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# ALLYLGLUCOSINOLATE BIOSYNTHESIS IN *BRASSICA CARINATA*

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**Key Word Index**—*Brassica carinata*, Brassicaceae, biosynthesis, glucosinolates, allylglucosinolate, methionine, aminotransferase

**Abstract**—Glucosinolate levels in leaves of *Brassica carinata* were found to increase acropetally This accumulation pattern was paralleled by the rate of [U-$^{14}$C]methionine incorporation into allylglucosinolate in radiotracer uptake experiments and by the distribution of an extractable methionine–glyoxylate aminotransferase activity. This enzymatic activity may catalyse the first step in the chain extension of methionine which is required for alkylglucosinolate biosynthesis in *Brassica*

## INTRODUCTION

Glucosinolates, which are characteristic of the Brassicaceae, have their biogenetic origins in a limited number of protein amino acids [for review, see 1–3] The glucosinolate profiles of seeds of the genus *Brassica* are dominated by allylglucosinolate (sinigrin), but-3-enylglucosinolate and 2-hydroxy-but-3-enylglucosinolate (progoitrin) [4]. These compounds have been proposed to be derived from methionine, and in turn homomethionine, by a set of chain extension reactions analogous to those involved in leucine biosynthesis [5] Tracer studies of allylglucosinolate formation in *Armoracia* [6, 7], and *B campestris* [8], and of 2-hydroxy-but-3-enylglucosinolate in *B napobrassica* [9] have supported this hypothesis, but the enzymes involved have yet to be detected or characterized

Conceptually, the entrypoint into methionine-derived glucosinolate biosynthesis could be viewed either as the step converting methionine to its keto-acid, 2-keto-4-methylthiobutyric acid (KMTB), or as the putative condensation in which KMTB is linked with acetyl-CoA. In order to begin to clarify this question, and to gain some insight as to the optimal tissue for studies of glucosinolate enzymology in *Brassica*, a study was undertaken of the pattern of glucosinolate biosynthesis and accumulation in various aerial tissues of *B. carinata*. At the same time, the distribution of extractable aminotransferase activity toward methionine was also examined

## RESULTS AND DISCUSSION

Alkyl glucosinolates account for 80–90% of the total glucosinolates in leaf tissue of the *B carinata* genotype used in the present study, while indole glucosinolates dominate (80%) the root glucosinolate profiles. Leaves closest to the stem apex exhibited higher allylglucosinolate levels than lower leaves (Fig. 1). An analogous pattern of thiocyanate distribution has previously been observed in kale [10]. Our results indicate that this is both a leaf age and leaf position dependent phenomenon. Glucosinolate concentration in individual leaves of *B. carinata* is generally higher during early stages of leaf development and declines following leaf maturation, but the lower leaves never attain the concentration of glucosinolates observed in the uppermost leaves (Fig. 2).

To establish whether the observed differences in glucosinolate accumulation at various positions on the plant were correlated with the relative rates of *in situ* biosynthesis, feeding experiments were conducted with individual leaves. Higher relative rates of L-[U-$^{14}$C]

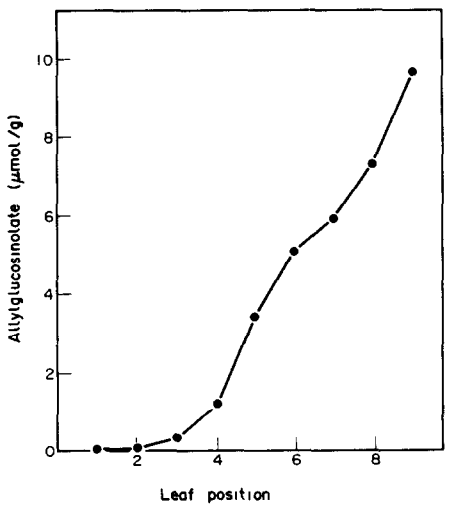


Fig. 1 Allylglucosinolate content (μmol/g dry wt) of leaves of six-week-old *B carinata* seedlings Starting with the first leaf above the cotyledon, the leaves are numbered consecutively according to their position on the stem

