Phytochemistry, Vol. 24, No. 9, pp. 1895–1898, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

THE OCCURRENCE AND ACTIVITY OF EPITHIOSPECIFIER PROTEIN IN SOME CRUCIFERAE SEEDS

ALEXANDER J. MACLEOD and JOHN T. ROSSITER*

Department of Chemistry, Queen Elizabeth College (University of London), Campden Hill Road, London W8 7AH, U.K.

(*Received* 10 *December* 1984)

Key Word Index—*Brassica napus*; *Brassica campestris*; *Lepidium sativum*; Cruciferae; epithiospecifier protein; cyanoepithioalkane; glucosinolates.

Abstract—Epithiospecifier protein (ESP) activity was determined in the seeds of two cultivars of *Brassica napus*, in *B. campestris* and in *Lepidium sativum*. All four types of seeds contained susceptible substrates for ESP (that is, glucosinolates with terminal unsaturation in their side-chain), although *L. sativum* contained only a very small amount of one. Results suggest that Fe^{2+} is essential for ESP activity, but its presence certainly promoted the effects of ESP to a considerable extent, and even at a very low level (e.g. 6×10^{-11} mol Fe^{2+}). Further evidence was gained for the intramolecular nature of the reaction which results in cyanoepithioalkane formation.

INTRODUCTION

On enzymic degradation, all glucosinolates can yield two main products, isothiocyanates and nitriles. A few can also form thiocyanates. In addition, those glucosinolates which possess terminal unsaturation in their side-chain are also capable of degradation to yield cyanoepithioalkanes. Since many of the most important and widespread glucosinolates do contain this necessary structural feature (e.g. allyl-, but-3-enyl- and 2-hydroxybut-3-enyl-glucosinolates), then clearly this is an important reaction and worthy of detailed study.

In 1973, Tookey identified a small protein in *Crambe abyssinica* involved in cyanoepithioalkane production, and showed it to be a necessary enzyme co-factor [1]. He named this the epithiospecifier protein (ESP). In the presence of ESP, thioglucoside glucohydrolase enzyme is capable of converting appropriate susceptible glucosinolates to corresponding cyanoepithioalkanes, but in its absence none is formed and only 'normal' products such as isothiocyanates and nitriles are obtained. On its own, ESP has no activity. It is thus an enzyme co-factor, but a very unusual one, in that it directs the enzyme to yield a different product. The only other of this type presently known is α-lactalbumin, which is involved as a specifier protein in the biosynthesis of lactose. ESP is worthy of much further investigation on these grounds alone, but since Tookey's original study [1] there has not been much further detailed work on its structure and/or activity.

Cole repeated Tookey's original work but on *Brassica campestris*, and obtained similar results, isolating an ESP from the seeds [2]. She also analysed a wide range of Cruciferae seeds and in so doing deduced ESP activity in many by detection of cyanoepithioalkanes on autolysis [3]. Interestingly, however, Kaoulla *et al.* could find no ESP activity in seeds of *Nasturtium officinale* [4]. They showed that appropriate extracts of the seeds converted allylglucosinolate to allyl isothiocyanate alone, whereas extracts of known ESP-containing seeds yielded 1-cyano-2,3-epithiopropane [4]. However, none of the four endogenous glucosinolates of *N. officinale* possesses terminal unsaturation, and the fact that the species also lacks ESP provoked the suggestion that ESP might have a truly specific function as enzyme co-factor, and that when no appropriate substrate was present, neither was the ESP (even though the enzyme was) [4]. Much further work would be necessary to substantiate this suggestion, but in the work described here a system has been studied (*Lepidium sativum*) which is intermediate between those previously investigated. Thus, *L. sativum* contains predominantly benzylglucosinolate but also a trace amount of allylglucosinolate [5], and the question was whether ESP would be present for such a minor constituent when absent from *N. officinale*.

Tookey showed that isolation of ESP from *Crambe abyssinica* seeds could only be achieved if extracts were stabilized by removing phenolic compounds with Polyclar AT, by adding ferrous ions and dithiothreitol, and by keeping the extract at 0° under nitrogen [1]. The co-factor could then be separated, especially from the thioglucosidase, and partially purified by ammonium sulphate precipitation and chromatography using Sephadex G-100 [1]. The addition of ferrous ions was considered to be essential, and this aspect was further studied in the work reported here.

