CHAPTER 2

MATERIALS AND METHODS

2.1) MATERIALS

2.1.1) Chemicals

Chemicals	Company
Absolute ethanol	Merck
Acrylamide	Sigma
Adenosine triphosphate	Sigma
Alcohol dehydrogenase	Sigma
Allylcyanide	Sigma
Allylisothiocyanate	Sigma
Amikacin	Abbot
Ammonium chloride	Merck
Ammonium persulfate	Sigma
Ammonium sulfate	Merck
L-Ascorbic acid	Sigma
Bactoagar	Difco
Barium chloride	Merck
Bis-acrylamide	Sigma
Boric acid	BDH
Bovine serum albumin	Sigma
Bromophenol blue	Sigma

Chemicals	Company
Brown mustard seed meals (*Brassica juncea*)	Lanna Products Co., Ltd.
Calcium chloride	Merck
Cellobiose	Sigma
Chymotrypsinogen	Sigma
Cobalt chloride	Merck
Comassie brilliant blue R-250	Sigma
Concanavalin-A Sepharose	Pharmacia Biotech
L-Cysteine	Sigma
L-Cystine	Sigma
DEAE Sephadex A-25	Pharmacia Biotech
Digitonin	Provided by Lanna Products Co., Ltd. research unit
Dithiothreitol	Sigma
D-glucose	Merck
Dinitrosalicylic acid	Merck
Dipotassium hydrogenphosphate	BDH
Disodium hydrogenphosphate	BDH
Ethanol 95 %	Vitdhayasom
Ethylenediamine tetraacetic acid	BDH
Ferric chloride	Merck
Ferrous sulfate	Merck
Gentamycin	Abbot
Glacial acetic acid	Merck

Chemicals	Company
Glycerol	Merck
Hexane (commercial grade)	-
Hexane (GC grade)	Merck
HK/G-6-PD mixtures	Sigma
Hydrochloric acid	Merck
Lentil lectin Sepharose	Pharmacia Biotech
Low molecular weight protein markers	Pharmacia Biotech
Magnesium chloride	Merck
Malt extract	Difco
Mercaptoethanol	Sigma
Mercuric chloride	Merck
Mercurous chloride	Merck
Myrosinase (*Sinapis alba*)	Sigma
Nickle chloride	Merck
NADP	Sigma
p-Nitrophenyl-β-D-arabinoside	Sigma
p-Nitrophenyl-β-D-galactoside	Sigma
p-Nitrophenyl-β-D-glucoside	Sigma
p-Nitrophenyl-β-D-maltoside	Sigma
p-Nitrophenyl-β-D-xyloside	Sigma
Nutrient broth	Difco
Ovalbumin	Sigma
Peptone	Difco

Chemicals	Company
Phenylisothiocyanate	Sigma
Phenylethylisothiocyanate	Sigma
Potassium dichromate	Merck
Potassium dihydrogenphosphate	BDH
Potato dextrose agar	Difco
Reagent kit for glucose assay (glucose oxidase)	Human
Sephadex G-100	Pharmacia Biotech
Sephadex G-200	Pharmacia Biotech
Sinigrin monohydrate	Sigma
Sodium azide	Sigma
Sodium bisulfite	BDH
Sodium borate	BDH
Sodium chloride	Merck
Sodium dihydrogenphosphate	BDH
Sodium dodecylsulfate	Sigma
Sodium hydroxide	BDH
Sodium potassium tartrate	Merck
Sodium sulfite	BDH
Sodium thiosulfate	Merck
Sulfuric acid	Merck
Starch	-
Stevioside	Provided by Dr. Duang Bduddhasukh

Chemicals	Company
TEMED	Sigma
Thimerosal	Sigma
Tris(hydroxymethyl)aminomethane	Sigma
Tryptone	Difco
Wheat germ agglutinin Sepharose	Pharmacia Biotech
Yeast nitrogen base	Difco
Zinc chloride	Merck

2.1.2) Instruments

Instruments	Company
Hewlett-Pack gas chromatograph 5890 series II	Hewlett-Packard
Hewlett-Pack GC/MS system 6890 series	Hewlett-Packard
Shimadsu 14A gas chromatograph	Shimadsu
U-2000 Spectrophotometer	Hitachi
UV-265 Spectrophtometer	Shimadsu
Column (1.0×15 cm)	Pharmacia Biotech
Column (2.6×30 cm)	Pharmacia Biotech
Column (1.6×100 cm)	Pharmacia Biotech
Peristatic pump	Eyela
Haemocytometer	Newbauer
Beckman J2-MC centrifuge	Beckman
Fraction collector	Pharmacia Biotech
Mini VE electrophoresis apparatus	Pharmacia Biotech

Instruments	Company
Stirred-cell ultrafiltration	Amicon
Ultrafiltration membrane	Millipores
Dialysis tube	Sigma
Flexi-Dry^{TM} freeze-dryer	FTS System

2.2) GENERAL METHODS

2.2.1) Preparation of sinigrin agar plate

A 100-ml agar medium comprising of 5 mM sinigrin, 5 mM ammonium chloride and 1.5 g agar was melt by heating in boiling water for 10 min. It was then autoclaved at 121 °C for 10 min. Then, 15 ml of the medium were poured into each petri disk.

2.2.2) Preparation of mustard extract medium

Two hundred grams of brown mustard seed meals (*Brassica juncea* var. Forge), obtained after fixed-oil expellation, were ground finely by a mortar and defatted three times with one liter hexane each. After the defatted mustard powder has been allowed to air-dry, it was then heated at 110 °C for 30 min, prior to being suspended in boiling water (2 l). During this time, it was vigorously stirred and heated further for 15 min.The slurry was stirred for an additional 3 h at room temperature and then filtered. The yellowed-brown mustard extract obtained after centrifugation (10,000×g, 20 min) was assayed for glucose and glucosinolate contents.

Cultured medium was made by diluting the mustard extract to a final glucosinolate concentration of 5.5 mM in 50 mM potassium phosphate buffer, pH 5.6

(50 mM sodium phosphate buffer at desired pH values in any cases). It was then autoclaved at 121 °C for 15 min.

