Enzyme-Linked Immunosorbent Assays for Alkenyl Glucosinolates

Ferah Hassan, Neil E. Rothnie, Stella P. Yeung, and Martin V. Palmer*

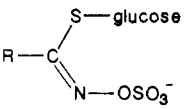
Enzyme-linked immunosorbent assays (ELISA's) were developed for the determination of alkenyl glucosinolates (sinigrin, gluconapin) in crude extracts of seed meal and vegetative tissues of Indian mustard (*Brassica juncea*). The assays were constructed from two types of high-titer anti-sinigrin polyclonal antibodies derived from antisera raised against a conjugate of sinigrin hemisuccinate with bovine serum albumin. An ovalbumin–sinigrin hemisuccinate conjugate was used as the ELISA plate-coating antigen. In cross-reactivity studies, antisera showed high specificities toward the alkenyl side chain and the thiohydroximate moiety of the glucosinolate molecule and did not bind to desulfoglucosinolates. All antisera showed 100% cross-reactivity with gluconapin, and one type cross-reacted completely with progoitrin, the principal glucosinolate of rapeseed (*Brassica napus*). The most sensitive ELISA had a linear inhibition curve over 1×10^{-8} to 1×10^{-13} mol of sinigrin, with 50% inhibition at 2.8×10^{-11} mol per assay. Parallel analyses of mustard seed and cotyledon extracts using both an ELISA and a standard HPLC method gave values identical within the error limits of the two procedures. The ELISA is, however, more sensitive, less expensive, and much faster than any other glucosinolate assay of comparable specificity.

The value of oilseed crops such as rapeseed (*Brassica napus, Brassica campestris*) and Indian mustard (*Brassica juncea*) would be doubled if their high-protein seed meals could be used in stock feed with no risk of glucosinolate toxicity (Kirk and Oram, 1978). Consequently, considerable effort has gone into the development of suitable screening methods for determining glucosinolates in seed samples, in batches of processed seed meal, and in individual plants in breeding programs. Until now the methods used in routine glucosinolate analyses have usually been a compromise between unequivocal but time-consuming physicochemical techniques such as high-performance liquid chromatography (HPLC) and rapid but less specific and less quantitative methods such as indicator paper tests for glucosinolate-derived glucose in crude seed extracts (McGregor et al., 1983; Sang and Truscott, 1984). Some recently developed colorimetric tests for glucose in partially purified, myrosinase-treated rapeseed meal are probably the best compromises presently available [e.g., Saini and Wratten (1987) and Smith et al. (1985)], but there are still severe limitations on the number of samples that can be processed daily. Furthermore, the colorimetric assay quantifies total glucosinolates in the extract, rather than just the five glucosinolates that are specified under the Canola rapeseed certification scheme (Fenwick, 1985). Consequently, there is still a demand for a method that is both efficient and highly specific, and this appears to be an instance where an immunoassay would be ideal.

Glucosinolate analysis of rapeseed is also complicated by the need to consider at least five different glucosinolates in the seed; these comprise three different structural types, having alkenyl, hydroxyalkenyl, or indolyl side chains (Sang et al., 1984; Table I). In contrast, the principal glucosinolates of Indian mustard seed are exclusively alkenyl; the commercially valuable, low erucic acid, yellow-seeded varieties contain only allyl glucosinolate (sinigrin; Table I), and brown-seeded varieties contain both sinigrin and butenyl glucosinolate (gluconapin; Table I) (Kirk and Oram, 1978; Sang et al., 1984; Palmer et al., 1987). In the present study an ELISA was developed for the simplest system—the quantification of sinigrin in yellow mustard seeds. The structural similarity between sinigrin and gluconapin also suggested a high probability of cross-reactivity between these two glucosinolates in this ELISA, which would result in an assay suitable for both types of mustard.

State Chemistry Laboratory, 5 Macarthur Street, East Melbourne, Victoria 3002, Australia.

Table I. Cross-Reactivity of Two Different Sources of Anti-Sinigrin Antisera with Various Glucosinolates and Related Compounds[a,b]



glucosinolate	R group	% cross-reactivity[c] rabbit 209	rabbit 576
sinigrin	$CH_2{=}CHCH_2$	100	100
gluconapin[d]	$CH_2{=}CHCH_2CH_2$	100	100
progoitrin[d]	$CH_2CHCH(OH)CH_2$	100	56
glucocapparin	CH_3	9.6	31.6
glucotropeolin	$C_6H_5CH_2$	10.0	10.0
glucosinalbin	$HOC_6H_4CH_2$	21.9	2.5
glucoerucin	$CH_3SCH_2CH_2CH_2CH_2$	<3.3	1.0
glucoiberin	$CH_3SOCH_2CH_2CH_2$	<3.3	0
glucocheirolin	$CH_3SO_2CH_2CH_2CH_2$	<3.3	0
succinic acid		0	0
glucose		0	0
desulfosinigrin		0	0
sinigrin hemisuccinate		5200	1000

[a] Cross-reactivity = (sinigrin concentration for 50% inhibition)/(glucosinolate concentration for 50% inhibition) × 100. [b] Antibody dilution factor, 1:60 000; sinigrin–ovalbumin coating antigen concentration, 5 ng/100 μL; IgGAPase dilution, 1:1000; incubation time with substrate, 1 h. [c] Values are means of triplicate analyses, each at five dilutions. [d] Cross-reactivities determined by comparative analysis of the meal extracts described in Table III.

