The effect of glucosinolates (mustard oil glycosides) and products of their enzymic degradation on the infectivity of turnip mosaic virus

J. ŠPAK*, L. KOLÁŘOVÁ*, J. LEWIS**, and G.R. FENWICK**

*Institute of Plant Molecular Biology, Academy of Sciences of Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic**
*AFRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich NR 47 UA, Great Britain***

Abstract

The effect of glucosinolates sinigrin, progoitrin, epiprogroitrin, gluconapin, gluconapoleiferin, glucobrassicanapin, glucotropaeolin and gluconasturtiin without and with the glucosinolate-degrading enzyme (myrosinase, EC:3.2.3.1.), on the infectivity of turnip mosaic virus was studied. Little or no effect was observed when the intact glucosinolate (2.5 μmol cm^{-3}) was added to the suspension of turnip mosaic virus (TuMV, isolate Ruzyně; 0.2 mg cm^{-3} in 0.01 M potassium phosphate buffer) at both pH 7 and pH 6. A significant decrease of virus infectivity was, however, observed when 0.25, 1.25 and 2.5 μmol cm^{-3} of the glucosinolate together with 0.31, 1.56 and 3.13 mg cm^{-3} of the myrosinase, respectively, was added to the virus suspension of both pH 7 and pH 6. The effect, which was greater at pH 6, was most intense with sinalbin and glucobrassicin substrates.

Introduction

Glucosinolates (mustard oil glycosides) as secondary metabolites play an important role in the metabolism of plant hormones (Helmlinger *et al.* 1983, 1987, Ludwig-Müller and Helmlinger 1977) and in the ecology of the family *Brassicaceae* (Feeny 1977). They determine, for example, the relationships between plants and herbivorous insects (Herrbach 1985). Recent researches have demonstrated an accumulation of glucosinolates in the green parts of plants infested with aphids (Lammerinck *et al.* 1984) and other insects (Koritsas *et al.* 1989, Birch *et al.* 1990). Similarly, the role of alkenyl and indole glucosinolates in the resistance mechanism of brassicas to diseases has been studied (Rausch *et al.* 1983).

The action of these substances on plant viruses is less understood, however, a previous study (Špak 1988) has revealed the virocidal effect of the sinigrin (2-propenyl glucosinolate) on the turnip mosaic virus (TuMV).

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Therefore we decided to study the effect of further glucosinolates on the purified virus under controlled conditions of pH and ionic strength, in order to gain further understanding of the role of glucosinolates and the products of their enzymic degradation in the defense mechanism of brassicas to plant viruses.

Material and methods

Virus purification: *Brassica pekinensis* (Lour) Rupr. cv. Nozaki plants were grown during the experiments in a greenhouse at 18 to 25 °C. They were inoculated at the stage of four true leaves with the turnip mosaic virus (TuMV), the isolate Ruzyně (Špak 1988).

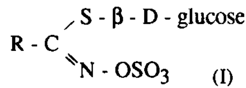
Leaves with developed symptoms of the infection were collected 17 - 20 d after inoculation and 100 g were homogenized for 3 min in a blender with chilled 0.5 M potassium-phosphate buffer, pH 7.5, containing 1 % Na_2SO_3 (1:2, m/v). Following filtration through a nylon cloth the homogenate was centrifuged at 7 000 *g* for 10 min in a *Janetzki K 24* centrifuge. A chloroform-*n*-butanol mixture (1:1, v/v) was added to the supernatant to a final concentration of 20 % and the mixture stirred at 4 °C for 1 h. The resulting suspension was separated by centrifugation (4 500 *g* for 10 min) and the clear yellow aqueous supernatant phase collected. The virus was precipitated from this fraction by addition of 8 % (m/v) PEG 6 000 and stirring at room temperature for 1 h. Pellets were concentrated by centrifugation (19 500 *g* for 10 min) resuspended overnight in 5 cm^3 of 0.1 M potassium-phosphate buffer pH 7.0 and recentrifuged (7 000 *g* for 10 min). The virus concentration of the resulting supernatant was determined by UV-spectrophotometry according to Choi *et al.* (1977). A freshly purified virus preparation was used in each experiment.