Various mechanisms have been proposed for the action of ESP. Cole first suggested that it simply caused rearrangement of alkenyl isothiocyanate [3], but this seems unlikely since it would necessitate conversion of a C–N bond at the isothiocyanate group to a C–C bond at the new cyanide function. Benn has suggested that cyanoepithioalkane formation arises from intramolecular capture of sulphur in the aglucone, this occurring within an enzyme cleft [6]. Indeed, one of the most interesting basic problems is whether the 'transfer' of sulphur to the terminal unsaturation does involve an intra- or inter-

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

molecular process, and this aspect was also briefly studied in the work described here. However, recently Brocker and Benn have reported convincing evidence in favour of the reaction being substantially intramolecular [7]. Using aqueous suspensions of *Crambe abyssinica* seed flour as the source of thioglucosidase plus ESP, they found that co-hydrolysis of but-3-enylglucosinolate labelled with ^{35}S at the thioglucosidic link and unlabelled allylglucosinolate led to the ^{35}S being found only in 1-cyano-3,4-epithiobutane and not in 1-cyano-2,3-epithiopropane [7].

2-Hydroxybut-3-enylglucosinolate, also known by the trivial name progoitrin, is one of the more important glucosinolates possessing terminal unsaturation in the side-chain. It is the major glucosinolate of rape and other economically important Cruciferae, and it is the precursor of goitrogenic 5-vinyloxazolidine-2-thione (goitrin) which is formed spontaneously by cyclisation of conventionally produced 2-hydroxybut-3-enyl isothiocyanate. Recently we have synthesized 2-hydroxybut-3-enylglucosinolate to provide a pure substrate for proper systematic study of its enzymic and non-enzymic degradation [8]. Thus, progoitrin provided the model glucosinolate for the majority of these studies on ESP occurrence and activity.

RESULTS AND DISCUSSION

The following seeds were analysed for ESP activity: *Brassica napus* cv. Quinta, *B. napus* cv. Panter, *B. campestris* cv. Manchester Market Early Green Top Stone and *Lepidium sativum*. The main glucosinolates of the first three were determined by Persson's method [9], and were found to be but-3-enylglucosinolate and 2-hydroxybut-3-enylglucosinolate (*ca* 10% and *ca* 90%, respectively, for both cultivars of *B. napus* and *ca* 70% and *ca* 30%, respectively, in *B. campestris*). This analytical method would not have been sensitive enough to detect the low levels of the important (in this case) minor glucosinolates of *L. sativum*, but from previous work [5] it was known that this species contains *ca* 95% benzyl-, *ca* 4% allyl- and *ca* 1% 2-phenethyl-glucosinolates. Therefore, all seeds examined contained glucosinolates capable of generating cyanoepithioalkanes, although *L. sativum* contained only a very small amount of only one.

Assessment of ESP activity in seed extracts was accomplished by removal of all endogeneous glucosinolates (partly to simplify subsequent analysis) and addition of known amounts of 2-hydroxybut-3-enylglucosinolate to the autolysis medium. Products of autolysis were analysed by GC and GC/MS, and the relative amounts obtained for the four types of seed are shown in Table 1. Here and throughout, figures for 1-cyano-2-hydroxy-3,4-epithiobutane are the totals for the produced diastereoisomers; these could be separated by GC but, of course, they gave identical mass spectra.

It is very clear from Table 1 that *L. sativum* does indeed contain ESP, in that a significant proportion of cyanoepithioalkane was produced from the added glucosinolate. On the basis of this and the previous result for *Nasturtium officinale* [4], it would appear that ESP is only absent from systems in which there are definitely no glucosinolates with terminal unsaturation, and the presence of only a very small amount of a susceptible glucosinolate is sufficient for there to be appreciable ESP activity.