MATERIALS AND METHODS

Chemicals. Freund's complete (FCA) and incomplete (FIA) adjuvant were purchased from Difco Laboratories, Detroit, MI. Radioimmunoassay-grade bovine serum albumin (BSA), ovalbumin, goat:antirabbit IgG-alkaline phosphatase conjugate (IgG-APase), *p*-nitrophenyl phosphate (PNP), glucose, succinic acid, polyoxyethylene sorbitan monolaurate (Tween-20), and Coomassie Blue G-250 were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-25 was purchased from Pharmacia,

0021-8561/88/1436-0398$01.50/0

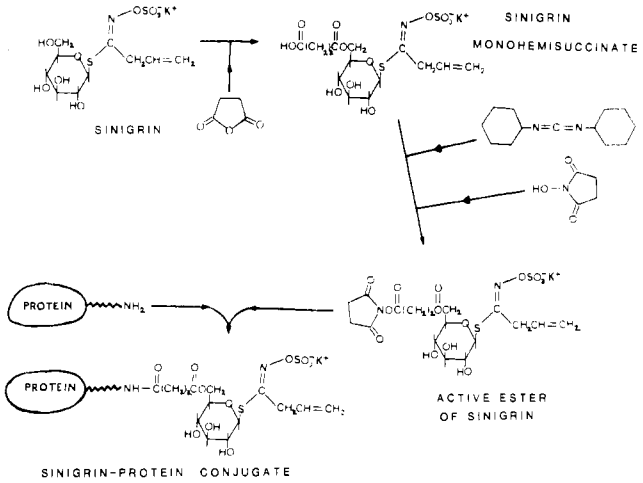


Figure 1. Schematic diagram illustrating the procedure developed for the synthesis of sinigrin–protein conjugates.

Uppsala, Sweden. *N*-Hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide were obtained from Merck-Schuchardt, Hoherbrunn, Germany. Trinitrobenzenesulfonic acid (TNBS) was a gift from the University of Melbourne. Sinigrin was purchased from Tokyo Chemical Industry, Tokyo, Japan. Glucocheirolin, glucosinalbin, glucocapparin, glucoiberin, and glucoerucin were obtained from Carl Roth, Karlsruhe, Germany. Glucotropeolin was a gift from Canola Council of Canada, Winnipeg, Manitoba, Canada. Desulfosinigrin was generously supplied by J. Sang (Sang and Truscott, 1984). Sinigrin hemisuccinate was synthesized as described below and dried in vacuo and its mass determined by weighing the powder. Certified (for uniform binding capacity and optical background) 96-well polystyrene microtiter plates, Nunc Immuno Plate I, were purchased from Nunc Inter-med, Copenhagen, Denmark.

Buffers. Sodium carbonate–bicarbonate buffer (0.1 M, pH 9.6) was used as the coating buffer. Phosphate-buffered saline (PBS), (0.15 M, pH 7.2) contained 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4/L of distilled water. A solution of 0.05% Tween 20 detergent in PBS (PBST) was used as the washing solution. Antibody diluent comprised washing solution containing 0.01% ovalbumin (PBSTO). Alkaline phosphate substrate consisted of 1 mg/mL PNP, 1.5 mM $MgCl_2$, and 1 mM $ZnCl_2$ in 0.1 M glycine buffer (pH 9.7).

Plant Material. Seeds of *B. juncea* Coss. were obtained from the Victorian Crops Research Institute, Department of Agriculture and Rural Affairs, Horsham, Victoria. The varieties investigated were Zem-2, a yellow-seeded, zero erucic acid line of European origin (Kirk and Oram, 1981), and the brown-seeded Indian lines CPI 81793 and IB 1337. For cotyledon analysis, seeds were germinated in a growth chamber, 14-h day length, light intensity 300 $\mu E\ m^{-2}\ s^{-2}$, 17–23 °C.

Synthesis of Sinigrin–Protein Conjugates. The protocol described below is summarized in Figure 1. Sinigrin (31.25 mg) was reacted with succinic anhydride (37.94 mg) and anhydrous pyridine (6.5 μL), in anhydrous dimethylformamide (250 μL), for 5 days at room temperature. Excess ether was then added to precipitate the sinigrin hemisuccinate product. The mixture was centrifuged, the supernatant removed, and the residue redissolved in a small volume of methanol. Excess ether was added again, and the mixture was centrifuged and separated as before. The procedure was repeated for a third time before final drying of the product in vacuo. The product (34 mg, yield 87%) was shown by ^{13}C NMR, ^1H NMR, and infrared spectroscopies to comprise a mixture of C^6-mono- and C^6,C^3-dihemisuccinates (*c* 9:1). Preparative thin-layer chromatography on cellulose, using a butanol–acetic acid–water (4:1:2, v/v) solvent was used to separate the two products. However, for practical purposes the reaction mixture could be used without further purification.

