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BENZYLGLUCOSINOLATE DEGRADATION IN HEAT-TREATED *LEPIDIUM SATIVUM* SEEDS AND DETECTION OF A THIOCYANATE-FORMING FACTOR

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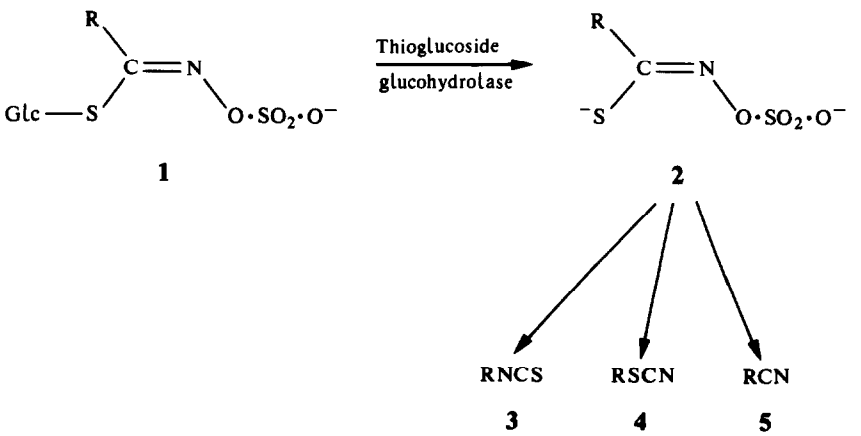
Key Word Index—*Lepidium sativum*; Cruciferae; cress; glucosinolate degradation; thiocyanate.

Abstract—*Lepidium sativum* seeds were dry heated at 125° for varying periods, and also for 30 min at various temperatures. Autolysates were then analysed for benzylglucosinolate degradation products. Whilst heating for 4 hr 20 min at 125° was sufficient to prevent formation of benzyl thiocyanate, just over 7.5 hr at 125° was required before benzyl isothiocyanate also ceased to be produced. This indicates the presence of a discrete, thiocyanate-forming factor in *L. sativum* seeds, separate from thioglucosidase. After 7.5 hr at 125°, benzyl cyanide continued to be formed, proving that it can be obtained (in relatively small amounts) directly from the glucosinolate even without the influence of any thioglucosidase. In general, isothiocyanate was the more favoured product of glucosinolate degradation following heat treatment of seeds, until the point of thioglucosidase inactivation was approached when nitrile formation took over. It is suggested that the thiocyanate-forming factor is an isomerase causing *Z–E* isomerization of the glucosinolate aglucone, but that only those glucosinolates capable of forming particularly stable cations are then able to undergo *E*-aglucone rearrangement to thiocyanate.

INTRODUCTION

Glucosinolates **1** are thioglucosides mainly located in the Cruciferae which undergo enzymic decomposition as shown in Scheme 1 to give three main types of product, isothiocyanates **3**, thiocyanates **4** and nitriles **5**. Occasionally, other products can be formed from particular glucosinolates under certain circumstances, but none of these is relevant to this paper. There are about 80 naturally occurring glucosinolates, differing mainly in the side chain, R. This can represent a number of structural possibilities, but all are derived from, or elaborated from, the side chains of α-amino acids. The major products of glucosinolate degradation have many important properties, and therefore understanding the mechanisms of their production is of particular importance. At this time, much information is available concerning the formation of isothiocyanates and nitriles, but the mechanism of thiocyanate production is completely unknown. Of all the naturally occurring glucosinolates, only three appear to undergo enzymic degradation to thiocyanate. These are allyl-, benzyl- and 4-(methylthio)butylglucosinolates. It would seem under the circumstances that this peculiar and most intriguing limitation would provide valuable information with regard to thiocyanate formation. However, although a num-



Scheme 1. Enzymic degradation of glucosinolates.

ber of theories have been advanced to explain this phenomenon, none has been substantiated by experiment.

