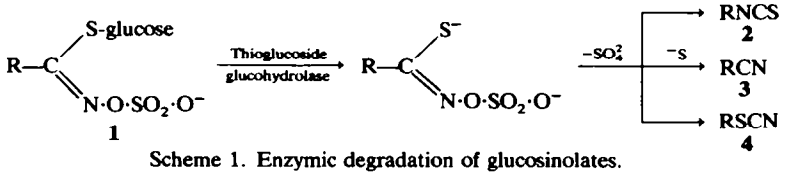
INTRODUCTION

Glucosinolates (**1**) are plant thioglucosides which undergo enzymic decomposition as shown in Scheme 1 to yield two main types of products, isothiocyanates (**2**) and nitriles (**3**). Thiocyanates (**4**) can also be produced occasionally, but of more than 70 known glucosinolates only three appear to be capable of this reaction. These are allyl- (**1**, $R = CH_2{=}CH{-}CH_2{-}$), 4-(methylthio)butyl- (**1**, $R = MeS(CH_2)_4$-) and benzyl- (**1**, $R = C_6H_5CH_2$-) glucosinolates. Many theories have been advanced to explain this odd limitation, but none is entirely convincing. Gmelin and Virtanen first proposed an enzyme-induced rearrangement from the glucosinolate itself [1]. Later, Virtanen and Saarivirta suggested that an isomerase acted on the initially formed isothiocyanate [2]. However, to date, all attempts to locate and isolate such thiocyanate-forming enzymes have failed, and Saarivirta subsequently abandoned the isomerase theory since certain model system experiments were unsuccessful [3]. In contrast, thiocyanate formation has been attributed to the particular properties of certain side chains (R in Scheme 1) which might favour thiocyanate production whilst others would not. In particular, glucosinolates capable of forming stable cations R^+ might then produce thiocyanates via an ion-pair mechanism. Bearing in mind the glucosinolates which do yield thiocyanates and the bidentate nature of the thiocyanate/isothiocyanate ion, this is an attractive theory, but attempts to substantiate it have not been entirely successful [4, 5].

Many of the studies of thiocyanate formation have been based on benzylglucosinolate in various *Lepidium* species. Numerous reports describe the identification of benzyl thiocyanate in extracts of seeds of *Lepidium sativum*, e.g. [1–3, 6–9]. However, it has never been located in extracts of the green leaves of this plant [3, 6, 10, 11], whilst it has been readily identified in extracts of both seeds and leaves of *L. ruderale* [1, 11], *L. virginicum* [11] and also *Coronopus didymus* [9, 11]. The peculiar behaviour of benzylglucosinolate of *L. sativum* in apparently only degrading to form thiocyanate in the seeds and not in the leaves has been seen by some as a basis for a further study of the mechanism of thiocyanate formation. It could be theorized that perhaps a thiocyanate-forming factor (enzyme ?) is present only in the seeds, or that some inhibitor is present only in the leaves. Many other possibilities exist, but obvious experiments could be undertaken in pursuit of these ideas. These were partly the objectives of this project. However, it seemed equally possible that previous analyses of *L. sativum* leaves had missed detecting benzyl thiocyanate, and therefore a careful search specifically for this compound was first undertaken.

RESULTS AND DISCUSSION

Young seedlings grown from authenticated *L. sativum* seeds were analysed for their glucosinolate degradation products, and evidence of the production of benzyl thiocyanate was obtained. However, subsequent repeat analyses of plants from the same crop



Scheme 1. Enzymic degradation of glucosinolates.

Table 1. MS of benzyl isothiocyanate and benzyl thiocyanate

m/e	149	117	116	103	102	101	91	90	89	77	65
Isothiocyanate, % rel. int.	11	3	2	3	1	1	100	3	10	1	9
Thiocyanate, % rel. int.	2	1	1	2	1	1	100	2	9	1	11

were not so convincing, and it was evident that the glucosinolate products varied with the age of the plant. It was also observed that the length of time the shredded plant material was allowed to autolyse at room temperature before analysis also affected the quantitative data. This could be an important variable in this project since insufficient time might not allow sufficient glucosinolate decomposition, whilst prolonged incubation might allow certain secondary reactions, such as isomerizations. Based on these preliminary experiments, a survey was carried out investigating the effects of both the age of the plant and the length of time of autolysis on the nature and ratio of glucosinolate products.