Table II. Assay Parameters for the ELISA Standard Curves[a]

detection limit, mol free sinigrin/assay	serum from rabbit 209		serum from rabbit 576	
	1[b]	5[b]	1[b]	5[b]
minimum	6.3×10^{-13}	1×10^{-11}	1×10^{-13}	2×10^{-12}
50% inhibn	5×10^{-10}	1.8×10^{-9}	2.8×10^{-11}	1.6×10^{-10}
maximum	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}

[a] See also Figures 3 and 4. [b] Nanograms of sinigrin–ovalbumin coating antigen bound/100-μL assay.

The *N*-hydroxysuccinimide active ester of sinigrin monohemisuccinate was then prepared by reacting *N*-hydroxysuccinimide (30 mg), *N,N'*-dicyclohexylcarbodiimide (27.6 mg), and sinigrin monohemisuccinate (55.8 mg) in anhydrous dimethylformamide (0.5 mL) at 4 °C over 12 h. The resulting mixture was filtered to remove the dicyclohexylurea precipitate. Sinigrin–protein conjugates were prepared by adding the filtrate to either a solution of BSA (21.22 mg) or ovalbumin (42 mg) in 2 mL of phosphate buffer (pH 7.6) (Figure 1). The mixture was stirred at 4 °C for 24 h.

Separation of free sinigrin from sinigrin–protein conjugate was achieved by column chromatography on Sephadex G-25, equilibrated with PBS. Protein-containing fractions were determined by measuring absorbance at 280 nm. Protein concentration was calculated from a calibration curve against crystalline BSA as a standard and with Coomassie Blue reagent (Sedmak and Grossberg, 1977). Sinigrin-BSA and sinigrin–ovalbumin conjugates were subdivided into 2.4-mg and 50- or 100-μg protein portions, respectively. These fractions were freeze-dried and stored at −4 °C. Subsequently, the sinigrin–BSA conjugate was injected into the rabbits, and the sinigrin–ovalbumin conjugate was used as the microtiter plate coating antigen.

Epitope Density Determination. Epitope density (moles of sinigrin/mole of protein) was measured with use of a modification of the trinitrobenzenesulfonic acid (TNBS) method of Habeeb (1966). Thus, 1 mL of protein or protein–sinigrin conjugate (known concentration of less than 1 mg/mL protein) solution in PBS, 1 mL of 50 mM carbonate–bicarbonate buffer (pH 9.6), and 1 mL of 0.1% TNBS were reacted at 40 °C for 3 h. Then 1 mL of 10% SDS was added, and the reaction was terminated with 0.5 mL of 1 M HCl. The extent of conjugation was determined by differences in absorbance at 335 nm. The molar extinction coefficients of one free amino group by the TNBS method in BSA (61 free amino groups) and ovalbumin (20 free amino groups) are 0.995×10^4 and 1.2×10^4, respectively (Habeeb, 1966).

Antibody Preparation. Two prebled female rabbits (209 and 576) were each injected subcutaneously with 1 mL (1.2 mg/mL) of an emulsion of sinigrin–BSA conjugate in FCA–PBS (1:1, v/v). Subcutaneous booster injections of 1.2 mg of sinigrin–BSA were administered in FCA–PBS (1:1) after 23 and 49 days from the first injection and in FIA–PBS (1:1) after 63, 78, 105, and 169 days. Rabbits were bled 2 weeks after boosting, and sera were separated and stored at −4 °C until use. The two serum types used in this study were collected on day 189, but usable titers were obtained as early as 23 days after primary immunization.

Adsorption of Coating Antigen. The lyophilized sinigrin–ovalbumin conjugate (antigen) was dissolved in coating buffer to give a concentration of 50 ng/mL, and 150 μL of this solution was added to each well of the plate. This gave 5 ng of bound sinigrin–ovalbumin/100-μL final assay volume. Unbound antigen was aspirated and the plate washed three times with PBST, using a Titertex Handiwash 110 (Flow Laboratories, North Ryde, Australia). Unoccupied protein binding sites were blocked by filling the wells with 300 μL of 0.05% ovalbumin in coating buffer for 90 min at room temperature. The plates were washed again with PBST, and then 300 μL of PBS was added to each well. Plates were frozen and stored in this condition at 0 °C. They were used within 1 month of preparation. Before incubation with antibodies the plates were brought to room temperature and washed once with PBST immediately before use.

Incubation Protocol. Two standard protocols were used. (a) Titration assay: 100 μL of antiserum diluted to required concentration in PBSTO was added to each well. (b) Competitive inhibition assay: First, 50 μL of appropriately diluted standard or sample solution in distilled water was added per well. Then, 50 μL of antiserum diluted in double-strength PBSTO was added. With both protocols, plates were incubated for 3 h at room temperature after which they were washed four times with PBST. A 110-μL portion of 1:2000 diluted IgGAPase in PBSTO was added to each well followed by incubation overnight at 4 °C.

