CHAPTER I

INTRODUCTION

1.1) BACKGROUND

Glycosyl hydrolases or glycosidases (EC 3.2.1.x - EC 3.2.3.x) are a wide-spread group of enzymes hydrolyzing the glycosidic linkage between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety [1]. In nature, 2 subgroups of these enzymes, O- and non-O-glycosyl hydrolases are responsible for oxyglycosides (O-glycosides) and non-oxyglycosides (non-O-glycosides), respectively [2]. All other O-glycosyl hydrolases are lack of hydrolyzing towards non-O-glycosides as thioglycosides (S-glycosides or glucosinolates) which naturally occur in plants of the order *Capparales* [3-5]. In respect of this, myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) is an only known enzyme able to hydrolyze glucosinolates [1, 6]. The enzyme usually co-exists in all glucosinolate containing plants [4, 7]. Glucosinolate hydrolysis leads to the production of D-glucose, sulfate, and a serie of sulfur and/or nitrogen containing compounds, nitrile, isothiocyanate, thiocyanate or cyanoepithioalkane. Such reaction products are formed exclusively as substrate specific, pH dependent or availability of some specific factors [8-16].

Since glucosinolates constitute more than 120 species distributing among *Capparales*, number of possible glucosinolate metabolites may be over 300 species [3, 17]. These compounds possess different flavouring and chemical properties being characteristics in such plant varieties [4, 18]. Despite of the co-existing myrosinase, the enzyme usually occurs as isoenzymes which are distributed exclusively as organ, age

and species specific [4, 8, 19]. Each isoenzyme exhibits different specificities among glucosinolate substrates [4, 20]. This indicates complexity of the myrosinase-glucosinolate system in plants. In term of plant physiology, the system has been established to function in defense mechanism against insects and phytopathogen, growth regulation and mobilization of sulfur and nitrogen [4, 21].

Among sixteen families of the *Capparales*, the family *Brassicaceae* (formerly *Cruciferae*) is named as economically important crops of the world [22]. Relationship of these plants, particularly the genus *Brassica*, to human is mentioned that *Brassica* oilseeds such as rape (*Brassica napus*), mustards (*B. juncea* or *B. nigra*) and canola (*Sinapis alba*) are economically important oil-producing crops [22]. The residue remaining after fixed-oil expellation from the seeds is protein-enriched which can be utilized further as animal feeds [23-25]. Different varieties of the genus *Brassica* such as cabbage (*B. oleracea* var. capitata), broccoli (*B. oleracea* var. botrytis), leaf mustard (*B. juncea*), turnip (*B. campestris* var. rapifera), *etc* are world-widely served as traditional vegetables [18]. Other *Brassica* crops such as kale (*B. oleracea* var. acephala) and swede (*B. napus* var. napobrassica) may also contribute indirectly to human food chains [18]. In addition, isothiocyanate compounds from brown and white mustard seeds (*B. juncea* and *S. alba*), generally known as mustard essential oils are responsibly exerted as condiments in several kinds of food products [18]. Some glucosinolate breakdown products offer anti-carcinogenic property that has been extensively studied for their therapeutic applications [3, 26, 27].

There were a number of works established that excessive consumption of *Cruciferous* plants or their by-products lead to the formation of toxic effects such as thyroid hypertrophy, liver haemorrhage, spleen enlargement and growth retardation in

experimental animals [28-31]. It has been elucidated that intact glucosinolates are non-toxic, whereas, their breakdown metabolites are toxic [32]. Although the endogenous myrosinase contained in such materials could be destroyed before consumption, these toxic effects still appeared [23, 32]. Several investigators suggested that intact glucosinolates could be biologically degraded within gastrointestinal tract [28, 29, 33]. In order to prove this hypothesis, a number of microflora were examined for glucosinolate-degrading activity. Gut bacteria, *Paracolobactrum aerogenoides* and *Escherichia coli*, isolated from fecal samples were established for their progoitrin-consuming capability [33]. In case of *P. aerogenoides*, myrosinase activity was also detected in cell-free extract [33]. Subsequently, a variety of bacterial strains such as *Bacillus subtilis* [33], *Bacillus cereus* [33], *Pseudomonas aeroginosa* [33], *Proteus vulgaris* [33], *Aerobacter aerogens* [33], *Alcaligenes faecilis* [33], *Staphylococcus epidermidis* [33], *Enterobacter cloacae* no. 406 [34], *Lactobacillus acidophilus* LEM220 [28], *Streptomyces* sp. [24], *Bacillus* sp. [24] and *Staphylococcus* sp. [24] have been isolated and established for their degradative capability for glucosinolates. In the field of food toxicology, the presence of undesirable glucosinolates in *Cruciferous* crops or their by-products affects directly to their usefulness as human diets or animal feeds [23, 31, 32].