The procedure removes any added myrosinase, too. After adding 1 mg of the glucosinolates to the virus suspension at either pH 7 or pH 6 and the mixture remained without a bad odour, indicative of the breakdown of glucosinolates.

Preparation of glucosinolates and myrosinase: The following glucosinolates (see Table 1 for structures) were isolated by methods of Hanley *et al.* (1983) and Peterka and Fenwick (1988) from plant sources. Sinigrin (potassium salt, molecular mass - M_r 397; from *Brassica juncea* seed), glucosinalbin (potassium salt, M_r 498; from *Brassica campetris* seed), glucobrassicanapin (potassium salt, M_r 425; from *Brassica campestris* seed), progoitrin (potassium salt, M_r 427; from *Brassica napus* seed), epi-progoitrin (potassium salt, M_r 427; from *Crambe abyssinica* seed), glucotropaeolin (tetramethylammonium salt, monohydrate, M_r 500; from *Lepidium sativum* seed), sinalbin (tetramethylammonium salt, monohydrate, M_r 498; from *Sinapis alba* seed), gluconasturtiin (potassium salt, M_r 461; from *Nasturtium officinale*) and glucobrassicin (potassium salt, M_r 486; from apical leaves of *Brassica oleracea*) plants). Purities were >98% as estimated by glucose release measurement (Heaney and Fenwick, 1981) and fast atom bombardment mass spectrometry (Fenwick *et al.* 1982). In addition, all glucosinolates were homogeneous by high performance liquid chromatography (Spinks *et al.* 1984).

Myrosinase was isolated from white mustard seed (*S. alba*) according to the procedures of Appelquist and Josefsson (1967).

Table 1. Glucosinolates examined in the present study



Trivial name	Side chain (R, I)	pH 6.7 hydrolysis products
Sinigrin	2-propenyl-	isothiocyanate
Gluconapin	3-butenyl-	isothiocyanate
Glucobrassicanapin	4-pentenyl-	isothiocyanate
Progoitrin	2-hydroxy-3-butenyl-	oxazolidine-2-thione
Epiprogoitrin	2-hydroxy-3-butenyl-	oxazolidine-2-thione
Glucotropaeolin	benzyl-	isothiocyanate
Sinalbin	*p*-hydroxybenzyl-	aromatic alcohol, thiocyanate ion
Gluconasturtiin	2-phenethyl-	isothiocyanate
Glucobrassicin	3-indolylmethyl-	indole-3-carbinol, diindolylmethane, thiocyanate ion

Estimation of the effect of the pH value on the infectivity of TuMV: The suspension of the purified virus in 0.1 M potassium- phosphate buffer (0.2 mg cm^{-3}) was dialysed at 4 °C for 1 h against Mc Ilvaine buffer (0.1 M citric acid + 0.1 M Na_2HPO_4, 800 ml, pH 7, 6, 5 and 4). The pH value was measured at the end of dialysis. Each sample was inoculated on half of a leaf of *Nicotiana tabacum* L. cv. Samsun, which had been previously dusted with carborundum 600 mesh. The sample at pH 7 was inoculated on the second half of the leaf as control.

In total, 30 leaves on 10 tobacco plants were inoculated in each experiment, which was conducted in triplicate. Local necrotic lesions were counted, which developed on inoculated tobacco leaves between seven to ten days after the inoculation.

The infectivity of the virus was calculated from the average numbers of lesions on the control and the experimental halves of each leaf and is expressed as a percentage of the control figure.

The concentration of the purified virus (0.2 mg cm^{-3}), which was established experimentally, corresponded to 30 to 100 lesions on half a leaf, this being optimal for both counting and statistical evaluation.