The results for *L. sativum* were remarkably similar to those for *B. napus* cv. Quinta, whilst the other two seeds also formed a similar pair, in that both yielded only 5-vinyloxazolidine-2-thione from added 2-hydroxybut-3-enylglucosinolate. From this result it might seem that these latter two types of seed do not contain ESP. However, on addition of relatively small concentrations of ferrous ions to the autolysis medium, major changes in the relative proportions of hydrolysis products were observed, and these data are also listed in Table 1. In *all* cases, Fe^{2+} caused considerable increases in the relative amounts of 1-cyano-2-hydroxy-3,4-epithiobutane at the expense of 5-vinyloxazolidine-2-thione. Thus, *B. napus* cv. Panter and *B. campestris* do, in fact, possess ESP, as would be expected, but it follows that Fe^{2+} is essential for proper ESP activity, and not merely for its stabilization during extractions, etc. Presumably *B. napus* cv. Quinta and *L. sativum* contain sufficient endogenous Fe^{2+} for natural activity, while the other seeds studied do not. However, the addition of extra Fe^{2+} still promoted the activity of ESP in *B. napus* cv. Quinta and *L. sativum*, to the extent that no 5-vinyloxazolidine-2-thione at all was then produced by those systems. Ferrous ions also promote the production of 'normal' nitriles (i.e. 1-cyano-2-hydroxybut-3-ene, not the epithionitrile), and that too is seen in Table 1, although there is no obvious explanation for the particularly high level in *B. napus* cv. Panter.

These results (Table 1) suggest that increased amounts of Fe^{2+} increase the extent of ESP activity. To test this deduction, the effects of a range of concentrations of Fe^{2+} on the autolysis products induced by one type of seed (*B. napus* cv. Quinta) were studied, and the results are given in Table 2. It can be seen that remarkably small amounts of

Table 1. Relative amounts of autolysis products of 2-hydroxybut-3-enylglucosinolate formed by the action of some seed extracts with and without the addition of Fe^{2+} (6×10^{-6} mol)

Seed	5-Vinyloxazolidine-2-thione (%)	1-Cyano-2-hydroxy-3,4-epithiobutane (%)	1-Cyano-2-hydroxybut-3-ene (%)
Brassica napus cv. Panter	100	—*	—
plus Fe^{2+}	17.8	23.4	58.8
Brassica napus cv. Quinta	54.3	32.4	13.3
plus Fe^{2+}	—	71.5	28.5
Brassica campestris	100	—	—
plus Fe^{2+}	6.3	70.9	22.8
Lepidium sativum	54.2	37.8	8.0
plus Fe^{2+}	—	81.0	19.0

*—, Not detected.

Table 2. Effects of added Fe^{2+} on the relative amounts of autolysis products of 2-hydroxybut-3-enylglucosinolate formed by the action of *Brassica napus* cv. Quinta seed extract

Added Fe^{2+} (mol)	5-Vinyloxazolidine-2-thione (%)	1-Cyano-2-hydroxy-3,4-epithiobutane (%)	1-Cyano-2-hydroxybut-3-ene (%)	epithioalkane / alkenyl cyanide
0	54.3	32.4	13.3	2.4
6×10^{-11}	21.8	54.9	23.3	2.4
6×10^{-10}	9.0	66.2	24.8	2.7
6×10^{-9}	—*	72.8	27.2	2.7
6×10^{-8}	—	70.6	29.4	2.4
6×10^{-7}	—	72.2	27.8	2.6
6×10^{-6}	—	71.5	28.5	2.5

*—, Not detected

added Fe^{2+} were sufficient to bring about a complete reversal of major product distribution. Thus, the addition of a mere 6×10^{-11} mol of Fe^{2+} was enough to change enzymic hydrolysis of 2-hydroxybut-3-enylglucosinolate to yield 1-cyano-2-hydroxy-3,4-epithiobutane as the major product (*ca* 55%) instead of 5-vinyloxazolidine-2-thione (*ca* 54% in the absence of added Fe^{2+}). This suggests that only catalytic quantities of Fe^{2+} are necessary to promote ESP activity. However, as the amount of added Fe^{2+} was increased, so too did the amount of cyanoepithioalkane produced, until it reached a roughly constant level at *ca* 6×10^{-9} mol Fe^{2+}. At this level no 5-vinyloxazolidine-2-thione was formed at all. It would seem, therefore, that the cyanoepithioalkane is the favoured normal product and that only lack of Fe^{2+} prevents its formation and results in the consequent formation of oxazolidinethione instead (or isothiocyanate in other cases). However, in this experiment (Table 2) the cyanoepithioalkane never became the sole product of autolysis, and the other nitrile was also formed, and proportional to the amount of Fe^{2+} added. In fact, the ratio of the one nitrile to the other did not change very much whatever the amount of Fe^{2+} present (and whether or not oxazolidinethione was also produced). This could mean that both of these nitriles arise from a common intermediate (presumably the aglucone), their formation being partly dependent on Fe^{2+}.