There have been attempts to improve nutritional quality of the by-products from *Brassica* oilseeds (traditionally known as oilseed meals). Several physico-chemical methods such as solvent extractions or steam stripping have been introduced to eliminate the undesirable glucosinolates from these oilseed meals [35-40]. However, all these processes have major drawback effects in reducing nutritive enrichment from the meals and could not be applied practically as industrial level [41]. Over the past 10

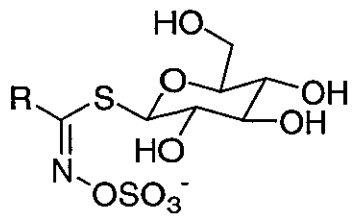
years, research on nutritional improvement of *Brassica* oilseeds meals have focused on biological processing [42-45]. Microbial strains with high glucosinolate-degradative potential are required for the detoxifying process. *Lactobacillus agilis* R16 [45], *Aspergillus clavatus* II-9 [43] and *Fusarium oxysporum* @146 [43] have been established to be high degradative potential for the glucosinolate sinigrin in liquid culture. The bacterium *Lactobacillus* also exhibited high activity for glucosinolate degradation in mustard extract medium (*Brassica juncea*). In the case of *Aspergillus* and *Fusarium*, both fungal strains demonstrated potential in degrading glucosinolates in mustard seed meals (*B. juncea* or *S. alba*) under solid-state fermentation. Fates of glucosinolate degradation by these three strains were rather different. Concerning with sinigrin degradation in liquid culture, allylisothiocyanate and allylcyanide were produced by whole cells of the *Lactobacillus* and *Aspergillus*, respectively. Whereas, none of degradation product was produced by the *Fusarium*. Myrosinase activity was only detected in cell-free extract of the *Aspergillus,* whereas, no enzyme activity was detected in the *Lactobacillus* and *Fusarium*. Cell-free extract of the *Aspergillus* showed capability to produce allylisothiocyanate from sinigrin hydrolysis at pH 6.2, while no liberation of allylcyanide was found. Several investigators suggested that sinigrin consumption by the *Aspergillus* was an intracellular process involving myrosinase [43]. While myrosinase did not involve in sinigrin degradation by the *Fusarium*. Isothiocyanate production by the *Lactobacillus* should be indicated from the action of myrosinase. However, the enzyme was very unstable [45]. These evidences indicate that mechanism(s) of glucosinolate degradation *via* intact microbial cells are complicated and varied among strains.

In term of chemoprevention, glucosinolate metabolites such as sulforaphane, phenylisothiocyanate, phenethylisothiocyanate, cyanohydroxybutane and indole-3-carbinol have been established for their anti-cancer properties *in vitro* [3, 26]. The occurrence of these metabolites *in vivo* has mentioned to be engaged by glucosinolate-degrading microflora. There is, however, limited knowledge concerning the *in vivo* metabolism of glucosinolates among a variety of microflora species. Recently, therapeutic applications of these anti-cancer compounds have been studied [3, 26].

1.2) GLUCOSIONOLATES

1.2.1) Definition and chemical structure

Glucosinolates (1-thiol-β-D-glucosides or β-thioglucoside-N-hydroxysulfates or S-glucopyranosyl thiohydroxymates or (Z)-(or cis)-N-hydroxyminosulfate esters) are a class of sugar anionic thioesters containing β-thioglucoside linkage [3]. In nature, glucosinolates are usually formed as potassium salt. Recently, more than 120 glucosinolate species have been discovered which differ by their aglycon substituents [3]. Chemical structure of glucosinolates is illustrated in Figure 1.1 [3, 17, 46].