The first theory of thiocyanate formation was proposed by Gmelin and Virtanen [1] who suggested an enzyme-induced rearrangement of the glucosinolate itself, but all attempts to isolate this enzyme merely resulted in a preparation with thioglucosidase activity. Later, Virtanen and Saarivirta [2, 3] proposed that an isomerase acted on the initially formed isothiocyanate and although this was a popular idea for some time, Saarivirta [4] later abandoned it on the basis of unsuccessful model system experiments. Miller [5] showed that a model aglucone of allylglucosinolate was converted to allyl thiocyanate by extracts of *Thlaspe arvense* seeds and this led to the suggestion that there was a thiocyanate-forming enzyme which acted on the aglucone (**2** in Scheme 1) rather than on the glucosinolate itself or the isothiocyanate [6]. This seems a more reasonable suggestion, but again all attempts to isolate this, or any other, thiocyanate-forming enzyme have failed. The main reason put forward to justify this is that the unknown enzyme is very labile, unlike the robust thioglucosidase [6, 7].

An alternative to the above enzyme theories is that thiocyanates are formed by a purely chemical mechanism (but possibly from the aglucone), and this could explain why only a few glucosinolates undergo this reaction. A common feature of the three glucosinolates concerned is that they all yield good stable cations, R^+ (Scheme 1), and this has led to the suggestion of thiocyanate formation via an ion-pair mechanism, which would also involve the bidentate nature of the isothiocyanate–thiocyanate ion [6]. This is an attractive theory, but efforts to substantiate it have not been successful [6, 7].

Thus, there are the two broad theories of thiocyanate production, one based on an enzyme or other biological 'factor', and one based on a specific structural feature of thiocyanate-forming glucosinolates. It is relevant to consider some other evidence in order to evaluate these theories further. Many studies of thiocyanate formation have been based on benzylglucosinolate in various *Lepidium* species. Numerous reports describe the identification of benzyl thiocyanate in extracts of the seeds of *L. sativum*, e.g. [1, 2, 4, 8–11], but it has never been located in extracts of the green leaves of this plant [4, 8, 12–14]. On the other hand, it has been readily identified in extracts of both seeds and leaves of *L. ruderale* [1, 13], *L. virginicum* [13] and also *Coronopus didymus* [13, 15]. These data provide strong evidence in favour of an enzymic theory of thiocyanate formation, since it would be expected that if the structural feature of glucosinolates was the sole requirement, then benzyl thiocyanate would always be produced, whatever the plant system or part thereof. The enzyme or biological factor presumably must be absent from *L. sativum* leaves, but present in the seeds, although it is possible that an inhibitor in the leaves could be the cause of this behaviour. Recently, Gil and MacLeod [16] succeeded in obtaining a crude enzyme preparation from *L. sativum* seeds which contained a thiocyanate-forming factor as well as thioglucosidase, since it converted pure benzylglucosinolate into both thiocyanate and isothiocyanate (as well as nitrile). However, this preparation produced only isothiocyanate and nitrile from pure 2-phenethylglucosinolate [16]. These results therefore indicate that there is also a structural requirement within the glucosinolate for thiocyanate formation. Some *in vivo* work supports these findings [17].

Our contention is thus that a combined theory is necessary for the mechanism of thiocyanate formation from glucosinolates, in which an enzyme or some other biological factor is required, but which functions only with glucosinolates which possess specific structural properties (i.e. those capable of forming stable cations, R^+) [16]. This paper describes work aimed at supporting some aspects of this proposal.