Experiments with pure glucosinolates: The suspension of the purified virus in 0.1 M potassium-phosphate buffer pH 7.0 was diluted with distilled water to a final concentration of 0.2 mg (virus) cm^{-3} in 0.01 M buffer. To 1 cm^3 of this suspension the glucosinolate was added to final concentration 2.5 μmol cm^{-3}; 1 cm^3 of the suspension alone served as control. Both samples were incubated at 25 °C for 1 h; the pH was measured before and after incubation and the inoculation on tobacco leaves carried out as described above.

Experiments on the effect of enzymatic degradation products of the glucosinolates: 0.5 cm^3 of the virus suspension (0.4 mg cm^{-3} in 0.02 M potassium-phosphate buffer pH 7.0) was diluted with 0.5 cm^3 of distilled water (control); to the remainder 0.5 cm^3 of the suspension the glucosinolate solution (0.3 cm^3) and the myrosinase solution (0.2 cm^3) in distilled water were added.

A range of the final concentrations of the glucosinolates 0.25, 1.25 and 2.5 μmol cm^{-3}, (approximately 0.1, 0.5 and 1 mg cm^{-3}) and of the myrosinase (0.31, 1.56 and 3.13 mg cm^{-3}) were used in the experiments conducted at pH 7 and pH 6.

In the experiments conducted at pH 6 the virus suspension was dialysed at 4 °C for 1 h against 0.1 M potassium-phosphate buffer pH 6 and then treated as described above.

Experimental and control samples were incubated at 26 °C for 1 h and the pH measured before and after incubation. The samples were inoculated on the leaves of tobacco plants as described above.

Experiments on the effect of ionic strength on the virocidal activity of the glucosinolates: The suspension of the purified virus (0.2 mg cm^{-3}), and the same containing sinalbin or glucobrassicin (0.25 μmol cm^{-3}) and myrosinase (0.31 mg cm^{-3}) were prepared in 0.5 M potassium phosphate buffer pH 7 and then treated in the same way as described above.

Results

The pH value of buffers lower than 7 decreased the infectivity of TuMV suspension. At pH 7, 6, 5 and 4 the infectivity of the virus was 100, 74.1, 13.8 and 0 %, respectively.

Table 2. Effect of the glucosinolates (2.5 μmol cm^{-3}) on the infectivity of TuMV (0.2 mg cm^{-3}) at pH 7 and pH 6, expressed in % of control

Glucosinolate	pH 7	pH 6
Sinigrin	81.9 ± 23.6	92.0 ± 17.0
Gluconapin	98.7 ± 18.3	105.9 ± 13.4
Glucobrassicanapin	122.1 ± 28.0	110.2 ± 14.8
Progoitrin	89.0 ± 12.8	103.3 ± 10.3
Epiprogoitrin	98.4 ± 10.1	100.0 ± 8.7
Glucotropaeolin	109.3 ± 13.6	109.2 ± 17.9
Sinalbin	98.7 ± 23.9	100.5 ± 28.1
Gluconasturtiin	113.2 ± 26.2	104.9 ± 18.7
Glucobrassicin	49.5 ± 18.9	70.5 ± 12.5

Because the viral nucleoprotein was precipitated at pH values lower than 6 and a marked decrease in the infectivity of the virus was observed we decided to study the effect of glucosinolates on TuMV only at pH 7 and pH 6.

No effect on the infectivity of TuMV was found in preliminary experiments when myrosinase (0.31, 1.56 and 3.13 mg cm^{-3}) was added to the purified virus.

The results of the experiments into the effect of intact glucosinolates on the virus are summarised in Table 2. With the exception of glucobrassicin (3-indolylmethyl glucosinolate) and sinigrin the effect of 1 mg of each glucosinolate was weak. There was no significant difference between the results obtained at pH 7 and pH 6.

As intact glucosinolates could only hypothetically affect the virus in plants, attention was focused on the effect of the products arising after treatment of glucosinolates with myrosinase in different concentrations. The results are shown in Table 3. It is evident that the products of enzymatic decay of the glucosinolates decrease the infectivity of TuMV significantly and the effect is more severe at pH 6.

Table 3. Effect of the products of enzymic degradation of the glucosinolates on the infectivity of TuMV at pH 7 and pH 6 expressed in % of control.

