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INTERACTIONS OF THIOGLUCOSIDE GLUCOHYDROLASE AND EPITHIOSPECIFIER PROTEIN OF CRUCIFEROUS PLANTS TO FORM 1-CYANOEPITHIOALKANES

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Key Word Index—Cruciferae; thioglucoside glucohydrolase; thioglucosidase; epithiospecifier protein; glucosinolate; 1-cyanoepithioalkanes; 1-cyano-2,3-epithiopropane.

Abstract—Allylglucosinolate is converted to 1-cyano-2,3-epithiopropane by interaction of the thioglucoside glucohydrolase and epithiospecifier protein from several genera: *Brassica*, *Crambe*, *Armoracia* and *Sinapis*. The interactions occur across genetic lines, indicating non-specific requirements for epithiospecifier protein and thioglucoside glucohydrolase.

INTRODUCTION

1-Cyanoepithioalkanes from glucosinolates have been implicated as toxicants to animals [1, 2]. These substances are formed from glucosinolates containing a vinyl moiety only when thioglucoside glucohydrolase (EC 3.2.3.1, also known as thioglucosidase) acts in conjunction with an epithiospecifier protein (ESP), which by itself does not hydrolyse glucosinolates [3, 4]. Previous studies have been conducted with both thioglucosidase and ESP from the same plant source [3–8]. We now report the conversion of the widely distributed allylglucosinolate (sinigrin) to 1-cyano-2,3-epithiopropane by interaction of thioglucosidase and ESP from several genera of crucifer vegetables: *Brassica*, *Crambe*, *Armoracia* and *Sinapis* (Fig. 1).

RESULTS AND DISCUSSION

Thioglucosidase and ESP were obtained from a variety of crucifer plants by adapting the procedures used by Tookey [3].

Thioglucosidase was found in all crucifers examined, but leafy vegetative sources, such as white cabbage (*Brassica oleracea* var. *capitata* L. f. *alba* DC.) or Brussels sprouts (*Brassica oleracea* var. *gemmifera* DC.), contained considerably lower amounts of enzyme than did horseradish root (*Armoracia rusticana*) or seed of white mustard (*Sinapis alba*), turnip (*Brassica campestris* subsp. *rapifera* (Metzg) Sinsk cv Purple Top Strap Leaf) or *Crambe abyssinica*. This finding is in agreement with results published by Iverson and Baggerud [9] in which thioglucosidase activity that was present in vegetative portions of one- to two-day-old seedlings decreased throughout the development of the plant.

ESP was found in Brussels sprouts, white cabbage vegetables, *C. abyssinica* and turnip seed but not in white mustard seed or horseradish. The lack of ESP in horseradish root was confirmed in a separate experiment in which the autolysis of the root, known to contain thioglucosidase and allylglucosinolate, did not result in the production of detectable amounts of 1-cyano-2,3-epithiopropane.

When allylglucosinolate was incubated with combinations of thioglucosidase and ESP from several crucifer genera, the aglucone was partially converted to 1-cyano-2,3-epithiopropane as well as to allyl cyanide and allyl isothiocyanate (Table 1). Formation of the last two products does not require the presence of ESP [3]. Allyl cyanide was not measured because of its extreme volatility and loss during sample work-up; partial loss of volatile allyl isothiocyanate may have occurred in some samples despite considerable care in sample handling. In all cases, the identity of the aglucone products was confirmed by GC/MS. The results, presented in Table 1, show that interactions of thioglucosidase and ESP occur across taxonomic lines, indicating non-specific requirements for both proteins. 1-Cyano-2,3-epithiopropane was also formed from allylglucosinolate using combinations of thioglucosidase prepared from white cabbage (which contains only trace amounts of enzyme) and ESP

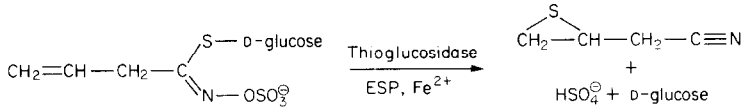

Fig. 1. Conversion of allylglucosinolate to 1-cyano-2,3-epithiopropane by interaction of thioglucosidase and ESP.

