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# INTERACTIONS OF A FUNGAL THIOGLUCOSIDE GLUCOHYDROLASE AND CRUCIFEROUS PLANT EPITHIOSPECIFIER PROTEIN TO FORM 1-CYANOEPITHIOALKANES: IMPLICATIONS OF AN ALLOSTERIC MECHANISM

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**Key Word Index**—*Crambe abyssinica*; Cruciferae; *Aspergillus sydowi*; thioglucoside glucohydrolase; thioglucosidase; epithiospecifier protein; crucifer; glucosinolate; 1-cyanoepithioalkanes; 1-cyano-2,3-epithiopropane.

**Abstract**—Allylglucosinolate is converted to 1-cyano-2,3-epithiopropane by interaction of the thioglucoside glucohydrolase from the fungus *Aspergillus sydowi* QM 31c and the epithiospecifier protein from *Crambe abyssinica*. The kinetic evidence presented supports the hypothesis that the epithiospecifier protein interacts with thioglucoside glucohydrolase in an allosteric manner.

## INTRODUCTION

Glucosinolates and their degradation products are undesirable toxic substances found in foods and feeds from cruciferous plants [1–3]. A widely distributed member of this series, allylglucosinolate (sinigrin), is hydrolysed by the action of thioglucoside glucohydrolase (EC 3.2.3.1, also known as thioglucosidase) at pH 5.9 to a mixture of products which include allyl cyanide, allyl isothiocyanate, D-glucose, $HSO_4^-$ and elemental sulfur [1, 4]. An additional but even more toxic product, 1-cyano-2,3-epithiopropane, is formed only when thioglucosidase acts in conjuction with ferrous ion and an epithiospecifier protein (ESP) that is found in many, but not all, cruciferous plants (Fig. 1) [4, 5]. ESP by itself does not hydrolyse glucosinolates [4, 5]. Interactions of thioglucosidase and ESP occur across taxonomic lines, indicating non-specific requirements for these substances in both proteins [6]. Previous studies have been conducted with both thioglucosidase and ESP from higher plant sources [3–10]. We now report the conversion of allylglucosinolate to 1-cyano-2,3-epithiopropane by interaction of a fungal thioglucosidase from *Aspergillus sydowi* QM 31c and ESP from *Crambe abyssinica*. The kinetic evidence presented supports the hypothesis that ESP interacts with thioglucosidase in an allosteric manner to produce 1-cyano-epithioalkanes from glucosinolates containing a vinyl moiety.

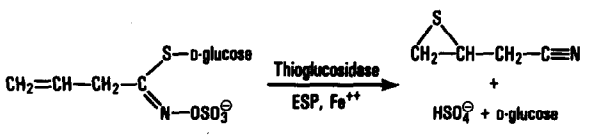


Fig. 1. Conversion of allylglucosinolate to 1-cyano-2,3-epithiopropane by interaction of thioglucosidase and ESP.

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## RESULTS AND DISCUSSION

Thioglucosidase is a constitutive enzyme in the fungus *Aspergillus sydowi* QM 31c [11]. The organism is easy to grow; however, we found that supplementing the medium devised by Reese *et al.* [11] with additional starch (10 g/l. vs 2.5 g/l.), urea (1.0 g/l. vs 0.25 g/l.) and yeast extract (0.25 g/l. vs 0.10 g/l.) was beneficial to both growth and enzyme production. We substituted white mustard flour for the Canadian brown mustard flour prescribed by Reese because of availability.

We wanted to determine whether the non-specific requirements for *Crambe* ESP [6] would extend to this fungal thioglucosidase, so we grew the organism and utilized the extracellular enzyme in our studies. When allylglucosinolate was incubated for 1 hr with combinations of fungal or higher plant thioglucosidase and *Crambe* ESP, 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 [4]. 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 (Table 1) show that interactions of fungal thioglucosidase and *Crambe* ESP do occur, and result in the formation of 1-cyanoepithioalkane. This finding extends the range of organisms whose thioglucosidases are known to interact with ESP, and suggests that thioglucosidases from both sources are highly conserved. Consistent with previous work [4, 6], the results shown in Table 1 suggest that the allylglucosinolate is converted to 1-cyano-2,3-epithiopropane in the presence of ESP, but to allyl cyanide in the absence of ESP. No ESP was detected in *Aspergillus sydowi* QM 31c.