Enzyme Assay. Plates were washed four times with PBST, and 200 μL of freshly prepared PNP substrate solution was added to each well at carefully timed (10-s) intervals. After incubation with substrate for a suitable length of time (30–180 min) at room temperature, the reaction was terminated at carefully timed (10-s) intervals with 50 μL of 2.5 M NaOH/well. Absorbance was measured at 405 nm on a Titertek Multiskan Plus ELISA plate reader (Flow Laboratories, North Ryde, Sydney).

ELISA Competitive Inhibition Reactions. Sinigrin was quantified by its capacity to inhibit the binding of anti-sinigrin antibodies to the sinigrin–ovalbumin adsorbed on the solid phase. A quantitative relationship was established between the amount of alkaline phosphatase activity on the wells and the amount of free sinigrin added to the well. This relationship can be represented by $B/B_0 = (A_B - A_{UB})/(A_{B0} - A_{UB}) \times 100$ where A_B is the absorbance at 405 nm in the standard with a known amount of sinigrin or in an unknown sample, A_{UB} is the absorbance in the presence of a large excess of sinigrin (3.16×10^{-7} mol/assay), and A_{B0} is the absorbance in the absence of free sinigrin. Linear standard curves were obtained by the logit-transformation of the relative binding parameter (B/B_0).

To determine the optimal concentrations of sinigrin–ovalbumin for plate coating and antibody concentration to be used in a competitive inhibition assay, checkerboard titrations of the antigen and of the antibody were made (Figure 2). Samples of 1 or 5 ng/100 μL per assay of sinigrin ovalbumin were routinely bound to microtiter plates for competitive inhibition assays. The antisera of both rabbits were tested. Final antiserum dilutions of 1:10 000–1:80 000 for both rabbits were found to be low enough to become limiting factors in the assay with an easily measurable response.

Extraction of Glucosinolates from Seed Meal and Vegetative Tissues. Although sinigrin (the principal glucosinolate in yellow mustard seeds) is available commercially, gluconapin (the main glucosinolate in brown

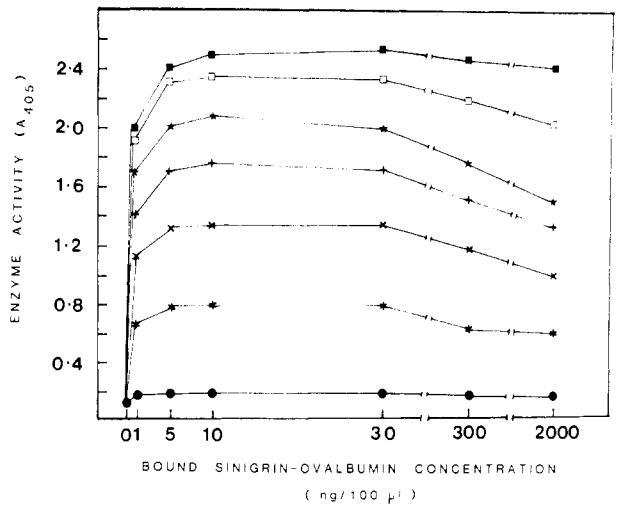


Figure 2. Checkerboard titration of sinigrin–ovalbumin coating antigen against the following dilutions of type 209 antiserum: $1:10^6$ (●), $1:10^5$ (*), $1:5 \times 10^4$ (×), $1:3 \times 10^4$ (+), $1:2 \times 10^4$ (★), $1:10^4$ (□), $1:4 \times 10^3$ (■). Incubation time with substrate, 45 min.

Table III. HPLC Analysis of Glucosinolate Content of Mustard Seed Meal Extracts

sample[a]	glucosinolate, μmol/mL			
	sinigrin	gluconapin	progoitrin	glucobrassicanapin
A	134.9	1.9	0.2	
B	6.6	0.1	0.1	
C	19.2	56.4	0.3	0.5
D	17.3	50.4	0.3	0.4
E	1.1	22.4	1.4	
F			1.4	
G	1.5			
H	0.4	1.1	0.1	

[a] Samples were partially purified seed meal extracts of the mustard lines Zem 2 (A, B, F), CPI 81793 (C, D), and IB 1337 (E) and cotyledon extracts of Zem 2 (G) and CPI 81793 (H), prepared as described in Materials and Methods.

mustard seeds) and progoitrin (the major rapeseed glucosinolate) are not. Cross-reactivity studies of gluconapin and progoitrin therefore relied on ELISA and HPLC analyses of purified extracts of mustard or rapeseed lines that are known to contain one or a combination of the three glucosinolates of interest. The material chosen for this purpose comprised the yellow mustard line Zem 2, and the brown mustards CPI 81793 and IB 1337, as described in Table III.