It has already been mentioned that failure to isolate a thiocyanate-forming factor has been attributed to its high lability compared with thioglucosidase [6, 7]. However, the cause of this apparent lability has never been positively characterized. When early attempts to isolate an isothiocyanate–thiocyanate isomerase failed, it was claimed that this was due to denaturation of the enzyme by compounds released when water was added to the seeds [4, 18]. Heat-lability of the thiocyanate-forming factor is another possibility, and it has been shown that in the autolysis of benzylglucosinolate in crushed *L. sativum* seeds a higher temperature (ambient or above) favoured isothiocyanate formation, whilst a lower one (near 0°) favoured thiocyanate formation [3, 4, 8, 10, 11, 18]. For this reason most work on *in vivo* thiocyanate formation has been carried out at low temperatures, but this is not absolutely necessary and benzyl thiocyanate is produced in reasonable amounts in *L. sativum* autolysates at ambient temperatures [11, 17]. Nevertheless, thiocyanate formation is particularly affected by temperature, and it has been further shown with *L. sativum* seed autolysates that whilst more benzyl thiocyanate is obtained at 0° than at ambient temperature, none is obtained if autolysates are heated at 100° for 2 hr [11]. Under the same circumstances isothiocyanate and nitrile production were almost constant over that temperature range [11]. It would seem from these data that variation in heat lability is thus a potential method of separating the effects of thioglucosidase and thiocyanate-forming factor, and therefore proving beyond doubt in a very simple manner than such a factor does indeed exist. All evidence to date on the existence of the factor is somewhat circumstantial and inconclusive. This paper describes the results of the appropriate experiments, which have been carried out in parallel with specific attempts to separate and isolate the factor.

RESULTS AND DISCUSSION

In one set of experiments, crushed *L. sativum* seeds were dry heated in an oven at a fixed temperature (125°) for measured periods of time. In a second series of experiments, seeds were similarly heated, but at varying temperatures for a fixed length of time (30 min). In all cases heating was followed by standardized autolysis of seeds in water, extraction of glucosinolate products, and high sensitivity analysis by GC and GC/MS. Table 1 gives results for the

Table 1. Amounts of benzylglucosinolate degradation products formed on autolysis of *Lepidium sativum* seeds following dry heat treatment of seeds at 125° for varying lengths of time

Time of heat treatment (hr)	Benzyl cyanide		Benzyl isothiocyanate		Benzyl thiocyanate		Total products ($mg \times 10^{-2}$)
	$mg \times 10^{-2}$	%	$mg \times 10^{-2}$	%	$mg \times 10^{-2}$	%	
0 (Blank)	341.1	69.7	65.9	13.5	82.2	16.8	489.2
0.25	97.0	51.4	75.2	39.8	16.6	8.8	188.8
0.50	91.9	47.0	85.5	43.7	18.1	9.3	195.5
1.00	83.7	41.5	104.1	51.6	14.0	6.9	201.8
2.00	67.3	31.4	137.8	64.3	9.1	4.3	214.2
3.00	51.6	23.2	165.6	74.4	5.5	2.4	222.7
4.00	32.3	27.0	85.7	71.6	1.7	1.4	119.7
4.33	24.7	52.9	22.0	47.1	—	—	46.7
5.00	27.6	58.2	19.8	41.8	—	—	47.4
6.00	21.3	92.2	1.8	7.8	—	—	23.1
7.00	26.4	92.6	2.1	7.4	—	—	28.5
7.50	32.9	93.5	2.3	6.5	—	—	35.2
7.60	44.8	100	—	—	—	—	44.8

constant temperature experiments and Table 2 gives those for the constant time experiments. Reproducibility of the results was better than ± 5% and Tables 1 and 2 give averages of duplicate determinations.

From Table 1 it is immediately obvious that the main objective of this project was achieved, in that the existence of a separate thiocyanate-forming factor or enzyme was proved. As expected, the factor was more heat-labile than the thioglucosidase, but it was nevertheless very much more stable than might have been supposed on the basis of previous reports e.g. [3, 4, 8, 10, 18]. It was necessary to dry heat the seeds at 125° for 4 hr 20 min before thiocyanate formation was prevented due to the inactivation of the factor. Thioglucosidase was, however, more robust in that isothiocyanate (and nitrile) was still produced under those conditions, and continued to be formed during a further 3 hr of heat treatment. A heating time of over 7.5 hr was necessary before the thioglucosidase in the seeds was also inactivated. It is significant and most interesting that nitrile was still produced after that period of heat treatment of seeds. This proves beyond doubt that nitrile can be obtained (although admittedly in relatively small amounts) directly from the glucosinolate even without the influence of any thioglucosidase. It is worth emphasizing at this point that the temperature adopted in these experiments was insufficient to cause direct thermal degradation of benzylglucosinolate [19].