Compounds positively identified in extracts of *L. sativum* leaves by means of GC–MS include the following non-glucosinolate products: toluene, butyl acetate, ethylbenzene, 2-methylbutanol, pentan-1-ol, styrene and *cis*-hex-3-en-1-ol. The glucosinolate products identified are as follows: benzaldehyde, benzyl alcohol, phenylacetonitrile (benzyl cyanide), benzyl isothiocyanate, benzyl thiocyanate and 3-phenylpropionitrile (2-phenethyl cyanide). Although they are included in this list, it has not yet been proved that aldehydes or alcohols are genuine glucosinolate products. Thus two glucosinolates occur in *L. sativum* leaves, benzyl- and 2-phenethyl-glucosinolates, although the latter is present in only trace amounts. From quantitative experiments on 7-day-old seedlings the amount of benzylglucosinolate in *L. sativum* leaves is *ca.* 1.2 mg/g based on the amounts of degradation products obtained.

Benzyl thiocyanate

This was positively identified in small amounts in extracts of very young seedlings. Its MS is very similar to that of the isothiocyanate; summaries of the spectra obtained are given in Table 1 for comparison. The only significant difference between the spectra is the relative intensities of the M^+. This agrees with previously reported differences between MS of thiocyanates and isothiocyanates [12]. Although the spectra are so similar as to render distinction between the two compounds somewhat uncertain, the two GC peaks giving rise to these spectra on GC–MS were well resolved with R_t under the conditions of analysis of 22 and 24 min. These R_t values furthermore agreed exactly with those of authentic standards (the thiocyanate is eluted later from the polar column). In addition, retrospective single ion monitoring (on *m/e* 149 and 91) using the data system after GC–MS confirmed the presence of two resolved peaks with virtually identical spectra. There is thus no doubt that benzyl thiocyanate was present in the samples as well as benzyl isothiocyanate.

Age of plant

Table 2 gives the approximate relative percentages of benzylglucosinolate products formed on autolysis by *L. sativum* seedlings of various ages (in days after sowing). Again it is assumed that benzaldehyde and benzyl alcohol are derived from the glucosinolate.

These data were obtained using a type of *L. sativum* marketed as 'curled cress', and the leaves of the mature plant do become curled. Certain changes in morphology were observed as the seedlings developed. Up to *ca.* 15 days only the cotyledons were present but at *ca.* 16 days two of the true leaves appeared and the outer leaves stopped growing. These new leaves rapidly became serrated and this subsequently caused the curling. Benzyl thiocyanate was only produced by the younger plants and even then only in trace amounts (less than 0.5% total glucosinolate products). It could not be detected in extracts of any seedlings or plants over 16 days in age despite considerable attention to this particular aspect of the analysis. In many respects it is not surprising that the cotyledons yield thiocyanate in the same manner as the seeds of which they form part, but it is intriguing that thiocyanate production seemingly ceases immediately the true leaves appear, although at this time, and for a number of days subsequently (usually at least a further 10 days), cotyledons were still present. This phenomenon was observed and confirmed in a number of trials

Table 2. Effect of age of *Lepidium sativum* seedlings on ratio of benzylglucosinolate degradation products

Age of plant (days)	Percentage of glucosinolate product				
	Benzaldehyde	Benzyl alcohol	Benzyl cyanide	Benzyl isothiocyanate	Benzyl thiocyanate
6	tr	tr	99	tr	tr
8	3	4	92	tr	tr
12	18	13	62	7	tr
17	30	20	32	18	ND
23	36	34	17	13	ND

tr = trace, ND = not detected.

when the time of development of true leaves varied between *ca.* 14 and 17 days. Some glucosinolate products do have growth-promoting abilities and it is tempting to theorize on some similar property in this instance.

The relative amounts of the other products also varied appreciably with the age of the plant, and in most cases approximately linearly, although this could well be fortuitous. Thus, whilst the production of nitrile decreased considerably as the plant aged, the formation of aldehyde and alcohol increased in compensation. Isothiocyanate production also increased but to a lesser extent and it is possible that a maximum was attained. Clearly there is no evidence to suggest why these changes occur, but superficially it might appear possible that as the plant ages perhaps the nitrile can be increasingly converted to aldehyde and alcohol as secondary products. Either that or some mechanism for their formation takes over from the more normal route to the cyanide.