2.2.3) Determination of protein content [108]

Reagent A was 2 % sodium carbonate in 0.1 N NaOH. Reagent B_1 was 2 % sodium potassium tartrate and reagent B_2 was 1 % copper sulfate pentahydrate. Reagent C was freshly prepared by mixing reagent A : B_1 : B_2 by the ratio 50 : 1 : 1 (v/v/v). Stock bovine serum albumin (1000 µg/ml) was diluted with distilled water to final concentrations of 100, 200, 300, 400, 500, 600 and 700 µg/ml, used as standards. One hundred µl of standard (or sample) and 1.0 ml of reagent C were mixed gently and incubated at room temperature for 10 min.Then, 100 µl of 50 % Folin-phenol reagent in distilled water were added and mixed gently. After incubating at room temperature for 30 min, an absorbance at 500 nm was then measured. Protein concentrations of every samples were determined directly from the calibration curve (as shown in appendix).

2.2.4) Glucosinolate assay

a) Determination of pure sinigrin content [56]

Absorbance values at 227 nm of different sinigrin concentrations in distilled water (25, 50, 125, 100 and 150 µM) were measured. Calibration curve was constructed by plotting between absorbance values and sinigrin concentrations. The determination of sinigrin content in liquid sample was done by diluting the sample to appropriate concentration and then monitoring an absorbance value. Sinigrin

concentration was determined directly from the calibration curve (as shown in appendix).

b) Determination of total glucosinolate content [43, 55]

A sample or standard (50 µl) was added to 1.0 ml reaction reagent (33 mM sodium phosphate buffer, pH 6.5 containing 3 mM $MgCl_2$, 1 mM L-ascorbic acid, 0.55 mM ATP and 0.72 mM NADP). After mixing, an absorbance at 340 nm was measured (A_1). Then, 5 µl of HK/G-6-PD mixtures (activity of 700 and 350 U/ml) was added and incubated for 15 min at 30 °C. The second absorbance (A_2) was then measured. In order to quantify glucosinolates, 10 µl of the *Sinapis alba* myrosinase in 33 mM sodium phosphate buffer, pH 6.5 (14 U/ml) was added into the reaction mixtures and incubated for 30 min at 30 °C before the third absorbance (A_3) was read. Glucose content could be calculated from A_2-A_1 by using a calibration curve for glucose (as shown in appendix). Glucosinolate level was determined from the absorbance difference, A_3-A_2, using the calibration curve for sinigrin (as shown in appendix).

2.2.5) Preparation of spore suspension [109]

Spores from the culture grown in potato dextrose agar slants were aseptically transferred into a screw-capped vial containing sterile distilled water. It was vigorously shaken for 3-5 min or until no cluster of spores was observed. The suspension was adjusted to desired concentration using a Newbauer haemocytometer. Normally, inoculum was made from 2-weeks old culture and the concentration was 1×10^6 spores/ml, unless stated otherwise.

2.2.6) Measurement of fungal cell biomass [109]

Fungal cell pellet was collected by filtering through Whatman filtered paper no. 93. It was then washed with distilled water (normally 4-5 volumes of cultured medium) until yellowed-brown color was disappeared. The mycelium was transferred to a pre-weighed watch glass and then heated at 70 °C until constant weight was obtained.

2.2.7) Disruption of fungal cell pellet [109]

Fungal mycelium was collected and washed as described in 2.2.6. Then, the mycelium was cooled at 4 °C in the presence of 0.1 M sodium phosphate buffer, pH 7.4 for 30 min. Disruption was carried out by a mortar in cold room (4 °C). Cell-free extract obtained after centrifugation (10,000×g, 20 min, 4 °C) was maintained at 4 °C, prior to being analyzed further.

2.2.8) Activity assay of enzyme myrosinase [55]

Enzyme activity of myrosinase was routinely determined by the coupled-enzyme assay with a slight modification. The assayed system was carried out on a double beam Shimadsu UV-265 spectrophotometer. Cuvette holders were connected to a circulating waterbath, equipped with a temperature control apparatus, which was adjusted to a desired temperature (normally 25 °C). One ml of buffered substrate (33 mM sodium phosphate buffer, pH 7.4 containing 5 mM sinigrin, 0.55 mM ATP, 0.72 mM NADP, 3.5 U hexokinase and 1.75 U glucose-6-phosphate dehydrogenase) was pre-incubated for a sufficient time at 25 °C (3-5 min). Then, appropriate amount of the enzyme was added. After mixing, the reaction rate was monitored at 340 nm. Myrosinase activity could be calculated from the rate of absorbance change, which was

due to the formation of NADPH (ε_{340nm} = 6220 $M^{-1}cm^{-1}$). One unit of myrosinase activity was defined as the amount of enzyme that catalyzed the liberation of 1 μmol of glucose per minute from sinigrin under the conditions which were described above.

2.2.9) Determination of reducing sugar [110]

The amounts of 0.5 ml of sample were added in 1 ml of DNS reagent (1 % dinitrosalicylic acid in 1 N NaOH) which were contained in a screw-capped tube. After mixing and capping, it was heated in boiling water for 10 min. An absorbance at 492 nm was then measured. The concentration of reducing sugar was determined from a calibration curve (as shown in appendix), using glucose at concentrations of 1, 2.5, 5 and 10 mM as standards.

2.2.10) GC analysis of sinigrin or glucosinolate breakdown products [43, 45]

The analysis of sinigrin (or glucosinolate) breakdown products was conducted using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard) or a Shimadsu 14A gas chromatograph (Shimadsu), equipped with a flame ionization detector (FID) and a carbowax column (50m×0.3mm ID), connected to the injector port. The carrier gas (helium) was operated constantly at a flow rate of 0.5 cm^3/min and a pressure of 50 kPa. Temperature of the column was initially maintained at 80 °C for 6 min and then programmed to increase at a rate of 10 °C/min for additional 6 min. The temperature of the injector and detector was maintained at 230 and 280 °C, respectively. Standard allylcyanide and allylisothiocyanate were prepared at concentrations of 1, 2.5, 5 and 10 mM in distilled water. Samples or standards (normally 1.0 ml) were extracted with an equal volume of chilled hexane (GC grade).

After adding approximately 2 g of solid ammonium sulfate, capping, shaking and centrifuging (3,000×g, 10 min), 1 µl of hexane layer was injected. Concentration of any sinigrin (or glucosinolate) breakdown products in the sample were determined directly from such calibration curves (as shown in appendix). In this assay, allylcyanide and allylisothiocyanate at concentration of 5 mM each, were used as internal standards.

2.2.11) Disruption of bacterial cells

Bacterial cells harvested by centrifugation (10,000×g, 20 min) were suspended in chilled 50 mM sodium phosphate buffer, pH 7.4. The cells were then washed twice by the same process. Then, the bacterial suspension was subjected to sonication which was operated according to instruction's manual. During sonicating process, the suspension was maintained in an ice-bath. After centrifugation, cell-free extract was assayed for myrosinase activity.