Glucosinolate concentration [μmol cm^{-3}]	pH 7 0.25	1.25	2.5	pH 6 0.25	1.25	2.5
Sinigrin	114.6± 0.1	77.8±1.9	23.3± 2.9	63.5±15.5	52.8±16.2	0.7± 0.6
Gluconapin	72.2±23.2	39.0	5.8± 4.8	48.3± 1.4	2.4± 0.7	2.5± 1.6
Glucobrassicanapin	71.5±15.5	87.5	79.2± 0.3	78.7±10.1	14.8	10.9±18.9
Progoitrin	76.2± 3.2	50.8±1.2	73.9± 1.5	66.8±24.6	13.9	9.9±17.2
Epiprogoitrin	82.3±12.1	63.5	79.1± 9.2	80.9± 7.1	6.3	0.2± 0.3
Glucotropaeolin	71.6±19.0	58.8±7.1	42.9±19.3	73.8±12.1	0	8.4± 4.5
Sinalbin	2.8± 1.7	0	0.1± 0.1	6.9± 1.8	2.8± 0.4	0
Gluconasturtiin	5.4± 2.2	62.3±9.1	40.1±21.8	85.4± 7.4	0	0
Glucobrassicin	0.1± 0.1	0	0.4± 0.4	1.2± 1.6	0	0

Table 4. Effect of the products of enzymic degradation of the glucosinolates on the pH values of TuMV suspension in 0.01 M potassium phosphate buffer pH 6.

Glucosinolate concentration [μmol cm^{-3}]	control	0.25	1.25	2.5
Sinigrin	6.1 ± 0.10	5.9 ± 0.03	3.5 ± 0.04	2.3 ± 0.03
Gluconapin	6.2 ± 0.15	5.8 ± 0.02	3.3 ± 0.01	3.2 ± 0.38
Glucobrassicanapin	6.2 ± 0.05	5.8 ± 0.01	3.7 ± 0.03	3.2 ± 0.02
Progoitrin	6.1 ± 0.04	5.7 ± 0.07	3.5 ± 0.03	3.1 ± 0.22
Epiprogoitrin	6.1 ± 0.01	5.9 ± 0.02	3.3 ± 0.01	3.0 ± 0.01
Glucotropaeolin	6.1 ± 0.05	5.6 ± 0.03	3.4 ± 0.03	3.1 ± 0.38
Sinalbin	6.1 ± 0.03	5.7 ± 0.02	3.1 ± 0.03	3.1 ± 0.33
Gluconasturtiin	6.1 ± 0.04	5.9 ± 0.01	3.5 ± 0.04	3.0 ± 0.04
Glucobrassicin	6.2 ± 0.03	5.6 ± 0.41	3.1 ± 0.14	3.0 ± 0.12

The effects of the hydrolysis products of sinalbin (*p*-hydroxybenzyl glucosinolate) and glucobrassicin were most evident being noticeable even at the lowest concentration (0.1 mg cm^{-3}). Only a weak effect was found with progoitrin, epiprogoitrin (both epimers of 2-hydroxy- 3-butenyl glucosinolate) and glucobrassicanapin (4-pentenyl glucosinolate) at pH 7.

Table 5. The effect of the products of enzymic degradation of sinalbin and glucobrassicin in the concentration 0.25 μmol cm^{-3} in 0.5 M potassium phosphate buffer (pH 7) on the infectivity of TuMV.

Glucosinolate	infectivity [% of control]	pH (start)	pH (end)
Sinalbin	28.55 ± 12.2	7.08	7.04
Glucobrassicin	9.14 ± 3.7	7.01	7.00

Discussion

A key element of this study may be the ionic strength of buffers used. It is difficult to predict and simulate the pH value, ionic strength and other conditions of the reaction in plants after their damage, since a complex mixture of compounds, including enzymes will be present. It is known that concentrated buffers (0.5 M) can protect the virus but simultaneously damage inoculated leaves (Špak 1988). At a lower buffer concentration (0.01 M) the pH values decreased from 7 or 6 to 3, respectively, as the result of the enzymatic reaction (see Table 4), in accordance with the work of Gil and MacLeod (1980).