The enzymatic degradation of glucosinolates, which results in the formation of 1-cyanoepithioalkanes, proceeds via an intramolecular migration of sulfur from the

Table 1. Conversion of allylglucosinolate to organic aglucone products by combinations of fungal or plant thioglucosidase with *Crambe* ESP

| Source of thioglucosidase | ESP | Extent of reaction (% completion) | % of hydrolysed allylglucosinolate converted to organic aglucone products* | |
|---|---|---|---|---|
| | | | 1-Cyano-2,3-epithiopropane | Allyl isothiocyanate |
| None | None | 0 | 0 | 0 |
| None | Present | 0 | 0 | 0 |
| *Aspergillus sydowi* | None | 77 | 0 | 39 |
| *Aspergillus sydowi* | Present | 68 | 58 | 47 |
| *Sinapis alba* | None | 86 | 0 | 40 |
| *Sinapis alba* | Present | 71 | 46 | 46 |

*Values were obtained by direct measurement by GLC; for conditions, see Experimental.

glucose moiety to the vinyl group [12]. In this process, ESP possibly functions by interacting with thioglucosidase in an allosteric manner [4, 13]. This hypothesis seemed plausible since ESP has the effect of reducing thioglucosidase activity [4]. In these studies, we found that *Crambe* ESP reduces the level of activity of thioglucosidases from *A. sydowi* and *S. alba* by 17 and 12%, respectively. Accordingly, additional supporting evidence of allosteric interaction was sought.

In order to reveal the nature of the inhibitory effect of ESP on thioglucosidase, a series of incubations were conducted with *S. alba* thioglucosidase over a range of substrate concentrations, utilizing different amounts of ESP from *Crambe abyssinica*. Three sets of data corresponding to three levels of ESP with several substrate concentrations were used to fit the model equation:

$$v = \frac{V S}{K_m + S}$$

where $v$ is the velocity of the enzymatic reaction in mmol glucose liberated per min $\times 10^3$, $S$ is the substrate concentration in mmol/l., $V$ is the maximal velocity that can be attained by elevating the substrate concentration, and $K_m$ is the substrate concentration permitting a half-maximal velocity. A summary of the results is shown in Tables 2 and 3. The values (Table 2) for $K_m$ remained relatively constant, whereas the values for $V$ decreased slightly with increasing ESP concentration; this result implies non-competitive inhibition of thioglucosidase by ESP. Thus, ESP should interact at a site on thioglucosidase other than the point of attachment of substrate.

The standard deviation, based on deviations about the fitted lines, was $s = 0.0135$. If a single line is fitted to all data ignoring ESP, $s = 0.0222$. Based on a comparison of variances, there was significant variation in the parameters among ESP groups. Three separate equations yield a significantly better fit of the data than a single equation.

Although thioglucosidases from *A. sydowi* QM 31c and *S. alba* appear to be highly conserved, differences exist between them. The fungal enzyme is less resistant to heat, acids, alkalis and chemical inhibitors than is the mustard enzyme [10]. In addition, gluconolactone inhibits the thioglucosidase from *A. sydowi* but not that from *S. alba*. These differences, plus the fact that fungal thioglucosidase interacts with *Crambe* ESP, provided us with a potentially useful experimental probe of the mechanism for formation of 1-cyano-2,3-epithiopropane.

Table 2. Summary of models and precision estimates

| Group | Parameters | | Sum of squares of deviations |
|---|---|---|---|
| | $K_m$* | $V$† | |
| No. ESP | 1.4885 | 0.6263 | 0.00035 |
| 0.5 ml ESP | 1.3696 | 0.5669 | 0.00072 |
| 1 ml ESP | 1.3956 | 0.5382 | 0.00040 |

$$s^2 = \frac{0.00035 + 0.00072 + 0.00040}{3+3+2} = \frac{0.00147}{8} = 0.00018$$

$s = 0.0135$

| | | | |
|---|---|---|---|
| All data | 1.4192 | 0.57704 | 0.00594 |

$s^2 = 0.00594/12 = 0.00050$

$s = 0.0222$

Test of variation of parameters among ESP groups

$$F_{4,8} = \frac{0.00594 - 0.00147}{4 \cdot (0.00018)} = 6.07‡$$

*$K_m$ is the substrate concentration, in mmol/l., permitting a half-maximal velocity.