2.3) SCREENING AND CHARACTERIZATION OF GLUCOSINOLATE-DEGRADING MICROORGANISMS

2.3.1) Sample collection

Samples suspected glucosinolate-degrading microorganisms were collected from decayed mustard seed meals (*Brassica juncea*), which were obtained from Lamphun, Thailand. Solid or semi-solid meal samples were bottled in sterile vials, whereas, liquid samples were in sterile tubes.

2.3.2) Screening and characterization of glucosinolate-degrading microorganisms

Each 1 g of solid or semi-solid meal samples was suspended with 10 ml sterile distilled water, whereas, 1 ml of liquid sample was diluted ten times with 10 ml sterile distilled water. After shaking, it was allowed to settle at room temperature for 2-3 h. Then, 100 µl of supernatant were spread on sinigrin agar plate (as described in 2.2.1). Incubation was taken at 30 °C and growths were observed daily.

Each distinct growing colony was picked and then plated onto nutrient agar plate (2 g beaf extract, 2 g tryptone and 1.5 g agar in 100 ml distilled water) or potato dextrose agar plate (3.9 g potato dextrose agar in 100 ml distilled water). Re-plating was made until pure isolate was obtained. Fungal isolates were maintained in potato dextrose agar slants and subcultured monthly. In case of bacterial isolates, it was maintained in nutrient agar slants and subcultured every two-weeks. Growth, colony morphology, gram staining (bacteria) and lactophenol-blue staining (fungi) were done for preliminary identification.

2.3.3) Preliminary examination of sinigrin degradation potential and myrosinase-producing ability of glucosinolate-degrading microorganisms in liquid culture [43, 45]

An overnight growth in nutrient broth (2 g beaf extract and 2 g tryptone in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0) of a glucosinolate-degrading bacterium, isolated from decayed mustard seed meal samples was harvested by centrifugation (10000×g, 10 min). After washing two times with 0.1 M sodium phosphate buffer, pH 7.0, bacterial cell pellet was suspended with the same buffer. Then, turbidity of the bacterial suspension was adjusted to be corresponded with that

of the MacFarland no. 2. Quantities of 100 µl of the suspension were inoculated in 1 ml sinigrin-glucose medium (5.5 mM sinigrin, 11 mM glucose, 6.7 g/l yeast nitrogen base in 0.1 M sodium phosphate buffer, pH 7.0) and incubated at 30 °C. Growth was monitored by measuring an absorbance at 660 nm. Samples were taken every 12 h. Supernatants obtained after centrifugation were assayed for glucose and sinigrin content. In this case, sinigrin was determined by the spectrophotometric method while glucose was determined by glucose-oxidase method.

Twenty-five µl of spore suspension of each fungal isolate (as described in 2.2.5) were inoculated in sinigrin-glucose medium (5.5 mM sinigrin, 11 mM glucose, 6.7 g/l yeast nitrogen base in 0.1 M sodium phosphate buffer, pH 5.6). Incubation was carried out at 30 °C under reciprocal shaking, operated at 150 rpm. Samples were taken periodically to assay for their contents of glucose and glucosinolates.

2.3.4) Scanning electron microscope as a tool for identification of a selected myrosinase-producing fungus [111-113]

a) Specimen preparation

Cellophane membrane (dialysis tube, molecular weight cut-off 12,000) was cut into 1 cm^2 pieces, prior to being autoclaved. Then, 3-4 pieces of the membrane were placed on potato dextrose agar plate. Quantities of 5-10 µl of spore suspension (as described in 2.2.5) were spread on each piece of membrane. Incubation was carried out at 30 °C.

b) Fixing, dehydrating and coating procedure

After growth at 30 °C for two days, each membrane piece with fungal growth was cut into 4-5 fractions before submerging in fixative solution I (1% v/v glutaral-

dehyde and 1% v/v formaldehyde in 50 mM sodium phosphate buffer, pH 7.3) at 4 °C for 1-2 h. Then, samples were rinsed three times with chilled rinsing solution (50 mM sodium phosphate, pH 7.3). Post-fixation was done by submerging the samples in fixative solution II (1% w/v osmium tetroxide in 50 mM sodoum phosphate buffer, pH 7.3) at 4 °C for 1-2 h. After rinsing, samples were subsequently soaked in a series of dehydrating solution I, II, III, IV and V (50 %, 70 %, 80 %, 90 % and 95 % acetone, respectively) at 4 °C for 15 min each. Then, dehydration was repeated for three times in dehydrating solution VI (100% acetone) before applying in a critical point drying apparatus for 1-2 h. When dehydration was complete, samples were coated with vapourized gold by an ion sputter mechine for 8-20 min.

c) Scanning electron micrograph

Specimens were placed properly in a specimen grid and then applied in a socket connected to the scanning electron micrograph mechine. Operation was done according to instruction's manual. Normally, suitable images were obtained at magnifications between 100 and 10,000 times, corresponding to the power supply of 10-25 kilovolts.

2.3.5) Pre-culture of *Aspergillus* sp. NR-4201

Induction media used in this experiment comprised of different concentrations of sinigrin and glucose (listed as follows) dissolved in 0.1 M potassium phosphate buffer, pH 5.6 with 6.7 g/l yeast nitrogen base. G-medium contained 11 mM glucose; SG-0.5/10.5 medium, 0.5 mM sinigrin and 10.5 mM glucose; SG-1/10 medium, 1 mM sinigrin and 10 mM glucose; SG-2.5/7.5 medium, 2.5 mM sinigrin and 7.5 mM

glucose; SG-5.5/5.5 medium, 5.5 mM sinigrin and 5.5 mM glucose and S-medium, 11 mM sinigrin.

The amounts of 25 µl inoculum (as described in 2.2.5) were placed in 25-ml erlenmeyer flask containing 2.5 ml of each SG-medium, G-medium or S-medium. Incubation was carried out at 28 °C in a waterbath, shaken at 150 rpm. Cultured filtrates were taken regularly to analyze for the amounts of glucose, sinigrin and myrosinase activity. Each mycelium collected at 32, 40 and 48 h was maintained at 4 °C for 30 min, prior to being disrupted by a mortar (as described in 2.2.7). Each cell-free extract sample obtained after centrifugation was recorded for volume and then determined for myrosinase activity by the coupled-enzyme assay.