Defatted seed meal was prepared according to the method of Sang et al. (1984). Meal samples B–E (Table III) were extracted in boiling water and centrifuged, and the supernatant was freeze-dried (Sang et al., 1984). The resultant powder was dissolved in a small volume of water to give stock solutions of partially purified glucosinolates. Sample A (Table III) was prepared according to the method of Hanley et al. (1983). This involved extraction with boiling 90% methanol, filtration through Celite, drying in vacuo, and redissolution in a small volume of water. Sample F (Table III) was prepared by purification of a portion of sample C on a column of acidic alumina, followed by chromatography on DEAE-Sephadex, eluted with 0.4 M pyridinium acetate (Hanley et al., 1983), and recrystallized from 96% aqueous ethanol–ether. The product was washed thoroughly with dry ether, dried in vacuo, and redissolved in a small volume of water. Seven-day-old cotyledons (1 g fresh weight) from Zem 2 and CPI 81793 seedlings were extracted in 70% methanol, as described previously (Palmer et al., 1987; Table III).

Preparation of Standard Solutions. All stock solutions and further dilutions were prepared in distilled water. A 50 mM stock sinigrin solution was used to prepare the standards by dilution. For cross-reactivity studies, 3 mM stock solutions of pure glucosinolates were prepared and diluted to five concentrations ranging from 1.5×10^{-7} mol/50 μL (mole/assay) to 2.1×10^{-11} mol/50 μL. Sinigrin–hemisuccinate stock solution (100 mM) was used to prepare nine dilutions ranging from 1×10^{-6} to 1×10^{-14} mol/50 μL. Extracts of seed meal and vegetative tissue (Table III) were diluted to 25 concentrations in water. All solutions were stored frozen, and they were brought to room temperature on the day of use.

RESULTS AND DISCUSSION

Small molecules such as glucosinolates must be conjugated to high molecular weight immunogenic carriers such as proteins in order to invoke an immune response. Since sinigrin and most other glucosinolates lack suitable functional groups capable of direct conjugation to proteins, a general procedure was developed for the addition of appropriately reactive bridging groups to glucosinolate molecules, such that subsequent conjugation would expose both the side-chain R group (Table I) and the thiohydroximate group as antigenic recognition sites. Figure 1 shows the pathway for sinigrin–protein conjugate synthesis.

The nature of the absorbance spectrum of sinigrin does not permit the facile differentiation of sinigrin from protein carrier by spectroscopy. Epitope density (moles of sinigrin/mole of protein) was therefore measured with use of TNBS, which reacts specifically and under mild conditions with free lysine amino groups to give trinitrophenyl derivatives. The number of unreacted lysine amino groups in the sinigrin–protein conjugate is then determined by comparison with the original carrier protein. Epitope densities ranged from 20 to 37 for BSA conjugates and 13 for the ovalbumin conjugate.

Antibody titer was determined by titration of serial dilutions (1:1000–1:1 000 000) of rabbit immune sera against a fixed concentration (5 ng/100 μL) of sinigrin–ovalbumin bound to the wells. A maximum absorbance of 2.0 and a minimum absorbance of 0.2 were observed over this range after only 30-min incubation with substrate. There was a direct relationship between the concentration of serum used and the measured absorbance. This relationship was linear within the antibody dilution range 1:10 000–1:100 000. Antibody titers were very high for both rabbits, with rabbit 209 being slightly higher than 576. Sera from both rabbits was reactive at dilutions as low as 1:1 000 000.

Several experiments were performed to quantify the background binding and to demonstrate that the increase in enzyme activity using sera from immunized rabbits was due to anti-sinigrin antibodies. When IgGAPase was titrated to wells coated with 0.05% ovalbumin only, a background absorbance of less than 0.1 was observed at all concentrations used, indicating that the enzyme tracer does not bind to ovalbumin. Titration of both test sera (209, 576) to wells coated with ovalbumin resulted in a constant background absorbance of less than 0.15, indicating that neither rabbit had antibodies to ovalbumin and that the antibodies in the test sera bind to the sinigrin portion of the sinigrin–ovalbumin conjugate. Preimmune serum obtained from the same rabbit prior to the immunization protocol was titrated on sinigrin–ovalbumin-coated (5 ng/100 μL) plates, and a constant background absorbance of less than 0.15 was observed.

To obtain maximal sensitivity in the assay, the amount of antigen used for coating was decreased as far as practical, with the amount of antibody added being low enough to become a limiting factor. It can be seen from Figure 2 that any antigen concentration below 10 ng/100 μL giving a measurable response can be used for coating in this ELISA. Also, at less than 5 ng/100 μL bound antigen, antiserum diluted less than or equal to 1:10 000 would be a limiting factor in a competitive inhibition assay. Both rabbits immunized with sinigrin–BSA produced similar checkerboard results. When large amounts (300–2000 ng/100 μL) of sinigrin–ovalbumin were bound to the wells, a decrease in enzyme activity was observed. This may be due to protein–protein interactions and stearic hindrance, resulting in less bound sinigrin being available for antibody binding.