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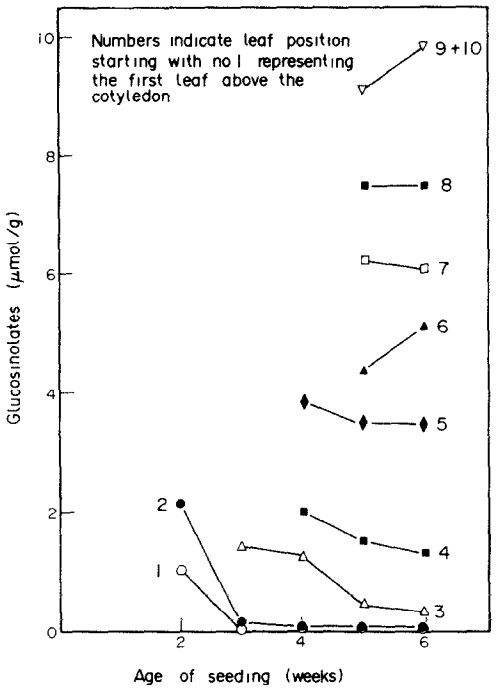


Fig 2 Time course of glucosinolate accumulation (μmol/g dry wt) in leaves of 2- to 6-week-old *B carinata* seedlings Numbers indicate position of sampled leaves as in legend of Fig. 1 Leaves from positions 9 and 10 were bulked together to ensure sufficient sample size for analysis

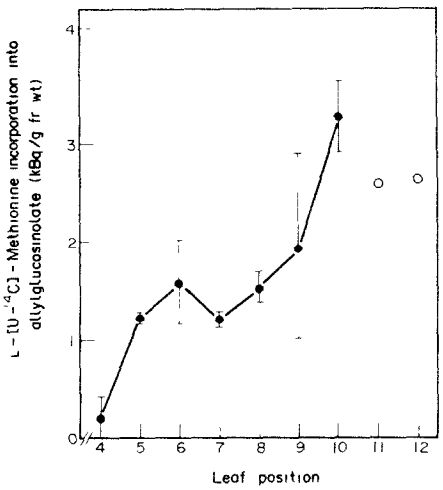


Fig 3 Incorporation of L-[U-$^{14}$C]methionine into allylglucosinolate in individual excised leaves of 6-week-old seedlings of *B carinata* Solid circles represent the mean of three feedings while vertical bars represent the standard deviation of the results Open circles indicate the results of single samples

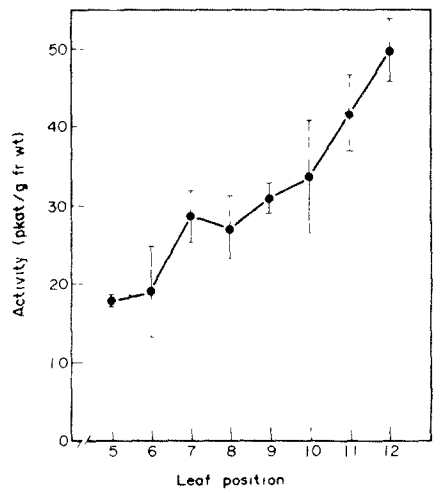


Fig 4 Methionine-glyoxylate aminotransferase activity in crude desalted extracts of individual leaves from 6-week-old *B carinata* seedlings Data points represent the means of three leaf extracts Standard deviation of the results are indicated by vertical bars

methionine incorporation into allylglucosinolate were observed in upper leaves (Fig 3), a pattern which parallels the relative accumulation levels. This suggests that the rate of *de novo* glucosinolate biosynthesis could be an important factor determining leaf glucosinolate concentration, although modulation of glucosinolate pool size by export or degradation, or by import from a remote site of synthesis, cannot be ruled out.

It has been shown previously that many glucosinolates are derived from non-protein amino acids which are chain-extended homologues of their protein amino acid precursors. The production of these amino acid homologues is thought to occur in a manner analogous to the biosynthesis of leucine from valine [5]. According to this model, the chain extension pathway for the biosynthesis of glucosinolates involves transamination of the parent amino acid to its keto acid prior to condensation with acetyl CoA Dehydration, rehydration, oxidative decarboxylation and transamination of the chain extended keto acid to the higher amino acid homologue would complete the chain extension pathway. The role of keto acids as biosynthetic intermediates in the chain extension reaction has, however, never been experimentally demonstrated When an excess of unlabelled KMTB was administered to *B carinata* leaves simultaneously with L-[U-$^{14}$C]methionine, the incorporation of label into allyl glucosinolate was reduced but the effect was not consistent (unpublished data). This may indicate that KMTB has poor access to a tightly coupled biosynthetic sequence It is also important to note, however, that KMTB is an immediate precursor of methionine in methylthio recycling from 5'-methylthioribose in ethylene-producing tissues [11] and that this pathway is probably functional in polyamine producing tissues as well [12] Depending on the metabolic flux through these pathways, administered KMTB may be diverted away from the glucosinolate biosynthetic pathway to a greater or lesser extent, contributing to variable results

To test the proposal that formation of KMTB is the initial step in the chain extension pathway, *B carinata* leaf enzyme extracts were assayed for the presence of an aminotransferase activity able to utilize methionine This enzyme activity increased acropetally (Fig 4) in parallel

with allyl glucosinolate accumulation (Fig 2) and glucosinolate biosynthetic rate as measured by L-[U-$^{14}$C]methionine incorporation (Fig. 3).