†$V$ is the maximal velocity of the enzymatic reaction, in mmol glucose liberated per min $\times 10^3$, that can be attained by elevating the substrate concentration.

‡Significant at 0.05 level.

We conducted a study to compare the rates of episulfide product formation utilizing ESP with equal activities of fungal and higher plant thioglucosidase (Fig. 2).

If fungal and higher plant thioglucosidases are somewhat different from one another, one would expect different conformations for species formed by interaction of ESP with thioglucosidases from the two sources. In principle, these two conformers could be detected if 1-cyano-2,3-epithiopropane were formed at different rates. The main question is, of course, whether the rate

Table 3. Observed and predicted velocity based on models in Table 2

| ESP (ml) | S (mM) | Velocity* Observed | Predicted |
|---|---|---|---|
| 0 | 0.1 | 0.035 | 0.039 |
| | 0.3 | 0.098 | 0.105 |
| | 1.0 | 0.247 | 0.252 |
| | 3.0 | 0.433 | 0.419 |
| | 10.0 | 0.538 | 0.545 |
| 0.5 | 0.3 | 0.091 | 0.101 |
| | 1.0 | 0.232 | 0.238 |
| | 3.0 | 0.410 | 0.389 |
| | 10.0 | 0.488 | 0.499 |
| 1 | 0.1 | 0.027 | 0.036 |
| | 0.3 | 0.085 | 0.095 |
| | 1.0 | 0.225 | 0.225 |
| | 3.0 | 0.380 | 0.367 |
| | 10.0 | 0.465 | 0.472 |

*mmol glucose liberated per min $\times 10^3$.

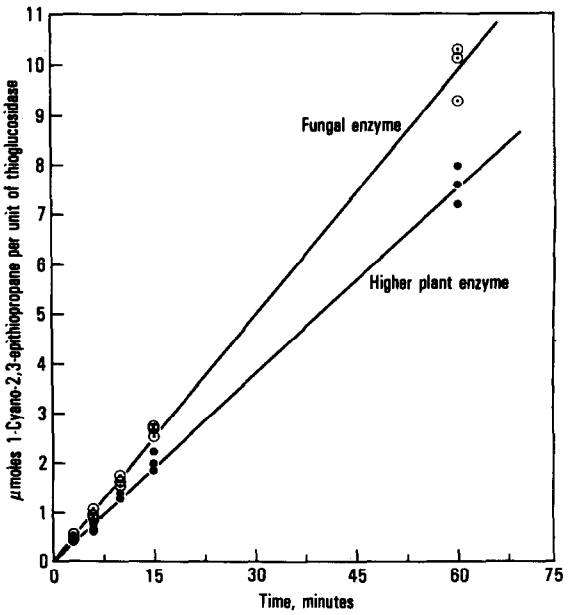


Fig. 2. Plot of μmol of 1-cyano-2,3-epithiopropane formed per unit of fungal and plant thioglucosidase vs time.

difference is large enough to be detected experimentally.

A plot of the μmol of 1-cyano-2,3-epithiopropane formed per unit of fungal and plant thioglucosidase vs time (Fig. 2) shows a distinct difference in the rates of episulfide product formation in the two cases: 0.166 μmol/min per unit of fungal thioglucosidase vs 0.127 μmol/min per unit of the corresponding higher plant enzyme. Thioglucosidase activity was not 'corrected' for inhibition by ESP. If such a 'correction' was done, a slightly greater difference in the rates of 1-cyano-2,3-epithiopropane formation would be observed: 0.200 μmol/min per unit of fungal thioglucosidase vs 0.144 μmoles/min per unit of the corresponding higher plant enzyme.