2.3.6) Sinigrin degradation by *Aspergillus* sp. NR-4201 in liquid culture

Pre-cultures of induced and non-induced fungal cells were done as follows. Twenty-five µl of inoculum (as described in 2.2.5) were placed in 2.5 ml of either induction medium (10 mM glucose, 1 mM sinigrin and 6.7 g/l yeast nitrogen base in 0.1 M potassium phosphate buffer, pH 5.6) or growth medium (11 mM glucose and 6.7 g/l yeast nitrogen base in 0.1 M potassium phosphate buffer, pH 5.6) which were contained in 25-ml erlenmeyer flasks. Incubation was performed at 28 °C in a waterbath with shaking at 150 rpm. Cultured filtrates were taken periodically to assay for glucose and sinigrin concentration.

Fungal cell pellet harvested at late log-phase (information from 2.3.5, normally 32 h after inoculation) was transferred to incubate further in sinigrin medium (5.5 mM sinigrin in 0.1 M potassium phosphate buffer, pH 5.6) or sinigrin-glucose medium (5.5 mM sinigrin, 5.5 mM glucose and 6.7 g/l yeast nitrogen base in 0.1 M

potassium phosphate buffer, pH 5.6). Cultured flasks were incubated at 28 °C in a waterbath shaker, operating at 150 rpm. Cultured filtrates were taken periodically to assay for their contents of glucose, sinigrin, myrosinase activity and sinigrin hydrolytic product(s). Fungal cell pellet was collected by filtering through Whatman filtered paper no. 93 and washed with distilled water, before subjecting for fungal cell disruption (as described in 2.2.7). Cell-free extract samples obtained after centrifugation (10,000×g, 10 min, 4 °C) were determined for myrosinase actvity.

2.3.7) Glucosinolate degradation by *Aspergillus* sp. NR-4201 in liquid culture

a) Two-step liquid culture

Induced or non-induced cells of the *Aspergillus* pre-cultured as described in 2.3.6 and information from 2.3.5, were placed in a 25-ml erlenmeyer flask containing 2.5 ml mustard extract medium (as described in 2.2.2). Incubation was carried out at 28 °C under reciprocal shaking at 150 rpm. As incubation progressed, cultured filtrates were taken periodically to measure for the contents of glucose, glucosinolate and glucosinolate hydrolytic product(s). Mycelial samples were also collected and processed for fungal cell disruption which led to determine myrosinase activity.

b) One-step liquid culture

This one-step culture was done by inoculating 25 µl of spore suspension of the *Aspergillus* in 2.5 ml mustard extract medium which was contained in a 25-ml erlenmeyer flask. Incubation was done as the preceding experiments. Cultured filtrates and cell-free extracts were analyzed regularly as performed in the two-step culture.

2.4) PRODUCTION OF INTRACELLULAR MYROSINASE FROM *Aspergillus* sp. NR-4201

Myrosinase production by *Aspergillus* sp. NR-4201 was carried out in mustard extract medium, in which cultured conditions such as pH of cultured medium, glucosinolate concentration, incubation temperature, inoculum size and spore age were studied. The effects of L-ascorbic acid or some inorganic salt supplement on enzyme production were also evaluated. In addition, stabilities of mycelium containing myrosinase and crude enzyme extract were also studied.

2.4.1) Effect of pH of cultured medium on myrosinase production

Quantities of 400 µl of inoculum (as described in 2.2.5) were placed in a 250-ml erlenmeyer flask containing 40 ml mustard extract medium of different pH values (5.5 mM glucosinolates contained in 50 mM sodium phosphate buffer, pH 5.6, 6.5 or 7.4). Incubation was carried out at 30 °C in a waterbath, shaken at 150 rpm. At regular intervals of incubation, cultured filtrates were taken to assay for their concentrations of glucose and glucosinolates. At that time, two mycelial samples were also harvested and washed (as described in 2.2.6). One mycelial sample was placed on a pre-weighed watch glass and dried at 70 °C until a constant weight was obtained. The other sample was maintained in 50 mM sodium phosphate buffer, pH 7.4 (30 ml) at 4 °C for 30 min, prior to being disrupted by a mortar (as described in 2.2.7). Cell-free extract obtained after centrifugation (10,000×g, 30 min, 4 °C) was determined for myrosinase activity.

2.4.2) Effect of glucosinolate concentration on myrosinase production

Optimum pH value of cultured medium, obtained from 2.4.1, was introduced to use in this experiment. Enzyme production in mustard extract medium was performed by varying glucosinolate concentrations to 2.8, 5.5, 8.3 or 11 mM. During growth of the *Aspergillus* in mustard extract medium, such parameters as glucose and glucosinolate concentrations in cultured filtrates, myrosinase activity in cell-free extract and mycelial dry mass were analyzed regularly as described in 2.4.1.

2.4.3) Effect of incubation temperature on myrosinase production

Optimal pH and glucosinolate concentration obtained from the preceding experiments were used in this study. Incubation was done either at 30 or 37 °C. All procedures were similar to those described in 2.4.1.

2.4.4) Effect of inoculum size and spore age on myrosinase production

To evaluate the effect of inoculum size on myrosinase production, 400 µl of inoculum at varying concentrations of 10^5, 10^6, 5×10^6 or 10^7 spores/ml were used. Whereas, spore age of 1, 2, 3 or 4 weeks grown in PDA slants were also investigated for the effect on enzyme production.

2.4.5) Effect of L-ascorbic acid or inorganic salt supplement on myrosinase production

In this study, L-ascorbic acid was supplemented in mustard extract medium to a final concentration of 1, 2.5, 5 or 10 mM. The cultivation was carried out at optimal

condition obtained from 2.4.1-2.4.4. In case of glucose, it was added in cultured medium as the same concentrations of L-ascorbic acid.

To study the effect of some inorganic salts on myrosinase production, magnesium chloride or calcium chloride or manganese chloride or cobalt chloride were added in mustard extract medium to a final concentration of 1 or 5 mM. Fungal biomass and myrosinase production obtained at incubation period of 48 h were determined.

2.4.6) Preservation of mycelium containing myrosinase

Fungal mycelium grown in mustard extract medium at optimal condition for 48 h were harvested and washed properly (as described in 2.2.7). Mycelial samples were maintained in 20 ml of 40 mM sodium phosphate buffer, pH 7.0 at 4 °C. These mycelial samples were disrupted after 1, 12, 24 and 48 h, respectively. While the other mycelial sample was kept at -40 °C for 24 or 48 h, before thrawing at room temperature and disrupting with the same procedure. Each cell-free extract was determined for myrosinase activity.

