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INVESTIGATION OF *BRASSICA OLERACEA* AND *NASTURTIUM OFFICINALE* SEEDS FOR THE PRESENCE OF EPITHIOSPECIFIER PROTEIN

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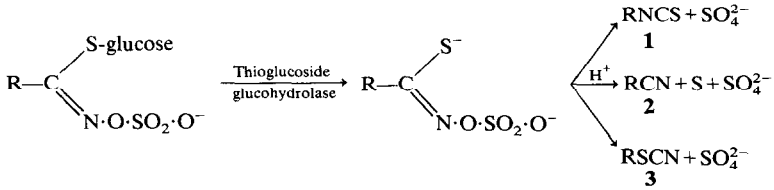
Key Word Index—*Brassica oleracea*; cabbage; *Nasturtium officinale*; watercress; Cruciferae; epithiospecifier protein; cyanoepithioalkane; glucosinolates.

Abstract—Cabbage seeds contain 5 glucosinolates and on autolysis produce, in particular, 1-cyano-2,3-epithiopropane. Watercress seeds contain 4 glucosinolates, but none capable of forming a cyanoepithioalkane. Cabbage exhibited behaviour commensurate with possession of an epithiospecifier protein (ESP) whilst watercress did not show any such activity.

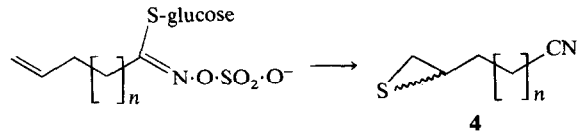
INTRODUCTION

Glucosinolates undergo enzymic decomposition, as shown in Scheme 1, to give two main types of products, isothiocyanates (**1**) and nitriles (**2**). Under certain circumstances some glucosinolates are also capable of forming thiocyanates (**3**). In addition to these there is a further type of product which has been obtained from those glucosinolates possessing terminal unsaturation in the R substituent. These products are cyanoepithioalkanes (**4**) and their formation is shown in Scheme 2, where *n* can be 0 (as in allylglucosinolate, otherwise known as sinigrin) or an integer. In many respects these compounds can be regarded as rearrangment products of the isothiocyanates, and indeed this origin has been suggested [1], but it seems unlikely. However, as yet there is no definite proven mechanism of formation of these compounds from glucosinolates. Bearing in mind the importance and widespread distribution of glucosinolates of the general structure shown in Scheme 2 (e.g. allyl- and but-3-enyl-glucosinolates) then the cyanoepithioalkane products could have much significance. Clearly they warrant more detailed study, and the question can be asked whether they are by-products of glucosinolate decomposition or whether they have some specific function for the plant; none has yet been suggested.

Although the pathway to formation of the products is uncertain, some evidence is available. Tookey found in the seeds of *Crambe abyssinica* a protein which, although it did not itself possess enzyme activity, was capable of acting as a co-factor with the appropriate thioglucoside glucohydrolase to produce cyanoepithioalkane [2]. This he termed epithiospecifier protein (ESP), and using fractional precipitation and gel filtration techniques he was able to substantially separate the ESP in the seeds from the thioglucosidase. In this way it was possible to show with the appropriate glucosinolate substrate that whilst the thioglucosidase enzyme alone produced only isothiocyanate, enzyme together with ESP gave the corresponding cyanoepithioalkane. On its own, ESP had no activity and could not accomplish any glucosinolate decomposition. Such an enzyme co-factor which affects the nature of the products of the reaction is unusual. The only other presently known is



Scheme 1. Enzymic decomposition of glucosinolates.



Scheme 2. Formation of cyanoepithioalkanes.

α-lactalbumin, which is involved as a specifier protein in the biosynthesis of lactose. ESP is worthy of much further study on these grounds alone. Cole recently repeated Tookey's work but on turnip (*Brassica campestris*) and was able to reproduce his findings isolating an ESP from the seeds [3].

The objectives of the work described here were two-fold. First, to investigate cabbage (*Brassica oleracea*) seeds for ESP activity; the main glucosinolate of cabbage is allylglucosinolate which is therefore a potential precursor of a cyanoepithioalkane. Second, to ascertain whether such activity is also present in watercress (*Nasturtium officinale*) seeds, where previous work has shown the absence of suitable substrates, in that only glucosinolates with saturated side chains have been found in this plant [4].