The analysis of variance is based on five different reaction times, measured in triplicate for each of the two thioglucosidases for a total of 30 observations (Table 4). The non-significance of the two terms labelled 'remainder' and 'E × remainder' indicates that the data are described satisfactorily by a linear model. There is a highly significant difference in the slopes of the lines, since 'E × linear' provides a comparison of slopes. The slopes are in μmol episulfide product formed per unit of thioglucosidase/min. The standard deviation per observation is 0.23, and the standard error of the mean of three points is 0.13 ( $= \sqrt{0.0518/3}$ ).

A summary of the equations (Table 5) shows that the intercept is not significantly different from zero, i.e. the lines pass through the origin. The slopes of 0.166 and 0.127 have a standard error of 0.003.

In addition to the mechanism of ESP's action, other factors were considered in accounting for the rate differences. The plots themselves (Fig. 2) tend to rule out selective inhibition or activation of one of the thioglucosidases by end products because the slopes are linear and pass through the origin. Deviations from linearity would be predicted if inhibition or activation of the enzymes were occurring. Reese *et al.* [11] observed that the products of the hydrolysis of allylglucosinolate did not inhibit the thioglucosidase from *A. sydowi* QM 31c, an enzyme known to be more labile and susceptible to

Table 4. Analysis of variance of enzyme assay results

| Source of variation | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Time ($T$) | 4 | | |
| Linear | 1 | 282.6408 | 282.6408 |
| Remainder | 3 | 0.1412 | 0.0471 n.s.* |
| Enzyme (E) | 1 | 3.7793 | 3.7793 |
| E × $T$ interaction | 4 | | |
| E × linear | 1 | 5.0634 | 5.0634† |
| E × remainder | 3 | 0.0274 | 0.0091 n.s.‡ |
| Variance | 20 | 1.0349 | 0.0518 |
| | 29 | 292.6870 | |

*n.s. = Not significant. There is no evidence that a more complex model than a linear one is needed.

†The significance of this mean square ($p < 0.01$) indicates that the lines for the two enzymes are not parallel.

‡The non-significance of the two remainder terms after fitting a linear model indicates that a linear model is adequate.

Table 5. Summary of equations relating μmol product ($p$) to time ($t$) in min

| Enzyme | Model $p = a + bt$ | Correlation $r$ |
|---|---|---|
| Fungal | $p = 0.0234^* + 0.1658t$ | 0.998 |
| Plant | $p = 0.0495 + 0.1267t$ | 0.998 |

*The standard error of an intercept '$a$' is 0.074. The standard error of a slope '$b$' is 0.0026.

inhibition than the corresponding enzyme from *S. alba*. We found that both thioglucosidases retained full enzyme activity under the temperature, pH and other reaction conditions employed for at least 2 hr; this is twice the time used in our longest incubations.

Since *Crambe* ESP converts allylglucosinolate to 1-cyanoepithioalkane in concert with thioglucosidase from either *S. alba* or *A. sydowi*, this enzyme appears to be highly conserved in both. Kinetic evidence implies that ESP acts as a non-competitive inhibitor of thioglucosidase. Distinctly different rates of episulfide formation were observed for the two thioglucosidases. Taken together, these two kinds of kinetic data support the hypothesis that ESP interacts with thioglucosidase allosterically. Studies are currently being conducted to elucidate the bio-organic reaction mechanism of this unique transformation fully.

## EXPERIMENTAL*

*Fermentation methods. Aspergillus sydowi* QM 31c was grown on Czapeks agar sants at 25° for 7 days, and stock cultures were stored in a refrigerator at 4° in sealed screw-cap tubes.

The standard liter of fermentation medium consisted of starch (10 g), *Sinapis alba* mustard flour (2.5 g), yeast extract (0.1 g), urea (1.0 g), $KH_2PO_4$ (2.0 g), $(NH_4)_2SO_4$ (1.4 g), $MgSO_4 \cdot 7H_2O$ (0.3 g), $CaCl_2$ (0.3 g), $FeSO_4 \cdot 7H_2O$ (5 mg), $MnSO_4 \cdot H_2O$ (1.5 mg), $ZnSO_4 \cdot 7H_2O$ (2.5 mg), $CoCl_2 \cdot 6H_2O$ (2.0 mg) and distilled $H_2O$, adjusted to pH 6.3 with aq. NaOH. The medium was sterilized in an autoclave at 121° for 15 min before use.