2.4.7) Stability of crude myrosinase extract

Crude enzyme extracts of the *Aspergillus* in 10-100 mM of various buffers were maintained at room temperature or at 4 °C. Anti-microbial growth agents such as sodium azide and thimerosal were added into enzyme solution at a final concentration of 0.02 % each. At regular intervals of time, myrosinase activity in each sample was determined.

2.5) PURIFICATION OF *Aspergillus* MYROSINASE [114, 115]

All purification works were done in cold room (temperature 3 - 6 °C) unless stated otherwise.

2.5.1) Ammonium sulfate fractionation

Crude enzyme extract obtained after centrifugation was subjected for partial purification by mean of ammonium sulfate fractionation. In order to keep pH constant during salting out, enzyme solution was adjusted to be contained in 50 mM sodium phosphate buffer, pH 7.0. Forty ml of the enzyme solution were placed in a beaker and cooled at 4 °C for 30 min. Then, solid ammonium sulfate pre-weighed and pre-cooled for at least 30 min was added slowly, whilst stirring, to a final concentration of 40 % saturation. When ammonium sufate salt was dissolved completely, it was then stirred for an additional 30 min. The solution was allowed to settle for 30 min, prior to being centrifuged (10,000×g, 20 min, 4 °C).

Then, protein pellet was dissolved in 5 ml of chilled 40 mM sodium phosphate buffer, pH 7.0 and dialyzed against the same buffer, three times, 1 liter each. Supernatant was subsequently fractionated in a series of ammonium sulfate concentrations of 50, 60 and 70 % saturation, respectively. Each protein pellet obtained after centrifugation was dissolved and dialyzed as described earlier.

2.5.2) Dialysis

Dialysis tube of appropriate diameter was cut into suitable lengths to contain the required volume. Then, it was submerged in distilled water for at least 24 h or boiled for 10 min. Before using, the dialysis tube was rinsed with dialysis buffer for several times. The bottom end of the tube was sealed with knot or clip. Then, enzyme

solution was poured into the tube and air bubbles over the solution were expelled out. Subsequently, the top end of tube was then sealed. The dialysis tube with enzyme solution was placed in a large volume (traditionally 20-50 times of enzyme volume) of chilled 40 mM sodium phosphate buffer, pH 7.0. Agitation was made gently by magnetic stirring. The first and second changes of dialysis buffer were performed usually within 6 h, whereas, the third change was 12 h. After complete dialysis process, enzyme solution was centrifuged (10,000×g, 20 min, 4 °C). The supernatant was determined for protein and myrosinase activity content.

2.5.3) DEAE Sephadex A-25 chromatography (I)

a) Swelling the resin

Ten grams of DEAE Sephadex A-25 powder was placed in distilled water. It was allowed to swell and settle at room temperature for 24 h or boiling at 90 °C for 1 h. Mixing the gel slurry was recommended by stirring with a glass rod, not by magnetic stirrer.The slurry was then filtered through filtered paper using a Bushner funnel and washed with 2 l of distilled water, and followed with 500 ml of 100 mM sodium phosphate buffer, pH 7.0. Then, the slurry was placed in 200 ml of 40 mM sodium phosphate buffer, pH 7.0 and allowed to settle at room temperature for 2 h. Floated particles were removed by sucking with a pasteur pipette connected to a vacuum pump. The slurry was adjusted to desired pH of 7.0 by adding 0.1 N NaOH, prior to cool at 4 °C for 4 h. Then, it was subjected to de-gas using a vacuum pump.

b) Preliminary study of separating conditions in small DEAE Sephadex A-25 columns

Swolen DEAE Sephadex A-25 slurry in 20 mM of sodium phosphate buffer, at different pH values (6.0, 6.5, 7.0 or 7.4) was packed in a 3-ml syringe to a packing volume of 1 ml. Each column was equilibrated with 5 ml of 10 mM sodium phosphate buffer, at the desired pH. Enzyme solution obtained from 50-60 % ammomnium sulfate precipitated fraction was dialyzed three times against distilled water. Then, this crude enzyme was mixed with an equal volume of 20 mM of sodium phosphate buffer, pH 6.5 or 7.0 or 7.4. Each enzyme sample of 1 ml volume was loaded on its corresponding column and then washed with 5 ml of the equilibrating buffer. Step-wise elution was performed with different concentrations of sodium phosphate buffer, in a series of 20, 40, 75, and 125 mM, respectively. Eluates from the column were collected and determined for protein content and myrosinase activity.

c) Packing and equilibrating the column

A column (2.6×30 cm) assemblied according to the instruction's manual and equipped with an outlet-tube connected to peristaltic pump, was casted properly to the stand. After rinsing with 10 ml of 40 mM sodium phosphate buffer, pH 7.0, 80 ml of gel slurry was poured into the column. The slurry was overlaid with 40 mM sodium phosphate buffer, pH 7.0 up to the top end of the column. After the gel was settled, an adapter was applied and adjusted to approximately 1 cm from bed-packed gel. The column was washed with 40 mM sodium phosphate buffer, pH 7.0 for at least 5 column volumes, operated at a flow rate of 2 ml/min. Then, distance between bed-packed gel and adapter was adjusted to approximately 5 mm. To equilibrate the

column, 5 column volumes of 40 mM sodium phosphate buffer, pH 7.0 were added at the same flow rate.

d) Separating procedure

After equilibration, enzyme solution (50-60 % ammonium sulfate precipitated fraction) was applied into the column, using three-way inlet adapter. Subsequently, the column was washed with the equilibrating buffer at the same flow rate. Eluates from the column of 10-ml fractions were collected and monitored spectrophotometrically at 280 nm for protein content. Equilibration was made until absorbance values of the eluates were nearly zero. Then, the column was eluted with a linear gradient of 40-100 mM sodium phosphate buffer, pH 7.0 (600 ml). Fractions of 10-ml were collected and then measured for protein content and myrosinase activity. Myrosinase active fractions were combined and concentrated by using ultrafiltration.

e) Method for measuring of enzyme activity in eluated fractions

Enzyme activity in eluated fractions was measured by the glucose released assay. In this case, 25 µl of 10 mM sinigrin in 50 mM sodium phosphate buffer, pH 7.0 was mixed with 25 µl of enzyme solution and incubated at 25 °C for 10 min. Enzymatic reaction was stopped by placing in boiling water for 2-3 min. After cooling, 1.0 ml of color reagent (glucose oxidase) were added. After mixing and incubating at 25 °C for 10 min, an absorbance at 546 was then measured. Glucose content was calculated from the calibration curve for glucose (as shown in appendix). One unit of myrosinase was defined as the amount of enzyme able to produce 1 µmol of glucose from sinigrin at the condition described above.