The data in Table 2 further confirm the existence of a separate thiocyanate-forming factor (and its lesser thermal stability compared with thioglucosidase), since it was inactivated on heating at 135° for 30 min, whilst it is obvious that thioglucosidase was still appreciably active under these conditions.

Considering in detail the data in Table 1, the following statements can be made. Compared with the blank determination (i.e. on unheated seeds), there was a very rapid decrease in glucosinolate degrada-

Table 2. Amounts of benzylglucosinolate degradation products formed on autolysis of *L. sativum* seeds following dry heat treatment of seeds for 30 min at various temperatures

Temperature °C	Benzyl cyanide		Benzyl isothiocyanate		Benzyl thiocyanate		Total products ($mg \times 10^{-2}$)
	$mg \times 10^{-2}$	%	$mg \times 10^{-2}$	%	$mg \times 10^{-2}$	%	
Blank	341.1	69.7	65.9	13.5	82.2	16.8	489.2
64	315.5	68.5	69.2	15.0	75.9	16.5	460.6
80	243.6	65.9	67.3	18.2	58.6	15.9	369.5
92	212.0	64.2	70.9	21.5	47.3	14.3	330.2
105	172.0	61.6	71.4	25.6	36.0	12.8	279.4
115	123.5	63.3	81.3	35.1	26.7	11.5	231.5
125	91.9	47.0	85.5	43.7	18.1	9.3	195.5
135	99.8	52.6	89.9	47.4	—	—	189.7

tion after heating the seeds for only a few min due mainly to a very large decrease in nitrile production, for which there is no obvious explanation. It was definitely not caused by any change in thioglucosidase, since isothiocyanate production was not decreased under these conditions. After this time, nitrile formation decreased fairly regularly with increased period of heat treatment of seeds, until eventually thioglucosidase was inactivated, whereupon the amounts of nitrile formed increased. This was presumably due in part to a relatively greater amount of glucosinolate being available for non-thioglucosidase-induced degradation to nitrile. Whilst thiocyanate production decreased fairly regularly with increased time of heat treatment until the thiocyanate-forming factor was inactivated, isothiocyanate production increased during most of that period and became the major product of autolysis.However, just before the inactivation of the factor, isothiocyanate formation also began to decrease, and this trend continued regularly until thioglucosidase was in turn inactivated. In percentage terms isothiocyanate increased at the expense of nitrile during the first 3 hr heat treatment, after which nitrile increased at the expense of isothiocyanate. Thus there was a peak of isothiocyanate production in both percentage and absolute concentration terms after about 3 hr heat treatment of seeds. It is possible to theorize on the reasons for all of these observations, but without further evidence it is not appropriate to do so here.

One point which should be discussed, however, relates to the high resistance of thioglucosidase to this type of heat treatment, and from some previous work it might appear that thioglucosidases from different sources have varying susceptibilities. Thus the enzyme from watercress [20], cabbage [21] and Brussels sprouts [22, 23] would seem to be particularly heat-labile, in that heating the leaves for a few min at 100° or less was sufficient to cause inactivation. On the other hand, the enzyme from *Brassica napus* and *Crambe abyssinica* would seem to be much more stable, in that it was still active after heating the defatted seeds for 1 and 4 hr, respectively at 120° [24, 25]. These findings are more in agreement with the results reported here for *L. sativum* seeds, but the other work mentioned [20–23] was carried out on the green parts of the plants. Thus it may be that the species origin of the thioglucosidase is not the major factor in its stability, and that it is the seed enzymes in the seed environment which are inherently more resistant.

The results in Table 2 show that the extent of glucosinolate degradation decreased slowly, but regularly, with increasing heat treatment of seeds. Again this was due mainly to the nitrile product. Isothiocyanate formation increased with increasing heat treatment, whilst thiocyanate decreased regularly until the thiocyanate-forming factor was inactivated. These results are in general agreement with those in Table 1 and both sets of data show that glucosinolate degradation to isothiocyanate was more favoured following heat treatment of seeds, until the point of thioglucosidase inactivation was approached when nitrile formation took over. This is an interesting finding which shows that commercial dry heat treatment of Cruciferae seeds to prevent subsequent isothiocyanate production could be counterproductive if the high thermal resistance of thioglucosidase was not taken into account.