Fermentations were conducted at 25° in cotton-plugged 250 ml Erlenmeyer flasks containing 50 ml of medium. The medium was aerated by a rotary shaker operating at 200 rpm, describing a 2-in. stroke. Fermentations were initiated by suspending the surface spores from slants in 5 ml of sterile medium and using the suspension to inoculate stage I cultures. Thick 72-hr stage I cultures were used to inoculate stage II (production) fermentations. The inoculum (5 ml) was 10% of the vol. held in stage II culture flasks. Sinigrin (20 mg in 1.0 ml $H_2O$) was aseptically added to 24-hr stage II cultures to induce thioglucosidase production.

*Recovery of fungal thioglucosidase.* Mycelium from 72-hr-old stage II cultures was separated from the broth by filtration and discarded. The extracellular thioglucosidase [11] was recovered from the broth by $(NH_4)_2SO_4$ fractionation in the cold, according to the monograph of di Jeso [14]. Precipitates were separated by centrifugation (15 min, 15 300 *g*) at 0°. The desired protein fraction, precipitated by $(NH_4)_2SO_4$ at 40–80% saturation, was resuspended in 12 ml 10 mM maleate buffer, pH 5.9, containing 200 mM NaCl and 1 mM dithiothreitol (DTT). From 330 ml of broth (8 flasks of stage II cultures), 32 units of thioglucosidase were obtained. (A unit of enzyme hydrolyses 1 μmol of allylglucosinolate per min at 25°.)

*Preparation of white mustard thioglucosidase and ESP.* White mustard thioglucosidase and ESP from *Crambe abyssinica* were prepared by the methods of Petroski and Tookey [6]. Briefly, both proteins were purified by $(NH_4)_2SO_4$ fractionation followed by Sephacryl S-200 chromatography twice.

*Incubation of allylglucosinolate with thioglucosidase and ESP.* Incubation mixtures contained allylglucosinolate (24.1 μmol), either fungal thioglucosidase (0.2 ml, 62 μg protein, 0.53 units) or plant thioglucosidase (0.2 ml, 37 μg protein, 0.40 units), ESP (1.0 ml, 150 μg protein) and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (7.2 μmol), all in a total vol. of 3.0 ml 0.05 M maleate buffer, pH 5.9, containing 0.2 M NaCl and $6.5 \times 10^{-4}$ M DTT. Prior to the initiation of the reaction by addition of substrate, solns were purged with $N_2$ to remove dissolved $O_2$ [4]. Incubations were conducted in triplicate at 25° for 3, 6, 10, 15 and 60 min. Reactions were terminated by a quick freezing of the reaction mixture in dry-ice–EtOH. The extent of enzymatic hydrolysis of allylglucosinolate at 60 min was measured by determination of liberated glucose, utilizing the Lancer Glucose Auto/Stat Kit Method based on the procedure of Trinder [15].

*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 [16]. Corrections were made for pre-formed aglucone products present in crude enzyme preparations. The episulfide product (1-cyano-2,3-epithiopropane) was independently determined by a colorimetric assay using 4-(*p*-nitrobenzyl)-pyridine [17].

*Kinetic study incubations.* Incubation mixtures contained allylglucosinolate (0, 0.1, 0.33, 1.0, 3.3 or 10.0 mM), white mustard thioglucosidase (0.2 ml, 30 μg protein, 0.60 units), ESP (0, 0.5 or 1.0 ml, 640 μg protein per ml) and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (6.0 μmol), all in a total vol. of 2.5 ml 0.05 M maleate buffer, pH 5.9, containing 0.2 M NaCl and $6.5 \times 10^{-4}$ M DTT. Prior to the initiation of the reaction by addition of substrate, solns were purged with $N_2$ to remove dissolved $O_2$ [4]. Incubations were conducted for 8 min. Reactions were terminated by immersion in a boiling $H_2O$ bath for 2 min. The extent of enzymatic hydrolysis of allylglucosinolate was measured by determination of liberated glucose, utilizing the Lancer method.

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*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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