2.5.4) Ultrafiltration

To remove any preservatives contained in an ultrafiltration membrane (molecular weight cut-off 50 kDa), a membrane disc with glossy side up was placed in distilled water for 4 h. Assembly of the membrane to the ultrafiltration apparatus was casted according to the instruction's manual, and connected to pressurized nitrogen valve. The ultrafiltration-stirred cell was then passed with 50 ml of chilled distilled water and followed by 50 ml of 40 mM sodium phosphate buffer, pH 7.0, operating at a pressure of 1-3 psi. During that time, stirring was operated at 100 rpm. Quantities of 10-100 ml of enzyme solution were poured in the stirred cells which then processed as the preceding description. The enzyme sample was ultrafiltrated to the required volume, normally not exceeded 5 % of the initial volume. Then, ultrafiltration was repeated for at least three times, using 50 mM sodium phosphate buffer, pH 7.0.

2.5.5) DEAE Sephadex A-25 chromatography (II)

a) Packing and equilibrating the column

In this experiment, myrosinase separation was done in a small column (1.0×15 cm). Packing and equilibrating the column was performed as described in 2.5.3. In this case, 50 mM sodium phosphate, pH 7.0 was used as an equilibrating buffer, operating at a flow rate of 1 ml/min.

b) Separating procedure

Protein sample obtained after ultrafiltration, which was contained in 50 mM sodium phosphate buffer, pH 7.0 was loaded onto the column. Then, the column was washed with the equilibrating buffer until an absorbance of the eluates was zero. Elution was performed with a concave gradient of 50-90 mM sodium phosphate

buffer, pH 7.0 at a flow rate of 1 ml/min (gradient-forming model is shown in appendix). Five-ml fractions were collected. Protein content and myrosinase activity were then measured. Myrosinase containing fractions were examined for their purities by SDS-PAGE. The combined enzyme solution indicated by SDS-PAGE result, was extensively ultrafiltrated to a final volume of about 1 ml contained in 40 mM sodium phosphate buffer, pH 7.0.

2.5.6) Sephadex G-100 gel-filtration

a) Packing and equilibrating the column

Twenty grams of Sephadex G-100 resin was swelled in distilled water at room temperature overnight. The gel slurry obtained after filtration using Buchner funnel, were suspended in 40 mM sodium phosphate buffer, pH 7.0. After allowed to settle for 2 h, floated particles were aspirated out. The degased-gel slurry was poured in a 1.6×100 cm column, connected to a peristaltic pump operating at a flow rate of 15 ml/h.The adapter was applied and adjusted to be adjacent to the top of packed gel bed. Equilibration was done with 40 mM sodium phosphate buffer, pH 7.0 using 5-10 times of the total column volume.

b) Calibrating and separating procedure

Void volume and total volume of the column were measured by using blue dextran 2000 and potassium dichromate. To evaluate an efficiency of the column packing, blue dextran, bovine serum albumin, ovalbumin and chymotrypsinogen were used. Concentrated myrosinase solution with appropriate volume was loaded into the Sephadex G-100 column. The flow rate was maintained at 15 ml/min and 1-ml fractions were collected. Eluates were assayed for protein content and myrosinase

activity. Homogeneity of interesting fractions was assessed by SDS-PAGE. Fractions containing highly purified myrosinase were pooled, concentrated and dialyzed against 10 mM sodium phosphate buffer, pH 7.0.

2.5.7) SDS-polyacrylamide gel electrophoresis [116]

a) Gel polymerization

A mini-VE vertical slab-gel unit was assembled in the casting stands according to the manufacturer's manual, using a 0.75 mm-thick spacer. The 10 % total acrylamide (%T) separating gel solution was made by mixing 10.0 ml of monomer solution (30.8 % T and 2.7 % cross link, C), 7.5 ml of separating gel buffer (1.5 M tris-HCl buffer, pH 8.8), 0.3 ml of 10 % SDS and 12.1 ml of deionized water to give a 30-ml total volume. Then, 150 µl of 10 % ammonium persulfate and 10 µl of TEMED were added and mixed gently. The solution was poured into the slab compartment approximately 1 cm, being below the upper level. The gel was overlaid carefully with deionized water and it was allowed to polymerize for about 1 h at room temperature. The complete polymerization was confirmed by observing a miniscus between the gel layer and deionized water.

Stacking gel solution comprised with 4 % T was made by mixing 1.33 ml of monomer solution, 2.5 ml of stacking gel buffer (0.5 M tris-HCl buffer, pH 6.8), 0.1 ml of 10 % SDS and 6.0 ml of deionized water. The amounts of 50 µl of 10 % ammonium persulfate and 5 µl of TEMED were added and mixed gently. Subsequently, the overlaid water was poured off from the gel unit and the about 2 ml of stacking gel solution were carefully filled up instead. Then, a comb was inserted between the glass sandwich. This had to be done carefully without an air bubble

formed below the teeth of the comb. It was allowed to set for about 1 h for polymerization completion.

b) Preparation of protein samples

Interesting protein fractions were concentrated by ultrafiltration, which were contained in low ionic strength buffer. Appropriate concentrations of crude protein samples were about 10-20 mg/ml, whereas, those of the partially purified samples were about 5-10 mg/ml. One part of protein sample was mixed with one part of the sample buffer (0.125 M tris-HCl, 4 % SDS, 20 % (v/v) glycerol, 0.2 M dithiothreitol and 0.02 % bromophenol blue, pH 6.8), prior to being heated in boiling water for 3 min.

c) Sample application

The comb was slowly removed from the gel and rinsed with running buffer. The gel was installed properly in the electrophoretic apparatus according to the manufacturer's instruction. A constant current of 20 mA was circulated to pre-equilibrate the buffer system for 15 min. Then, the amounts of 5-10 µl of samples and molecular weight protein markers were loaded carefully into each lane by using a suitable microsyringe.

d) Separating procedure

During separation within the stacking gel, a constant current of 1.5 mA per lane was supplied until the bromophenol blue tracking-dye entered into the separating gel. For protein separation in the separating gel, the current was supplied constantly at 2 mA per lane. When the tracking-dye front reached the gel bottom, the power supply turned off. After pouring out the running buffer (0.025 M tris, 0.192 M glycine and 0.1 % SDS, pH 8.3), the gel was de-assembled and was removed from the sandwich glasses.

e) Staining and destaining

Staining was performed at room temperature by placing the gel in staining solution (0.025 % coomassie brilliant blue R-250, 40 % methanol and 7 % acetic acid). It was shaken on a rotary shaker, operating at 30-40 rpm for 4 h. Destaining protocol was done as follows. The gel was shaken with destaining solution I (7 % acetic acid and 40 % methanol) for 30 min, prior to being replaced with destaining solution II (7 % acetic acid and 5 % methanol). The destaining solution II was changed every 6-12 h until the gel background was clear.