Table 1. Conversion of allylglucosinolate to organic aglucone products by combinations of thioglucosidase and ESP from various crucifer plant sources

Source of thioglucosidase	Source of ESP	Conversion (%) to organic aglucone products*	
		1-Cyano-2,3-epithiopropane	Allyl isothiocyanate
Brussels sprouts†	None	0	66
Brussels sprouts	Brussels sprouts†	10	97
Brussels sprouts	White cabbage‡	—	—
Brussels sprouts	*C. abyssinica*§	6	87
Brussels sprouts	Turnip	4	69
C. abyssinica§	None	0	57
C. abyssinica	Brussels sprouts	51	48
C. abyssinica	White cabbage	35	30
C. abyssinica	*C. abyssinica*	48	57
C. abyssinica	Turnip	44	31
White mustard§	None	0	54
White mustard	Brussels sprouts	31	48
White mustard	White cabbage	19	50
White mustard	*C. abyssinica*	23	82
White mustard	Turnip	8	77
Turnip§	None	0	47
Turnip	Brussels sprouts	55	58
Turnip	White cabbage	42	61
Turnip	*C. abyssinica*	41	57
Turnip	Turnip	36	74
Horseradish‖	None	0	40
Horseradish	Brussels sprouts	35	27
Horseradish	White cabbage	42	43
Horseradish	*C. abyssinica*	22	43
Horseradish	Turnip	7	32

*Values were obtained by direct measurement by GLC; for conditions, see Experimental.
†Bud.
‡Leaf.
§Seed.
‖Root.

from any source, but the small amounts of product allowed identification only by GC/MS.

It should be noted, however, that differences in the extent of interaction between thioglucosidase and ESP do occur. For example, when allylglucosinolate was incubated with combinations of thioglucosidase from Brussels sprouts and ESP from any source, small amounts of 1-cyano-2,3-epithiopropane were formed. Also, combinations of ESP from turnip and thioglucosidase from either *C. abyssinica* or turnip gave rise to greater amounts of 1-cyano-2,3-epithiopropane from allylglucosinolate than did combinations of ESP from turnip and thioglucosidase from white mustard, Brussels sprouts or horseradish root. The reasons for these differences are still unclear, partly because thioglucosidase and ESP activity varied with different curcifer plant sources.

In several instances where enzyme and ESP interact, the sum of 1-cyano-2,3-epithiopropane and allyl isothiocyanate represents about 100% conversion of the glucosinolate (Table 1). This fact implies that no allyl cyanide was formed. However, in the absence of ESP, the per cent allyl isothiocyanate remains nearly the same. These results suggest that the allylglucosinolate, which is converted to 1-cyano-2,3-epithiopropane in the presence of ESP, is converted to allyl cyanide in the absence of ESP, as would be expected from previous work [3].

The results of studies reported in this paper imply that 1-cyanoepithiopropanes are formed from glucosinolates by essentially the same mechanism in all crucifer vegetables. Studies are currently being conducted to elucidate the bio-organic reaction mechanism of this transformation.

EXPERIMENTAL

Preparation of white mustard thioglucosidase. Thioglucosidase was extracted from defatted flakes of white

mustard seed (100 g) with 500 ml 0.2 M acetate buffer, pH 5.9, containing 50 mg dithiothreitol (DTT) at 4°. The crude enzyme extract was fractionated by precipitation with EtOH. The 30–75% EtOH-precipitated fraction was resuspended in 50 ml chilled, deionized H_2O containing 5 mg DTT. The mixture was lyophilized to afford a white powder (4.15 g). Portions (300 mg) were taken up in extraction buffer (6 ml) and further purified by gel filtration as described under general protein separation methods. Active fractions were pooled, dialysed against chilled (4°), deionized H_2O containing DTT (100 mg/l.), and lyophilized to yield *ca* 50 mg of enzyme, which was stable for several months when stored at 4°.

Preparation of thioglucosidase and ESP from seed. Polyclar AT [insoluble poly(vinylpyrrolidone)] (2.9 g) was suspended in 75–80 ml of extraction buffer. The mixture was centrifuged (10 min, 2000 *g*) and the supernatant was discarded. Defatted seed meal (5.0 g), prepared as previously described [10], was added to the moistened Polyclar AT followed by 50 ml extraction buffer. After mixing, the slurry was exposed to ultrasound as described [11] and centrifuged (10 min, 2000 *g*) to remove solids. Thioglucosidase was separated from ESP by $(NH_4)_2SO_4$ fractionation of the supernatant followed by gel filtration as described in the section below on general protein separation methods.

Preparation of thioglucosidase or ESP from vegetative plant material. Fresh plant material (100 *g*) was blended with extraction buffer (150 ml), and the mixture was filtered through cheesecloth. The filtrate was centrifuged (15 min, 15 300 *g*) at 0°. Thioglucosidase was separated from ESP by $(NH_4)_2SO_4$ fractionation of the supernatant and subsequent gel filtration, as described in the section on general protein separation methods.