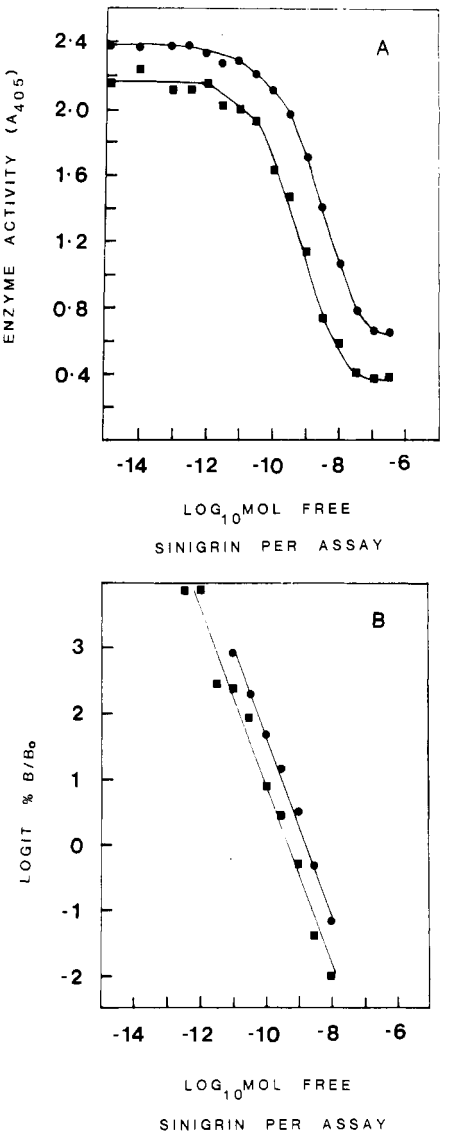


Figure 3. (A) Standard curve for the sinigrin competitive inhibition ELISA using serum type 209, with sinigrin–ovalbumin coating antigen concentrations of 1 ng/100 μL (■) and 5 ng/100 μL (●). Antiserum titer, 1:40 000; incubation time with substrate, 75 min. Data are mean values of triplicate analyses. (B) Logit transformation of data shown in Figure 3A.

Two different ELISA's were developed from antiserum from two rabbits. Standard curves for the sinigrin competitive inhibition assay using sera from rabbits 209 and 576 are shown in Figures 3 and 4, respectively. Assay parameters of these standard curves are summarized in Table II. Sinigrin exhibited strong inhibition with the 50% inhibition point as low as 2.8×10^{-11} mol/assay, making detection of sinigrin by ELISA the most sensitive method

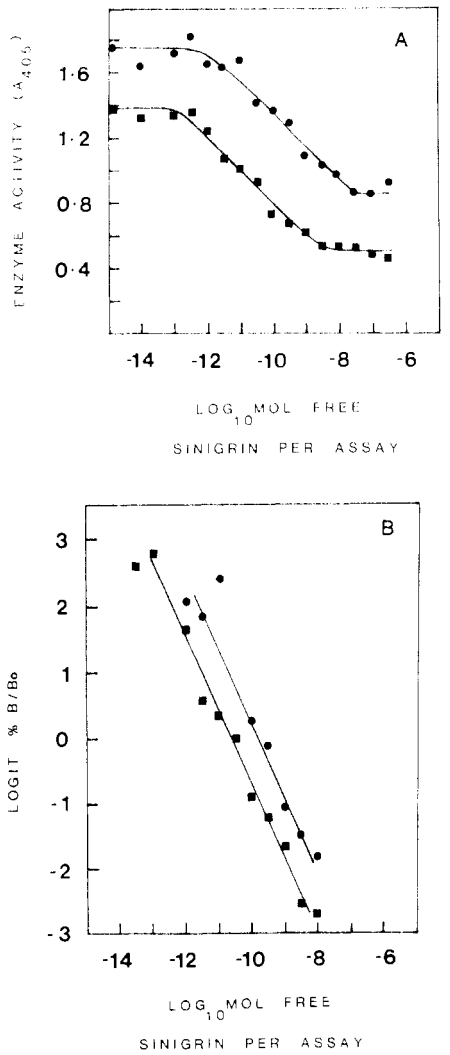


Figure 4. (A) Standard curve for the sinigrin competitive inhibition ELISA using serum type 576, with sinigrin–ovalbumin coating antigen concentrations of 1 ng/100 μL (■) and 5 ng/100 μL (●). Antiserum titer, 1:40000; incubation time with substrate, 90 min. Data are mean values of triplicate analyses. (B) Logit transformation of data shown in Figure 4A.

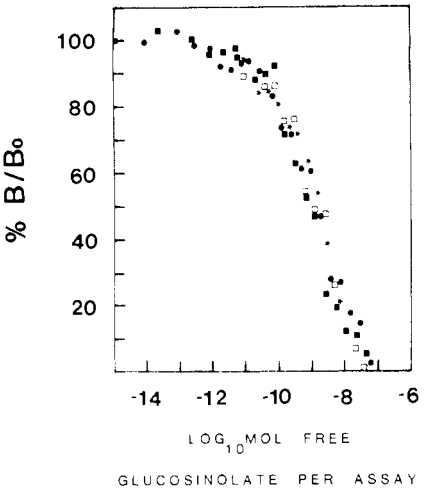


Figure 5. Comparison of inhibition curves obtained from ELISA of standard sinigrin (●) and the partially purified mustard seed meal extracts A (■), D (□), and F (*) described in Table III. Glucosinolate content of meal extracts was determined by the HPLC procedure of Sang and Truscott (1984). Values represent the means of triplicate analyses. Type 209 antiserum was used, as described for Figure 3A.