2.5.8) Sephadex G-200 gel-filtration

To assess of molecular mass of native enzyme, Sephadex G-200 chromatography was performed in a 1.6×100 cm column. Concentrated solution (0.4 ml) of the purified enzyme were loaded into the column which was operated at a flow rate of 12.5 ml/h. One-ml fractions were collected. Total volume and void volume of the column were determined by loading blue dextran 2000 and potassium dichromate, operating at the same flow rate. Alcohol dehydrogenase (M.W. 150 kDa), bovine serum albumin (M.W. 67 kDa), ovalbumin (M.W. 43 kDa) and chymotrypsinogen (M.W. 26 kDa) at a concentration of 1 mg/ml each, were used to calibrate the column.

2.6) CHARACTERIZATION OF THE PURIFIED *Aspergillus* MYROSINASE

2.6.1) Methods for activity assay of the purified *Aspergillus* myrosinase

All methods used to determine enzyme activity of the purified *Aspergillus* myrosinase were based on a kinetic-monitoring assay, which were determined at the zero-ordered reaction. An acceptable reaction rate ($\Delta A/\Delta t$) should be monitored

constantly for at least 5 min. The assay was traditionally carried out at 25 °C, comprising of 1.0 ml of reaction reagent and 10 µl of enzyme solution. Enzyme should be in a solution with low ionic strenght. Aliquots of enzyme with appropriate dilution should be kept at the desired temperature for at least 5 min, before starting the assay.

a) Determination of enzyme activity by a spectrophotrometric method [86, 95]

The assay was carried out on a double beam Shimadsu UV-265 spectrophotometer. Cuvette holders were connected to a circulating waterbath equipped with a temperature control apparatus which was adjusted to a desired temperature. The buffered substrate of 1.0 ml total volume (100 µM sinigrin in 33 mM sodium phosphate bufer, pH 7.4) was maintained in quartz cuvettes (1.0 ml light path) at a desired temperature for sufficient time (3-5 min) before adding the enzyme. After mixing, the rate of sinigrin hydrolysis was monitored directly by following the decrease of absorbance by time ($\Delta A/\Delta t$) at 227 nm. A constant reaction rate should be observed for at least 5 min. One unit of β-thioglucosidase activity was expressed as the amount of enzyme that catalyzed the hydrolysis of 1 µmole of sinigrin per minute at the conditions described above.

b) Determination of enzyme activity by a coupled-enzyme method [43, 55]

The assay was carried out on a double beam Shimadsu UV-265 spectrophotometer. The cuvette holders were connected to a circulating waterbath equipped with a temperature control apparatus in which was adjusted to a desired temperature. One ml of buffered substrate (33 mM sodium phosphate buffer, pH 7.4 containing 5 mM sinigrin, 3 mM $MgCl_2$ 0.55 mM ATP, 0.72 mM NADP, 3.5 U hexokinase and 1.75 U glucose-6-phosphate dehydrogenase) was pre-incubated for a sufficient time (3-5 min)

at 25 °C. Then, appropriate amounts of the enzyme were added. After mixing, the reaction rate was monitored at 340 nm. Myrosinase activity was calculated from absorbance increasing rate, which was due to the formation of NADPH (ε_{340nm} = 6220 $M^{-1}cm^{-1}$). One unit of myrosinase activity was defined as the amount of enzyme that catalyzed the liberation 1 μmol of glucose per minute from sinigrin under the conditions describe above.

2.6.2) Absorptibility of sinigrin

In this study, extinction coefficient of sinigrin was determined from average absorbance value at 227 nm of 100 μM sinigrin in deionized water (ten samples).

2.6.3) Effect of enzyme concentration on activity assay

Aliquots of the purified *Aspergillus* myrosinase in 10 mM sodium phosphate buffer, pH 7.4 were maintained at the assayed temperature for at least 5 min, before starting the assay. In this study, the reaction was carried at 25 °C. The amounts of the enzyme which exhibited the zero-ordered reaction would later be used in further experiments.

2.6.4) Optimum pH

Optimum pH for activity of the purified *Aspergillus* myrosinase was determined by the spectrophotometric assay (as described in 2.6.1.a). In this case, pH values of buffered substrates were varied from 4 to 9 (4-6 : sodium boric acid-phosphate buffer, 6-8 : sodium phosphate buffer and 8-9 : tris buffer).

2.6.5) Optimum temperature

Optimum temperature for activity of the *Aspergillus* myrosinase were assessed by the method described in 2.6.1.a. Temperatures of the assayed system were varied from 25 to 37 °C.

2.6.6) pH stability

To study pH stability, enzyme samples in various 10 mM buffer (4-6 : sodium boric acid-phosphate buffer, 6-8 : sodium phosphate buffer and 8-9 : tris buffer) were kept at 4 °C for 24 h. The samples were then diluted 5-times with 100 mM sodium phosphate buffer, pH 7.4 and then measured for remained activity by the coupled-enzyme method.

2.6.7) Temperature stability

Samples of the purified enzyme in 40 mM sodium phosphate buffer, pH 7.0, were maintained at different temperatures for 15 min. Then, samples were determined for remained activity by the coupled-enzyme method.

2.6.8) Preservation of the purified *Aspergillus* myrosinase

Samples of the purified enzyme in different buffers (10-40 mM sodium phosphate buffer, pH 6.0-8.0) contained in screw-capped vials were kept at 4 °C, 25 °C and room temperature (about 30 °C). Anti-microbial agents such as sodium azide and thimerosal were added in enzyme solution to a final concentration of 0.02 % (w/v). Enzyme samples were taken periodically to determine the remaining activity.

Enzyme solutions were dialyzed against deionized water or 5 mM sodium phosphate buffer, pH 7.0 at 4 °C overnight (about 14 h). Each enzyme sample was poured in a freeze-dry bottle and frozen at -40 °C, prior to being subjected to the freeze-dryer machine. Freeze-dried enzyme powder was kept at -70 °C for several weeks. Reconstitution was done by dissolving the protein powder with chilled 10 mM sodium phosphate buffer, pH 7.0, prior to assay the remaining activity.