A simple method of gaining some insight into an enzymic reaction is to use an appropriate inhibitor. Since ESP requires a terminal unsaturated bond as a substrate, a range of autolysis experiments was also carried out as before and using *B. napus* cv. Quinta, but with the addition of varying amounts of allyl alcohol (prop-2-en-1-ol). However, this compound failed to inhibit ESP over a range of 1–4 equivalents of allyl alcohol to 2-hydroxybut-3-enylglucosinolate. This implies that the addition of sulphur to alkene unsaturation does not occur in an intermolecular reaction, but occurs intramolecularly, probably in a thioglucosidase–ESP complex. This supports the recent findings of Brocker and Benn [7].

Attempts were made to solubilize and isolate the ESP of *B. napus* cv. Quinta using Tookey's method [1], but when assayed as before for ESP activity the extract gave only 1-cyano-2-hydroxybut-3-ene and no 1-cyano-2-hydroxy-3,4-epithiobutane. The problem was traced to the sonication stage of the procedure, when some heat developed. Elimination of this stage yielded a product which did give mainly the cyanoepithioalkane, indicating that ESP activity had been maintained. However, all efforts to include sonication (e.g. with stirring, with cooling, in sonic bursts) failed, and it may be that ESP from *B. napus* cv. Quinta is more labile than that from *Crambe abyssinica* (used by Tookey [1]). Indeed, it was found that the isolated ESP (without use of sonication) entirely lost its activity over a period of 24 hr, even though stored at *ca* 2°.

EXPERIMENTAL

Lepidium sativum ('curled cress') seeds and *Brassica campestris* seeds (cv. Manchester Market Early Green Top Stone) were obtained from Suttons Seeds, Reading, U.K. and were authenticated by basic seed microscopy studies. Authenticated seeds of *Brassica napus* (cv. Quinta and cv. Panter) were obtained from Karlshamns AB, Karlshamn, Sweden.

Synthesis of standards. 2-Hydroxybut-3-enylglucosinolate was prepared by a multi-stage synthesis from propenal and α-D-glucose [8]. 5-Vinyloxazolidine-2-thione was prepared from 1,2-epoxybut-3-ene in three stages by Ettlinger's method [10]. 1-Cyano-2-hydroxybut-3-ene was prepared in two steps from buta-1,3-diene and calcium hypochlorite using standard procedures [11].

Assay of glucosinolates in seeds. The method used was basically that of Persson [9]. Glucosinolates were extracted (ice-cold Me_2CO) from defatted (hexane) ground seeds. The extract was dried, concd, and a sample (15 mg) heated at 110° for 40 min in a soln (1.5 ml) of hexamethyldisilazane, trimethylchlorosilane and pyridine (2:1:10) containing 3 mg allylglucosinolate internal standard. The TMS-glucosinolates were then analysed by FID-GC using a 1.5 m × 4 mm i.d. glass column packed with 3% OV 17 coated on Chromosorb Q 80/100; temp. programme, 195–235° at 2°/min; injection port and detector temp., 250°; flow rate, 30 ml N_2/min. Quantification was by reference to the internal standard, and identification of individual glucosinolates was by comparison of GC retention data with standards and by routine GC/MS using a Kratos MS 25 instrument equipped with a DS 50S data processing system.