to date. The inhibition curve is linear over a wide range of free sinigrin concentrations, thus allowing between 1×10^{-8}–1×10^{-13} mol of sinigrin to be readily quantified. An additional advantage of this method is that the desired sensitivity and linear response range of the ELISA can be varied readily by varying the antigen coating concentration. Lower detection limits of sinigrin are obtained when 1 ng instead of 5 ng of antigen is bound to the wells/assay. Increasing the amount of bound sinigrin resulted in higher absorbance readings due to the equilibrium reaction between the antibodies and the free and bound sinigrin. Comparison of the standard sinigrin curves for the two rabbits shows rabbit 576 has lower detection limits than standard curves from rabbit 209. However, a change in enzyme activity on the wells in response to changes in free sinigrin added to the well is higher using 209 antiserum. It is possible to get an even more sensitive ELISA for sinigrin by decreasing the bound antigen and antibody concentrations lower than 1 ng/100 μL and 1:40000 dilution, increasing the IgGAPase concentration, and having longer incubation times with the substrate.

Cross-reactivity studies of antiserum from both rabbits were performed with various related glucosinolates and other compounds to determine the specificity of the antisinigrin antibodies (Table I). Although the antibodies from the two rabbits have different specificities, they also have some similar binding characteristics. Antibodies from both rabbits have higher affinity for sinigrin hemisuccinate (the sinigrin derivative used for conjugation with proteins) than sinigrin alone. It would appear that changes in the sinigrin structure caused alterations in spatial orientation that enhanced antibody recognition of sinigrin hemisuccinate. Succinic acid and glucose did not bind to sinigrin antibodies at all, indicating that the succinate bridging group is not recognized. Desulfosinigrin is not recognized by sinigrin antibodies from either rabbit, indicating that the anionic sulfate group plays an important role in antibody recognition. Other closely related glucosinolates such as glucoerucin, glucoiberin, and glucocheirolin were found to be poor inhibitors, indicating that the R group (Table I) also plays a very important role in antibody recognition. Sinigrin antibodies showed slight cross-reactivity with glucocapparin, glucotropeolin, and glucosinalbin. The antisera from the two rabbits have different specificities because they differ in their recognition of glucocapparin, glucotropeolin, glucosinalbin, and progoitrin.

Cross-reactivity of gluconapin and progoitrin with sinigrin antibodies was determined on seed meal extracts because these pure glucosinolates are unavailable. These extracts were also used for parallel analysis and comparison of sinigrin determination by HPLC and ELISA (Figure 5). HPLC analysis (Sang et al., 1984) results are shown in Table III. Competitive inhibition ELISA of samples A–E using antiserum from both rabbits gave 100% cross-reactivity (cf. Table I). In other words, the amount of total glucosinolates in samples A–E giving 50% inhibition was equal to the amount of pure sinigrin (standard curve) needed to give the same response. Sample F contained only one glucosinolate, progoitrin, and using antibodies from rabbits 209 and 576 we found 100% and 56% cross-reactivity, respectively, with this sample. Hence, progoitrin cross-reacts to 100% and 56% with sinigrin antibodies from rabbits 209 and 576, respectively, and antibodies from either rabbit cannot distinguish between sinigrin and gluconapin (Table I). In conclusion, these cross-reactivity results indicate that the allyl R group of sinigrin, the sulfate, and the thiohydroximate moieties play a critical role in antibody–antigen recognition.

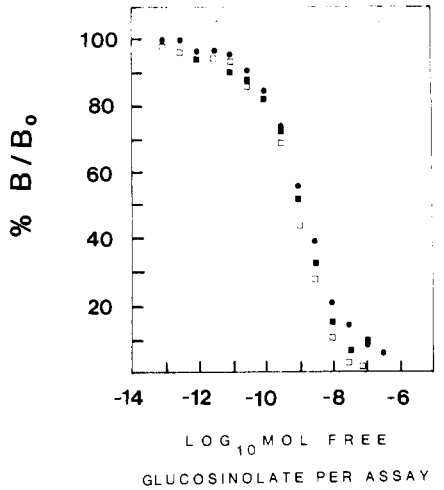


Figure 6. Comparison of inhibition curves obtained from ELISA of standard sinigrin (●) and the crude extracts of 7-day-old cotyledons of mustard lines Zem 2 (□) and CPI 81793 (■) described in Table III. Glucosinolate content of cotyledon extracts was determined by the HPLC procedure described by Palmer et al. (1987). Type 209 antiserum was used, as described for Figure 3A.