The partially purified (Glover, J. and Ellis, B. E., manuscript in preparation) aminotransferase utilized methionine and glutamine most effectively as substrates (Table 1). When tested for keto acid specificity with methionine as the amino donor, glyoxylate proved to be the best co-substrate (Table 2).

We therefore conclude that the first step in allyl glucosinolate biosynthesis in *Brassica* may be catalysed by a methionine–glyoxylate aminotransferase. It is also possible that this activity functions in the direction of methionine formation in the final step of the recovery pathway for the methylthio moiety of methionine following polyamine and/or ethylene biosynthesis, or that it serves as the first step in a methionine catabolic sequence similar to that described in mammalian systems [13], but which has not been thoroughly investigated in plants. Whether isoenzymic forms of methionine: glyoxylate aminotransferase activity exist in *Brassica* to carry out each of these potential functions remains to be determined. Further purification of the aminotransferase, and the identification and characterization of other enzymes in the chain extension pathway are expected to provide a more definitive answer to this question.

Table 1. Substrate specificity of methionine· glyoxylate aminotransferase from *B carinata* with respect to keto acids

| Amino donor | Amino acceptor | Relative activity* |
|---|---|---|
| Glutamine | KMTB | 187 |
| | Glyoxylate | 100 |
| Methionine | Glyoxylate | 100 |
| | KMTB | 90 |
| | Pyruvate | 35 |
| | Oxaloacetate | 24 |
| | α-Ketoglutarate | 19 |

*Results expressed as a percentage of activity obtained with glyoxylate.

Table 2. Substrate specificity of methionine. glyoxylate aminotransferase from *B carinata* with respect to amino acids

| Amino donor | Relative activity* |
|---|---|
| L-Methionine | 100 |
| L-Methionine sulphoxide | 54 |
| L-Methionine sulphoximine | 35 |
| L-Methionine sulphone | 29 |
| *S*-Methyl-L-cysteine | 49 |
| L-Homoserine | 56 |
| L-Glutamine | 106 |
| L-Histidine | 16 |
| Others† | <10 |

*Results expressed as a percentage of activity obtained with methionine

†D,L-Homomethionine, L-alanine, α-aminobutyrate, L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine, L-tryptophan, glycine, L-serine, L-threonine, L-cysteine, L-asparagine, L-aspartic acid, L-glutamic acid, L-lysine, L-arginine, L-ornithine, L-citrulline

## EXPERIMENTAL

*Plant material.* Seeds of *Brassica carinata* var 4218 (Agriculture Canada Research Station, Saskatoon, Saskatchewan) were sown at weekly intervals, maintained at 22° on a day/night cycle of 16/8 hr and watered regularly with a commercial fertilizer soln (Plant Prod, Plant Products Ltd, Bramalea, Ont). For determination of glucosinolate profiles, leaves from each position along the stem were harvested from plants in each age group, frozen in liquid $N_2$ and lyophilized The dried samples were stored at room temp in a desiccator until extractions were performed

*Purification of* [Me-$^3$H]*methionine* Upon storage, $^3$H-methionine was found to slowly oxidize to methionine sulphoxide. Methionine preparations were therefore purified before use by a modified reduction procedure reported in [12] After incubation in 0.7 M 2-mercaptoethanol, 10 mM Na-Pi buffer, pH 7 5, for 1 hr at 100°, the methionine was purified by descending PC on pre-washed Whatman 3 MM paper in *n*-BuOH–HOAc–$H_2O$ (20 5 11) The methionine band was eluted with $H_2O$ at 4° and stored frozen

*Radiotracer feeding experiments* Leaves from 3- to 5-week-old plants were cut from plants while the petiole was submerged in $H_2O$ Purified L-[U-$^{14}$C]methionine ($3.7 \times 10^4$ Bq, 10 nmol, 200 μl) was administered via the transpiration stream, followed by $H_2O$ Two hr after beginning the feeding, leaves were extracted in MeOH as described below