The results presented here not only prove the existence of a thiocyanate-forming factor, but they also confirm that it is not an isothiocyanate–thiocyanate isomerase [2–4]. If it were, then after inactivation it would be expected that there would be a build-up in isothiocyanate concentration, whereas Table 1 shows that there was in fact a decrease in concentration.

Nevertheless, on the basis of these and the previously mentioned results, we propose that the thiocyanate-forming factor is indeed an isomerase, which acts upon the aglucone (2 in Scheme 1) after its formation by thioglucosidase and which causes *Z–E* isomerization about the C=N bond. This is what was suggested by Benn [6], based partly on Miller's observations [5]. In the natural configuration (*Z*), the aglucone spontaneously, and rapidly, undergoes the Lossen rearrangement to give isothiocyanate. However, should the unknown factor or enzyme cause isomerization to occur to give the *E*-form (which would have to be a swift reaction to compete with the Lossen rearrangement), then the sulphate grouping would block attack by R (see Scheme 1). Under these circumstances R might migrate instead to the anionic sulphur forming a thiocyanate. Conversely, the process could be regarded as nucleophilic attack on R. However, whichever way the mechanism, it is proposed that rearrangement of the *E*-form would occur only if the R group was capable of generating a strong cation (as are the three thiocyanate-forming glucosinolates). This approach too has been invoked in the ion-pair theory of thiocyanate formation [6, 7], and it has been shown that with allylglucosinolate (only) a regiospecific rearrangement occurs such that the terminal carbon of the allyl group (C-3) becomes C-1 of the resultant allyl thiocyanate [7], but this does not conflict with the current proposal and an S_N2-type rearrangement can be envisaged [7]. Thus this overall theory would adequately explain all the current and recent observations with regard to glucosinolate catabolism to thiocyanates, but obviously proof requires isolation of the thiocyanate-forming factor and a study of its mode of action.

EXPERIMENTAL

Lepidium sativum seeds were obtained from Suttons Seeds, Reading, U.K. ('curled cress') and were authenticated by basic seed microscopy studies.

Heat treatment of seeds and autolysis. L. sativum seeds were freshly ground to a fine powder in a coffee grinder. A sample of seed powder (1 g) was weighed into a beaker and the beaker placed in a conventional laboratory oven maintained at a steady temp. for a recorded period of time. Seed autolysis was carried out immediately following removal of the sample from the oven, after cooling. H_2O (50 ml) was added to the seed powder and the mixture shaken at room temp. for 15 min. Seed residue was removed by centrifugation and the aq. layer was extracted with CH_2Cl_2 (2 × 30 ml). Following centrifugation, the organic layer was separated, dried (dry Na_2SO_4) and carefully reduced in vol. to less than 5 ml using a rotary evaporator without the application of heat.

Analysis by GC. Extracts (1 μl) were examined by routine GC using a Pye-Unicam model 104 instrument with heated

FID and a 1.5 m × 4 mm i.d. glass column packed with 3% Carbowax 20 M coated on 100–120 BSS mesh acid-washed Diatomite C. The carrier gas was N_2 (42 ml/min) and the column temp. was 160°. An attenuation setting of 200 (i.e. 2×10^{-10} A full-scale deflection) was necessary. RR_ts were measured from the onset of the solvent peak. Peak area measurements were accomplished manually. Absolute amounts of compounds produced were determined by standard injections of pure samples of benzyl cyanide, benzyl thiocyanate and benzyl isothiocyanate. The recovery of the sample was determined by submitting standard amounts of these pure compounds to the whole autolysis procedure.

GC/MS. Glucosinolate degradation products in the samples were identified by GC/MS using a Kratos MS 25 instrument (in the EI mode) equipped with a Kratos DS 50S data processing system. The same GC conditions as above were used, but with He as carrier gas. MS conditions were: ionization potential, 70 eV; ionization current, 300 μA; source temp., 230°; resolution, 600; scan speed, 3 sec/decade (repetitive throughout run).

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