RESULTS AND DISCUSSION

Both cabbage and watercress seeds were allowed to autolyse and were examined in detail for their glucosinolate products. The results for cabbage are given in Table 1 and those for watercress in Table 2. In both tables components from a common glucosinolate precursor are bracketed together. In all instances the compounds were identified by GC–MS. MS of all components have been previously reported, including those of the nitriles of the long chain ω-(methylthio)alkylglucosinolates from watercress seeds [4, 5], but this is the first report of the production of the isothiocyanates by watercress. Thus, their spectra are as follows: 7-(methylthio)heptyl isothiocyanate *m/e* (%) 203 (M^+, 25), 170 (18), 156 (15), 121 (15), 72 (18), 67 (30), 61 (75), 55 (100), 54 (35), 41 (92), 39 (40); 8-(methylthio)octyl isothiocyanate 217 (M^+, 32), 184 (8), 170 (10), 136 (18), 98 (18), 81 (27), 72 (18), 67 (29), 61 (68), 55 (69), 41 (100), 39 (35). These spectra agree with those previously published for these compounds [6]. From the approximate percentage relative abundances of products it is interesting to note that for all glucosinolates from both sources, the isothiocyanate was always the major product and not the corresponding nitrile under these conditions of analysis. No thiocyanates were detected.

For cabbage (Table 1) 5 glucosinolates were positively identified, although pent-5-enonitrile could not be detected. These results compare well with previous work and particularly with the detailed survey of cabbage leaves recently carried out by Buttery *et al.* [7]. A minor difference is that these workers found a very small percentage of phenylacetonitrile (but not the corresponding isothiocyanate) in addition to degradation products from the 5 glucosinolates listed in Table 1. However, suprisingly, they did not detect any allyl cyanide, an omission on which they comment in particular. More significant is the fact that the cyanoepithioalkane from the relatively abundant precursor allylglucosinolate is lacking from the list of compounds identified by Buttery *et al.* [7]. The compound, 1-cyano-2,3-epithiopropane, was however detected in our work, and it was produced by allylglucosinolate in reasonable amounts. On the other hand, no trace could be found of 1-cyano-3,4-epithiobutane which would be expected from but-3-enylglucosinolate also located in cabbage seeds.

Table 1. Glucosinolate degradation products of autolysed cabbage seeds

Component	R_t (min)	Approximate relative percentage abundance
But-3-enonitrile(allyl cyanide)	9.25	7.3
Allyl isothiocyanate	12.2	30.2
1-Cyano-2,3-epithiopropane	16.9	6.1
But-3-enyl isothiocyanate	13.0	3.4
4-(Methylthio) butanonitrile	16.4	16.2
3-(Methylthio)propyl isothiocynate	20.3	21.6
5-(Methylthio)pentanonitrile	18.4	2.5
4-(Methylthio)butyl isothiocyanate	23.3	5.6
3-Phenylpropanonitrile	21.1	1.3
2-Phenethyl isothiocynate	26.9	5.9

Table 2. Glucosinolate degradition products of autolysed watercress seeds

Component	R_t (min)	Approximate relative percentage abundance
Phenylacetonitrile (benzyl cynide)	19.5	tr
3-Phenylpropanonitrile	21.1	8.8
2-Phenethyl isothiocyanate	26.9	90.1
8-(Methylthio)octanonitrile	29.5	tr
7-(Methylthio)heptyl isothiocyanate	40.0	0.7
9-(Methylthio)nonanonitrile	33.2	tr
8-(Methylthio)octyl isothiocyanate	49.3	0.3

tr = trace.

However, since this glucosinolate was present in much lesser amounts it is probable that any epithioalkane produced was below the limit of detection.

Although the MS of allyl isothiocyanate and the isomeric 1-cyano-2,3-epithiopropane are similar there are significant differences such that they can be distinguished. Furthermore, the two components are quite clearly resolved on GLC (the isothiocyanate was additionally confirmed by comparison of R_t with an authentic standard), and retrospective single ion monitoring (on *m/e* 99, M^+) using the data system following GC–MS also clearly showed the two separate components. The spectra of the isomers are given below for comparison:

Allyl isothiocyanate

m/e	99	41	39	72	45	40	38	71	98
% rel. int.	100	98	79	30	10	10	8	3	2

1-Cyano-2,3-epithiopropane

m/e	99	59	72	41	39	54	71	58	57
% rel. int.	100	72	65	35	32	21	18	15	11

Coincidentally the characteristic peak of most isothiocyanates at *m/e* 72 due to α-fission ($CH_2{=}\overset{+}{N}CS$) is also seen for the nitrile, but in this instance it is presumably due to the characteristic nitrile rearrangement fragmentation, $M^+ - HCN$. Since the allyl group is no longer present in the epithiocompound the peaks at *m/e* 41 and 39 are appreciably decreased, but the most obvious and significant difference between the two spectra is the presence of an intense peak at *m/e* 59 in the epithioalkane, absent in the spectrum of the isothiocyanate. This presumably is due to α-fission leaving the fragment ion CH_2——CH.
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S^+