General protein separation methods for ESP and thioglucosidase. The extraction buffer contained 200 mM NaCl, 1 mM DTT and 10 mM acetate, pH 5.8–5.9, dissolved in ice-cold, N_2-purged, deionized H_2O. $(NH_4)_2SO_4$ fractionation was conducted in the cold according to the nomograph of di Jeso [12]. Precipitates were separated by centrifugation (15 min, 15 300 *g*) at 0°. The desired fraction, 40–70% $(NH_4)_2SO_4$ saturation, was resuspended in 6 ml fresh extraction buffer. Gel filtration was carried out on Sephacryl S-200 (2.6 × 72 cm) suspended in extraction buffer containing 5 ppm Zephiran chloride as a preservative. The resuspended protein fraction (6 ml) was added to the top of the column and eluted with extraction buffer containing 5 ppm Zephiran chloride. The column, which had a void vol. of 105 ml, was eluted at 4° with a flow rate of 0.25 ml/min; the eluate (5 ml/tube) was monitored by absorbance at 280 nm.

Assay of fractions for thioglucosidase and ESP. Fractions containing thioglucosidase were assayed by incubating 1.5 ml with allylglucosinolate (25.2 μmol) and titrating released HSO_4^- at constant pH (5.8). Fractions containing ESP were assayed by adding 2 ml to white mustard thioglucosidase (2 ml) and hydrolysing allylglucosinolate to 1-cyano-2,3-epithiopropane, as described in the sections on incubation of allylglucosinolate with thioglucosidase and ESP and on assay of organic aglucone products from allylglucosinolate.

Incubation of allylglucosinolate with thioglucosidase and ESP. Incubation mixtures contained allylglucosinolate (25.2 μmol), thioglucosidase (2.0 ml), ESP (2.0 ml), and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (12.0 μmol), all in a total vol. of 5.0 ml 0.01 M acetate buffer, pH 5.8, containing 0.2 M NaCl and 6.5×10^{-4} M DTT. Incubations were carried out at 25° in a N_2 atmosphere at pH 5.8 until the substrate was consumed or for 6 hr (when enzyme activity was very low). The extent of enzymatic hydrolysis of allylglucosinolate was measured by titration of released HSO_4^- at constant pH or by determination of liberated glucose utilizing the Pierce Glucose Auto/Stat kit method (Pierce Chemical Co., Rockford, IL) which is based on the method of Trinder [13].

Assay of organic aglucone products from allylglucosinolate. Solns containing hydrolytic products from allylglucosinolate were extracted with CH_2Cl_2 (15 ml), and the CH_2Cl_2 extract was reduced in vol. and subjected to GLC [14]. Corrections were made for pre-formed aglucone products present in crude enzyme preparations.

Autolysis of horseradish root. Horseradish root (40 *g*) was pulped with cold (4°) extraction buffer (60 ml) in a blender and the mixture was filtered through cheesecloth. An aliquot (7.5 ml) was autolysed by titration at a constant pH of 5.8. After the consumption of base (NaOH) ceased, CH_2Cl_2 extractables were analysed by GLC [14].

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REFERENCES

1. Nishie, K. and Daxenbichler, M. E. (1980) *Food Cosmet. Toxicol.* **18**, 159.
2. Gould, D. H., Gumbmann, M. R. and Daxenbichler, M. E. (1980) *Food Cosmet. Toxicol.* **18**, 619.
3. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1654.
4. Cole, R. A. (1978) *Phytochemistry* **17**, 1563.
5. Daxenbichler, M. E., Van Etten, C. H. and Wolff, I. A. (1966) *Chem. Commun.* **15**, 526.
6. Daxenbichler, M. E., Van Etten, C. H., Tallent, W. H. and Wolff, I. A. (1967) *Can. J. Chem.* **45**, 1971.
7. Kirk, J. O. and MacDonald, C. G. (1974) *Phytochemistry* **13**, 2611.
8. Luthy, J. and Benn, M. (1979) *Phytochemistry* **18**, 2028.
9. Iverson, T. H. and Baggerud, C. (1980) *Z. Pflanzenphysiol.* **97**, 399.
10. Tookey, H. L. and Wolff, I. A. (1970) *Can. J. Biochem.* **48**, 1024.
11. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1305.
12. di Jeso, F. (1968) *J. Biol. Chem.* **243**, 2022.
13. Trinder, P. (1969) *J. Clin. Pathol.* **22**, 246.
14. Daxenbichler, M. E. and Van Etten, C. H. (1977) *J. Assoc. Off. Analyt. Chem.* **60**, 950.