2.6.9) Effect of some inorganic salts or organic compounds on enzyme activity

To study the effect of some inorganic salts or organic compounds on myrosinase activity, it was employed by the spectrophotometric method using sinigrin as a substrate. Quantities of 1.0 ml of 33 mM sodium phosphate buffer, pH 7.4 containing 1 mM each inorganic salts or organic compounds were poured in a cuvette and kept at 25 °C for 3-5 min. Then, 10 µl of the purified enzyme with appropriate activity was added into the cuvette. After mixing for 5 sec, 10 µl of 10 mM sinigrin in deionized water were added. It was mixed immediately and then monitored the rate of sinigrin hydrolysis at 227 nm.

Some metal ions such as manganese, nickel, cobalt, zinc, *etc* were capable of forming insoluble salts with phosphate that interfered the absorbance at 227 nm. In this case, sodium phosphate buffer was replaced with deionized water. Relative activity of the enzyme with such metal ions could be compared with activity of the enzyme in deionized water.

2.6.10) Substrate specificity

For assessment of substrate specificity towards the purified *Aspergillus* enzyme, p-nitrophenyl derivatives were used in the spectrophotometric method, as described in 2.6.1.a. In this case, p-nitrophenyl-O-glycosides were used at a concentration of 2 mM each. Reaction rate was monitored at 430 nm due to the liberation of p-nitrophenol. Relative activity could be compared with the activity towards pNPG. In case of non p-nitrophenyl glycosides, 500 µl each (1 % contained in 50 mM sodium phosphate buffer, pH 7.4) and 50 µl of the purified enzyme (0.3 U) were incubated at 25°C for 2 h. The amount of reducing sugar released was measured by using the dinitrosalicylic method (as described in 2.2.9).

2.6.11) Kinetic constants

Kinetic constants for sinigrin-hydrolyzing activity (β-thioglucosidase) of the purified *Aspergillus* myrosinase were determined both by the spectrophotometric assay (25-150 µM sinigrin) and the coupled enzyme assay (0.1-5.0 mM sinigrin) depending on sinigrin concentrations. In case of β-glucosidase activity towards pNPG, it was determined spectrophotometrically at 430 nm using different pNPG concentrations (0.25-5.0 mM). Apparent K_m and V_{max} values were calculated from the double-reciprocal plots of Lineweaver and Burk method.

2.6.12) Inhibition of sinigrin hydrolysis by pNPG

Quantities of 950 µl of 50 mM sodium phosphate buffer, pH 7.4 containing different concentrations of pNPG (50, 100 or 150 µM) were placed in a cuvette and incubated at 25 °C for 3-5 min. Then, 10 µl of enzyme with appropriate activity were

added and mixed gently for exactly 5 sec. After mixing, 50 µl of sinigrin dissolved in deionized water were immediately added into the reaction mixtures to final concentrations of 25, 50, 75, 100, 125 or 150 µM. The reaction rate of sinigrin hydrolysis was monitored at 227 nm for at least 5 min.

2.6.13) Inhibition of pNPG hydrolysis by sinigrin

Quantities of 950 µl of the pre-incubated buffer (50 mM sodium phosphate buffer, pH 7.4) containing different concentration of sinigrin (200, 400 or 600 µM) were mixed gently with 10 µl of enzyme with appropriate activity for exactly 5 sec. After mixing, 50 µl of pNPG in 50 mM sodium phosphate buffer, pH 7.4 were added in the reaction mixtures. Final concentrations of pNPG added were desired to be 0.25, 0.5, 1, 2, 2.5 and 5 mM. The hydrolysis of pNPG was monitored at 430 nm for at least 5 min.

2.6.14) Inhibition of sinigrin hydrolysis by D-glucose

This study was performed as in experiment 2.6.12 but different concentrations of D-glucose (5 or 10 mM) were used instead of pNPG.

2.7) ANALYSIS OF GLUCOSINOLATE BREAKDOWN PRODUCTS BY GAS CHROMATOGRAPHY

2.7.1) Products from sinigrin degradation by the purified *Aspergillus* myrosinase and *Sinapis alba* myrosinase

Reaction mixtures of 1.0 ml total volume were carried out in screw-capped vials (10 × 50 mm) containing 0.1 M designed buffer (sodium phosphate or sodium

citrate-phosphate or tris), 10 mM sinigrin and 0.3 U of the purified *Aspergillus* enzyme (or *Sinapis alba* myrosinase). During incubation at 25 °C, samples were taken periodically to assay glucose and sinigrin concentrations. After incubation for 1-2 h, the vials were placed in an ice-bath. Samples and standards were extracted with an equal volume of chilled hexane (GC grade). After adding approximately 2 g of solid ammonium sulfate, capping, shaking and centrifuging (3,000×g, 10 min), the hexane layer was subjected for GC analysis, as described in 2.2.10.

2.7.2) Products from sinigrin degradation by disruted fungal cells of the *Aspergillus*

Fungal cell pellet of the *Aspergillus* grown in mustard extract medium was collected after 48 h. After washing with deionized water, the mycelium was disruted immediately at room temperature without adding any buffers. Then, 0.5 ml of the fungal suspension was incubated with 0.5 ml of 20 mM sinigrin in deionized water at 25 °C. After incubation for 1 or 2 h, supernatant obtained after centrifugation (10000×g, 10 min) was assayed for the contents of sinigrin, glucose and sinigrin hydrolysis product(s).

2.7.3) Products from glucosinolate degradation (*Brassica juncea* seeds) by crude *Aspergillus* myrosinase

This experiment was performed as described in 2.7.1. In this case, mustard extract (*Brassica juncea*) at the corresponding concentration was used instead of sinigrin. Crude *Aspergillus* myrosinase with the same activity was used.

2.7.4) GC-MS analysis of sinigrin breakdown products

The major or minor products, generated from sinigrin hydrolysis were achieved by a Hewlett-Packard 6890 series GC-system, equipped with a (5 %)-diphenyl-(95 %)-dimethylpolysiloxane column (50m×0.3mm ID), connected to the Agilent 7683 series injector port. The carrier gas (helium) was operated constantly at a flow rate of 1.5 cm^3/min and a pressure of 50 kPa. Temperature of the column was initially maintained at 80 °C for 6 min and then programmed to increase at a flow rate of 10 °C/min. Each compound was analyzed for its spectral pattern by a Hewlett-Packard 5973 mass selective detector (electron impact).

In case of chemical impact analysis, it was performed with the system and conditions as precedingly described, but a polydimethylsiloxane column was used instead.