Assay of ESP activity in seeds. Seeds (5 g) were ground in a coffee grinder to give a fine powder which was then defatted by repeated extraction with dry hexane (10 × 100 ml). The powder was air-dried and glucosinolates were removed by extraction with

ice-cold Me_2CO (2 × 100 ml). The dried seed powder (0.25 g) was added to 5 ml of NaOAc buffer (0.2 M, pH 5.3) containing 15 mg of 2-hydroxybut-3-enylglucosinolate. The mixture was shaken at room temp. for 1 hr to allow autolysis to occur, after which it was centrifuged and the supernatant extracted with CH_2Cl_2 (2 × 20 ml). The extract was dried (anhyd. Na_2SO_4) and concd carefully by vacuum distillation. The product was analysed by FID-GC using a 1.5 m × 4 mm i.d. glass column packed with 5% Apiezon L coated on 100–120 BSS mesh acid-washed Diatomite C; temp. programme 60–190° at 3°/min; flow rate, 30 ml N_2/min. 5-Vinyloxazolidine-2-thione and 1-cyano-2-hydroxybut-3-ene were identified and quantified by comparison with the synthesized reference standards. Identifications were confirmed by GC/MS using the same GC conditions, and GC/MS was used to identify 1-cyano-2-hydroxy-3,4-epithiobutane in autolysates. The cyanoepithioalkane was quantified by direct comparison with the isomeric vinyloxazolidinethione standard.

Effects of Fe^{2+} *on ESP activity in seeds.* Standard ferrous ion solns were prepared in NaOAc buffer (0.2 M, pH 5.3) using ferrous ammonium sulphate, and were added at the beginning of autolysis to the autolysis medium at the range of concns indicated in Table 2. Otherwise, assay of ESP activity was exactly as described above.

Effects of allyl alcohol on ESP activity in seeds. Allyl alcohol (1, 2, 3 or 4 equivalents with respect to 2-hydroxybut-3-enylglucosinolate) was added at the beginning of autolysis to autolysis solutions containing 6×10^{-7} mol δf Fe^{2+}. Otherwise, assay of ESP activity was exactly as described above.

Attempted solubilization of ESP. Brassica napus cv. Quinta seeds were defatted and had their endogeneous glucosinolates removed as described above. The air-dried seed powder (5 g) was then added to 80 ml of extractant (0.2 M NaCl, 10^{-3} M dithiothreitol, 10^{-2} M NaOAc buffer pH 5.3, 0.2×10^{-3} M Fe^{2+}) at 0° purged with N_2 and containing 2.9 g of Polyclar AT, previously washed with extractant. The soln was stirred, cooled, and given short bursts of sonication totalling 20 min using a 0.25″ probe and ensuring that the temp. did not exceed 2°. The soln was then centrifuged at 13000 *g* at 0° for 15 min and the supernatant removed and stored under N_2 at 0°. ESP activity was assessed by mixing 0.2 ml of the supernatant with 1.6 ml of a salt soln (0.2 M NaCl, 0.2×10^{-3} M Fe^{2+}, 0.2 M NaOAc buffer pH 5.3) containing 10 mg of 2-hydroxybut-3-enylglucosinolate, and allowing autolysis to proceed for 1 hr at room temp. Products of autolysis were then extracted and analysed as described above.

Extraction of ESP. The method was as for solubilization above, except that sonication was omitted and in its place the seed powder and the extractant medium were allowed to stand for 10 min at 0° with stirring.

Acknowledgements—We thank the Agricultural and Food Research Council for support, Karlshamns AB, Karlshamn, Sweden for provision of authenticated seeds of *Brassica napus*, and Mr. W. G. Gunn and Mr. A. E. Cakebread for running the GC/MS.

REFERENCES

1. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1654.
2. Cole, R. A. (1978) *Phytochemistry* **17**, 1563.
3. Cole, R. A. (1976) *Phytochemistry* **15**, 759.
4. Kaoulla, N., MacLeod, A. J. and Gil, V. (1980) *Phytochemistry* **19**, 1053.
5. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1369.
6. Benn, M. H. (1977) *Pure Appl. Chem.* **49**, 197.
7. Brocker, E. R. and Benn, M. H. (1983) *Phytochemistry* **22**, 770.
8. MacLeod, A. J. and Rossiter, J. T. (1983) *J. Chem. Soc. Perkin Trans.* 1, 717.
9. Persson, S. (1974) *Proc. IVth Intl. Rapeseed Conf.* 381.
10. Ettlinger, M. G. (1950) *J. Am. Chem. Soc.* **72**, 4792.
11. Bissinger, W. E., Fredenburg, R. H., Kadesch, R. G., Kung, F., Langston, J. H., Stevens, H. C. and Strain, F. (1947) *J. Am. Chem. Soc.* **69**, 2955.