As in previous work (Špak 1988), experiments using 0.5 M phosphate buffer showed a certain protective effect on the virus of high ionic strength; the values of the virus infectivity being somewhat higher than in experiments with 0.01 M buffer (compare Table 5 with Table 3). It is, however, evident that even highly concentrated buffers did not prevent or eliminate the virocidal effect of the products of enzymic breakdown of the glucosinolates. Thus while the decrease of pH values was similar for all glucosinolates (Table 4), the decrease of the virus infectivity varied greatly (compare results in Table 3).

The formation of potentially toxic cleavage products upon the hydrolysis of glucosinolates by myrosinase has led to a proposal that the myrosinase-glucosinolate system might have a role as a non-specific defense system in the plant (Höglund *et al.* 1991, Lüthy and Matile 1984).

Because of the lack of studies on plant viruses our results can be compared only with those obtained with fungal pathogens. Butcher *et al.* (1984), using radio-chromatographic procedures established the concentration of glucobrassicin in root tissues of swede infected with *Plasmodiophora brassicae* Woron to be more than doubled during the growth of clubs while the concentration of neoglucobrassicin decreased slightly. The results of Rausch *et al.* (1983) demonstrate both an increased synthesis and a dramatically changed metabolism of glucobrassicin in *P. brassicae* - infected tissue leading to a specific route for indol-3-yl-acetic acid biosynthesis,

usually not encountered in the healthy root tissue of brassicas. The results of the present study support the suggestion of Buchwaldt *et al.* (1985) that the products formed by enzymic hydrolysis of glucosinolates with myrosinase following the disruption of the plant cell by fungal pathogens may be more important than the intact glucosinolates in the interaction between pathogens and their brassica host plants.

The results obtained with glucobrassicin appear to be interesting with respect to the significant role in the defense mechanism of brassicas of 3-indolylmethyl glucosinolates which predominate in green parts of plants. Koritsas *et al.* (1989) found greatly increased levels of indole glucosinolates and especially that of glucobrassicin in *Psylliodes chryocephala* L. infested tissue of oilseed rape. Similarly, Birch *et al.* (1990) found considerably modified both concentrations and relative proportions of individual glucosinolates in *Delia floralis* Fall. attacked roots of forage and oilseed rape and increased levels of indolyl-containing glucosinolates.

It is difficult, however, to directly relate our results to the processes occurring in freshly disrupted cells after attack by an insect and the resultant transmission of the virus. The length of time for the glucosinolate breakdown products to affect the transmitted virus is much shorter. On the other hand, under such circumstances there are several glucosinolates and their products acting on the virus whose complex effect could be synergistic, and therefore different than the results presented here for individual glucosinolates and their hydrolysis products. Moreover, the effect could be enhanced in different glucosinolates by other compounds, *e.g.* ascorbic acid (ascorbate) which is present in cell vacuoles together with glucosinolates (Lüthy and Matile 1984).

The ratio of the total and individual glucosinolates, and of the virus could differ markedly in brassicas. For example, the content of glucobrassicin in the green parts of brussels sprouts varied between 0.1 and 3.2 mmol kg^{-1} fresh mass (McDanell *et al.* 1988) and according to Wolfson (1980) sinigrin content in *Brassica nigra* (L.) Koch amounts to 0.04 - 0.32 % of fresh leaf matter. The yield of purified TuMV varied from 15 to 40 mg kg^{-1} fresh mass of infected plants (Choi *et al.* 1977). In the present experiments concentrations of glucosinolates 0.25 - 2.5 μmol cm^{-3} and 0.2 mg (virus) cm^{-3} were used.

The results emphasize the limitations of analysing the whole content of glucosinolates in plants as a means of predicting the resistance or susceptibility of cultivated brassicas to pathogens and insects. A study on the changes of glucosinolate concentration in TuMV infected rape cultivars with different content of glucosinolates is in progress.

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