The results for watercress seeds (Table 2) compare very well with the only previously reported detailed analysis of the glucosinolates of watercress (leaves) [4], except that in this instance the isothiocyanates of the long chain ω-(methylthio)alkylglucosinolates were detected as well as the previously determined nitriles [4, 5]. It can be seen that 2-phenethylglucosinolate is by far the most abundant glucosinolate, but significantly no glucosinolate is present with terminal unsaturation in the side chain. Consequently no cyanoepithioalkanes are produced by watercress.

However, the question remains as to whether watercress, in common with all of the few other Cruciferae already studied, possesses ESP activity, despite the absence of an appropriate substrate. This question was resolved as follows.

Two identical samples of defatted cabbage seed meal were prepared and to one was added pure allylglucosinolate; the other was a blank. The samples were allowed to autolyse and then were extracted and analysed as before. The results showed a marked increase in the 1-cyano-2,3-epithiopropane peak on GLC of the sample to which extra allylglucosinolate had been added. This merely proved that this approach is a satisfactory method of assay for ESP activity.

The same experiment was repeated with watercress seeds. One extra peak was obtained on GLC of the sample to which allylglucosinolate had been added. This was proven by R_t and GC–MS to be due to allyl isothiocyanate. This was not unexpected since although it is not a normal product from watercress, it was obviously formed by the action of the cress thioglucosidase on the added glucosinolate. However, more important is the fact that no 1-cyano-2,3-epithiopropane was produced, although clearly particular efforts were made to detect it, including single ion monitoring (e.g. on *m/e* 99) by GC–MS. Comparison of Tables 1 and 2 shows that no major GLC peaks are produced in watercress samples at the R_t of the epithioalkane, so the sensitivity of the search was high. Considering the large amounts produced in excess during the identical experiment on cabbage seeds, it is a reasonable deduction that there is no ESP in watercress.

Although superficially this might not seem surprising because of the lack of appropriate substrate, it does mean that ESP occurrence is dependent on the presence of alkenylglucosinolate, i.e. presumably it is not a component present for some other purpose which happens also to function in this peculiar manner. In other words, it is specific for the formation of cyanoepithioalkane and this indicates that these products might well have some particular important function for the plant system. Such possible properties of these compounds are presently being examined.

EXPERIMENTAL

Preparation of seed extracts. Seeds (cabbage, *B. oleracea* or watercress, *N. officinale*) were ground to a powder in a coffee grinder. Dry hexane was added and the mixture shaken gently at room temp. for 15 min. After filtration the filtrate was rejected. More hexane was added to the seed powder and the procedure repeated $\times 5$. The defatted seed meal was then air-dried. About 7 g of seed meal were blended with *ca* 16 ml H_2O and the mixture was allowed to autolyse for 15 min at room temp. CH_2Cl_2 (220 ml) was added and the mixture shaken vigorously. Following centrifugation the organic layer was separated, dried and finally carefully reduced in vol to 2 ml using a rotary evaporator.

Assay for ESP activity. Two samples (1 g each) of defatted seed meal (prepared as above, from cabbage or watercress seeds) were taken. Allylglucosinolate (100 mg) was added to one; the other was a control. Both samples were then allowed to autolyse and the autolysis products extracted and concd as described above.

GLC. Autolysed seed samples prepared as above were analysed first by conventional GLC. A 1.5 m$\times$4 m mid glass column was used, packed with polyethyleneglycol (10%) coated on acid-washed Diatomite C. After experimentation the following temp. programme was found to provide best resolution, using N_2 carrier gas (flow 30 ml/min), 60° for 5 min, then raised by 16° /min to 195° until the completion of the run. Injection of 1 μl at an attenuation setting of 500 (5×10^{-10} A mp f.s.d.) was adequate to detect all glucosinolate products.

GC–MS. Components in the samples were identified by GC–MS. The GLC was linked to the MS via a heated membrane separator and the same GLC conditions were employed as described above, using a He carrier gas. MS conditions were: ionization potiential, 70 eV; ionization current, 300 μAmp; source temp., 200°; resolution, 1500; scan speed, 3 sec/decade (repetitive throughout run). At various times the

retrospective single ion monitoring facility of the data system was employed.

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