Another type of garden cress is marketed as 'plain cress' and again seeds were authenticated as *L. sativum*. Seedlings of these were grown and a similar, but less detailed, survey carried out on these. Much the same leaf development was observed, but the true leaves were barely serrated and they did not curl. Again the onset of the true leaves coincided almost exactly with the last detection of benzyl thiocyanate as the plant developed. Much more thiocyanate was produced by plain cress and 10-day-old leaves gave *ca.* 3%. Again the relative amounts of the other benzylglucosinolate products varied with age of the seedlings, but the results were different from those for curled cress. Whilst aldehyde and alcohol production again increased with age and thiocyanate decreased, eventually to disappear, with plain cress isothiocyanate was the major component of extracts of young leaves, decreasing from *ca.* 70% at day 8 to *ca.* 20% at day 19. In contrast, nitrile increased over the same period from *ca.* 8 to 25%. This reversal of the curled cress behaviour is inexplicable, and is contrary to the idea of the aldehyde and/or alcohol being simple secondary products directly formed from one of the main primary products. If aldehyde and alcohol are produced by benzylglucosinolate in *L. sativum* seedlings then it is by some route (or routes) independent of the main products which becomes more dominant as the plant ages.

Period of autolysis

Table 3 gives results for the variations in the relative percentages of benzylglucosinolate degradation products dependent on the length of time allowed for autolysis of the shredded plant material. Data are given both for curled cress and plain cress. Much the same trends were observed for all seedlings of whatever age, so representative results have been selected here, in one instance showing detection of thiocyanate and in the other case at an age when thiocyanate production had ceased. Both types of *L. sativum* showed much the same behaviour although that exhibited by curled cress was consistently more extreme. Within this particular time period the proportions obtained of aldehyde, nitrile and isothiocyanate all decreased. In compensation the amount of alcohol formed increased considerably. Thiocyanate also increased appreciably, when produced at all. From these results it would appear that alcohol and aldehyde formation are not linked. It might be that alcohol is formed, with time, from one or other of the main glucosinolate products, but as already indicated, other data are against this. The same would apply to the thiocyanate, and the isomer would seem the obvious precursor, but again some earlier results conflict [3].

EXPERIMENTAL

L. sativum seeds were obtained from Suttons Seeds Ltd., Reading, U.K. ('curled cress', lot No. 6561 and 'plain cress', lot No. 187) and were authenticated by microscopy. They were sown indoors during July 1978 in trays containing John Innes potting compost No. 2, and with occasional watering they germinated rapidly at ambient temp. No specific light regime was adopted but samples were always collected for analysis at the same time of day.

Preparation of extracts. Aerial parts of seedlings (40 g) of a particular age (in days after sowing) were chopped in a Waring blender containing 100 ml of a citrate–Pi buffer (pH 6.75) for 5 min. The mixture was then allowed to autolyse by standing in a beaker at room temp. for 1 hr (or 2 or 3 hr). The plant material was then removed by filtration

Table 3. Effect of time allowed for autolysis on the ratio of benzylglucosinolate degradation products from two types of *Lepidium sativum* seedlings

Time allowed for autolysis (hr)	Percentage of glucosinolate product				
	Benzaldehyde	Benzyl alcohol	Benzyl cyanide	Benzyl isothiocyanate	Benzyl thiocyanate
	'Curled cress' (17 days old)				
1	30	20	32	18	ND
2	23	45	21	11	ND
3	9	73	8	10	ND
	'Plain cress' (10 days old)				
1	6	10	66	15	3
2	3	18	57	18	4
3	4	23	49	16	7

ND = not detected.

through a Buchner funnel using glass wool. This removed most of the residue. Centrifugation for 5 min deposited a further small amount of sediment which was rejected. The supernatant liquid was extracted with CH_2Cl_2(3 × 75 ml). An emulsion formed which had to be separated by centrifugation. The organic layer was collected, dried, and concd carefully *in vacuo* to 1 ml.

GC. Samples were analysed first by routine GC with heated FID. A 1.5 m × 4 mm i.d. glass column was packed with Carbowax 20 M (10%) coated on 100–120 BSS mesh acid-washed Diatomite C. N_2 carrier gas was employed (30 ml/min) and the most successful temp. programme was a 16°/min rise from an initial setting for 5 min of 60° to a final temp. of 200° for the remainder of the run. R_ts were measured using int. standards.

GC–MS. Components were identified using a medium resolution instrument equipped with a data system. A GC was linked to the MS via a heated membrane separator and the same conditions were employed as described above, but with He as carrier. Relevant MS parameters were: ionization potential, 70 eV; ionization current, 300 μA; source temp. 230°; resolution, 1500; scan speed, 3 sec/decade (repetitive throughout run). To ensure positive identification of benzyl thiocyanate in particular, the background subtraction facility and the retrospective single-ion monitoring facility of the data system were extensively employed.

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