*Extraction and analysis of glucosinolates.* Isolation of desulphoglucosinolates followed a modified procedure of ref [14] For glucosinolate profile analyses, 40 μl of 5 μM benzylglucosinolate was added to the initial extract as an int standard Leaves were extracted (2 × 15 min, 100% MeOH, 1 × 15 min, 70% MeOH) at 70° and the pooled extracts were dried under a stream of $N_2$ The residues were each taken up in 1 ml $H_2O$ and, for radiotracer feeding experiments, extracted (× 3) with 5 ml hexane The aq phase was made to 1 5 ml, brought to 15 mM Ba/Pb acetate and centrifuged The supernatant was applied to a 500 μl column of DEAE Sephadex A-25 (acetate form) and washed with 2.5 mL $H_2O$ 100 μl arylsulphatase preparation was added and the column was sealed and incubated overnight at 37° Desulphoglucosinolates were eluted with 1 5 ml $H_2O$, and passed through a 0 45 μm filter prior to analysis by HPLC For radiotracer feeding experiments, 1 ml fractions of the HPLC effluent were collected for liquid scintillation counting

Desulphoglucosinolate analysis was carried out on a Techsphere 3 μm C-18 column using a solvent program which consisted of 1% MeCN in $H_2O$ for 5 min, a gradient of 1% to 23% MeCN in 10 min, and a constant 23% for an additional 10 min (detection: UV absorbance at 226 nm) Allyldesulphoglucosinolate was identified by co-chromatography with arylsulphatase-treated sinigrin. Indole desulphoglucosinolates were tentatively identified by their absorbance at 280 nm and their labelling from administered $^3$H-tryptophan Quantitation of allyl glucosinolate was by comparison of the integrated peak areas with that of the internal standard using the response factors reported in [15]

*Enzyme extraction* Leaves were frozen in liquid $N_2$ and stored at −60° until extracted Individual leaves were ground in a cold mortar in liquid $N_2$ and the frozen powder was transferred to test tubes containing cold extraction buffer (0 2 M Tris–HCl adjusted to pH 7.4 at 25°) After 30 min extraction, the brei was filtered through glass wool and centrifuged (10 min, 20 000 g, 4°)

1 ml aliquots were desalted on Biogel P-6-DG equilibrated with assay buffer (0 02 M Tris–HCl adjusted at pH 8 2 at 25°) Protein concentrations were determined according to [16]

*Enzyme assays* The assay mixture contained 200 $\mu$l desalted crude extract, assay buffer, 10 $\mu$M pyridoxal-5′-phosphate, 10 mM glyoxylate, and 1 mM L-[Me-$^3$H]methionine (1 0 $\times 10^{10}$ Bq/mol) in a final vol of 400 $\mu$l The enzyme was first preincubated in the presence of unlabelled methionine for 30 min at 30 Preliminary experiments showed that this step was necessary to ensure linearity within the assay period The assay was initiated with the addition of keto acid and labelled methionine After 60 min the assay was stopped by the addition of 20 $\mu$l 5 M HCl and 10 $\mu$l 40 mM KMTB The mixture was extracted with 1 ml toluene–EtOAc (1 · 1), and a 0 8 ml aliquot of the organic phase was removed for liquid scintillation counting

To test the efficacy of potential amino donors, a partially purified enzyme preparation (40 8 pkat/mg; 66 8 fold purification over the crude extract) was desalted into the assay buffer (0 05 M TAPS, pH 8 2) by passage through pre-equilibrated Biogel P-6-DG 50 $\mu$l of this enzyme was preincubated with an amino donor (1 mM) and the assay initiated with the addition of 20 $\mu$l of 20 mM [1-$^{14}$C]glyoxylate (4 2 $\times 10^9$ Bq/mol) in a final vol of 400 $\mu$l After 60 min the assay was terminated with 20 $\mu$l 7% trichloroacetic acid The entire mixture was passed through a 0 5 ml bed of SP-Sephadex C-25, washed with 2 ml $H_2O$ and eluted with 0 6 ml 2 M $NH_4OH$ The eluate was neutralized with HOAc and a 200 $\mu$l aliquot submitted to liquid scintillation counting In one set of assays [Me-$^3$H]KMTB (1 3 $\times 10^{10}$ Bq/mol) (prepared by oxidation of L-[Me-$^3$H]-methionine with L-amino acid oxidase) was substituted for glyoxylate

Assays were carried out in triplicate, controls contained boiled enzyme. The identities of the incubation products were confirmed by co-chromatography with authentic compounds on TLC

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