The results of parallel analysis and comparison of sinigrin determination in meal and cotyledon extracts by HPLC and ELISA are shown in Figures 5 and 6. All the meal extract samples (Table III) were tested with use of antiserum from both rabbits, which gave very similar inhibition responses. The results presented in Figure 5 are a representative example. Percent enzyme tracer activity was plotted against a variable concentration of free standard sinigrin or free total glucosinolates in plant sample extracts, as determined by HPLC. It can be seen from Figures 5 and 6 that the inhibition response of total glucosinolates in both types of sample was identical with that obtained with standard sinigrin, within the limit of errors of the two procedures. The ELISA is therefore valid for the analysis of endogenous glucosinolates in both seed and vegetative tissues, over a wide range of concentrations. The coefficient of variation (CV) for the determination of sinigrin in mustard seed meals was 3.0% (nine replicates). This is significantly lower than the CV of 9.5% calculated for HPLC analysis of similar samples (Sang and Truscott, 1984).

ELISA analysis of glucosinolate samples from plant extracts does not involve the extensive purification routinely needed for chromatographic methods like HPLC. Glucosinolates were extracted from plant tissue in a single simple step in water or methanol and diluted to a concentration within the linear range of the standard curve. The accuracy and precision of the sinigrin ELISA compares favorably with the standard HPLC procedure, and it is also more sensitive, less expensive, and much faster. The chemical procedures developed to construct these ELISA's could be readily applied to develop similar assays for glucosinolates in rapeseed and other cruciferous crops. Indeed, the fortuitous cross-reactivity data for type 209 antisera (Table I) indicate that it may be possible to further develop this system for rapeseed analysis with little or no modification. The adoption of this new technology is likely to have a dramatic impact on the efficiency of screening procedures in meal processing and in plant breeding programs.

ACKNOWLEDGMENT

We thank the Reserve Bank of Australia for financial support, A. Stewart for technical assistance, J. Sang for advice on glucosinolate analysis, and Dr. C. Chapman for assistance with immunization procedures.

Registry No. Sinigrin, 3952-98-5; gluconapin, 19041-09-9; progoitrin, 585-95-5; glucobrassicanapin, 19041-10-2; glucocapparin, 497-77-8; glucotropeolin, 499-26-3; glucosinalbin, 19253-84-0; glucoerucin, 21973-56-8; glucoiberin, 554-88-1; glucoheirolin, 15592-36-6; sinigrin monohemisuccinate, 112968-95-3; succinic anhydride, 108-30-5; sinigrin monohemisuccinate succinimide, 112968-96-4; *N*-hydroxysuccinimide, 6066-82-6.

LITERATURE CITED

Fenwick, G. R. "The Analysis of Glucosinolates in Rapeseed". Bulletin No. 2; Groupe Consultatif International de Recherche sur le Colza: Paris, 1985; pp 110–112.

Habeeb, A. F. S. A. "Determination of Free Amino Groups in Proteins by Trinitrobenzene Sulphonic Acid". *Anal. Biochem.* **1966**, *14*, 328–336.

Hanley, A. B.; Heaney, R. K.; Fenwick, G. R. "Improved Isolation of Glucobrassicin and other Glucosinolates". *J. Sci. Food Agric.* **1983**, *34*, 869–873.

Kirk, J. T. O.; Oram, R. N. "Mustards as Possible Oil and Protein Crops for Australia". *J. Aust. Inst. Agric. Sci.* **1978**, *44*, 143–156.

Kirk, J. T. O.; Oram, R. N. "Isolation of Erucic Acid-Free Lines of *Brassica juncea*". *J. Aust. Inst. Agric. Sci.* **1981**, *47*, 51–52.

McGregor, D. I.; Mullin, W. J.; Fenwick, G. R. "Analytical Methodology for Determining Glucosinolate Composition and Content". *J. Assoc. Off. Anal. Chem.* **1983**, *67*, 825–849.

Palmer, M. V.; Yeung, S. P.; Sang, J. P. L. "Glucosinolate Content of Seedlings, Tissue Cultures, and Regenerant Plants of *Brassica juncea*". *J. Agric. Food Chem.* **1987**, *35*, 262–265.

Saini, H. S.; Wratten, N. "Quantitative Determination of Total Glucosinolates in Rapeseed and Meal Digests". *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 141–145.

Sang, J. P. L.; Truscott, R. J. W. "Liquid Chromatographic Determination of Glucosinolates in Rapeseed as Desulfoglucosinolates". *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 829–833.

Sang, J. P. L.; Minchinton, I. R.; Johnstone, P. K.; Truscott, R. J. W. "Glucosinolate Profiles in the Seed, Root and Leaf Tissues of Cabbage, Mustard, Rapeseed, Radish and Swede". *Can. J. Plant Sci.* **1984**, *64*, 77–93.

Sedmak, J. J.; Grossberg, S. E. "A Rapid, Sensitive and Versatile Assay for Protein using Coomassie Brilliant Blue G250". *Anal. Biochem.* **1977**, *79*, 544–552.

Smith, D. B.; Parsons, D. G.; Starr, C. "A Simple and Rapid Method of Quantitatively Measuring the Glucosinolate Concentration of Rapeseed". *J. Agric. Sci. Camb.* **1985**, *105*, 597–603.

Received for review June 17, 1987. Accepted November 9, 1987.