R = side-chain group

Figure 1.1 Chemical structure of glucosinolates

1.2.2) Classification of glucosinolates

On the basis of side-chain variation, glucosinolates can be classified into 4 major classes *e. g.* aliphatic, aromatic, indole and glycated glucosinolates [3].

a) Aliphatic glucosinolates

This class of glucosinolates comprises 6 subclasses, including aliphatic straight, aliphatic branched, aliphatic alcohol, aliphatic ketone, olefin and alkyl-thioalkyl glucosinolates, as shown in Table 1.1.

b) Aromatic glucosinolates

There are two subclasses of the aromatic glucosinolates , aromatic and benzoyl derivatives, as shown in Table 1.2.

c) Indole glucosinolates

Examples are shown in Table 1.3.

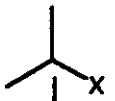
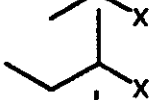
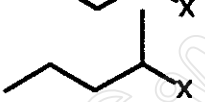
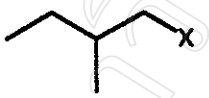
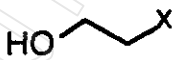
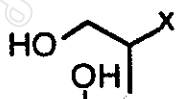
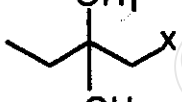
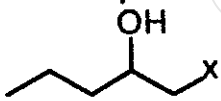
d) Glycated glucosinolates

Examples are shown in Table 1.4.

1.2.3) Distribution of glucosinolates

Glucosinolates are naturally present in plants of the order *Capparales*, including the families *Alkaniaceae, Bataceae, Brassicaceae, Bretschneideraceae, Capparaceae, Caricaceae, Euphorbiaceae, Gyrostemonaceae, Limnanthaceae, Moringaceae, Pentadiplantdraceae, Resedaceae, Salvodoceae, Tropaeolaceae* and *Tovariaceae* [3, 5]. Among these families, the *Brassicaceae* is mentioned as traditional vegetables of the world, especially the genus *Brassica* [18]. This genus comprises of three original species, *Brassica nigra*, *Brassica oleracea* and *Brassica campestris*.

Table 1.1 Aliphatic glucosinolates (derived from reference 3)

Side chain group	Scientific name	Common name
Aliphatic straight		
	Methylglucosinolate	Glucocapparin
	Ethylglucosinolate	-
	n-Propylglucosinolate	-
	n-Butylglucosinolate	-
Aliphatic branched		
	1-Methylethylglucosinolate	Glucoputranjivin
	1-Methylpropylglucosinolate	Glucocochlearin
	1-Methylbutylglucosinolate	-
	2-Methylbutylglucosinolate	-
Aliphatic alcohol		
	2-Hydroxyethylglucosinolate	-
	1-Methyl-2-hydroxyethylglucossinolate	Glucosisymbrin
	2-Hydroxy-2-methylbutylglucosinolate	Glucocleomin
	2-Hydroxypentylglucosinolate	-

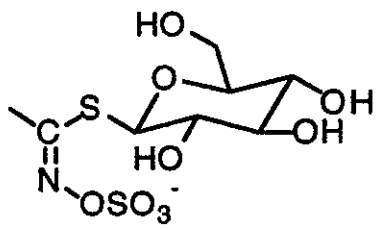
X = 

Table 1.1 Aliphatic glucosinolates (continued)

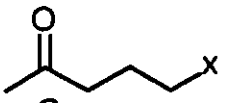
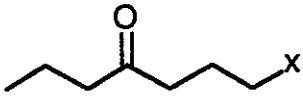
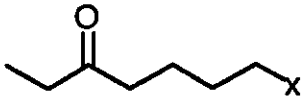
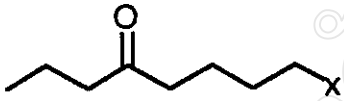
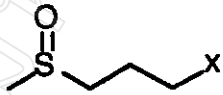
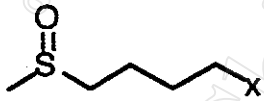
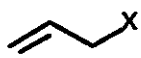
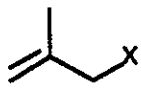
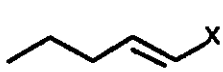
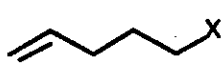
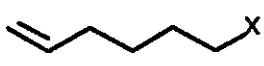
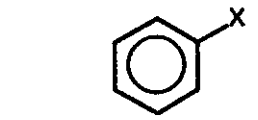
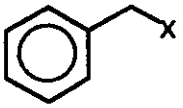
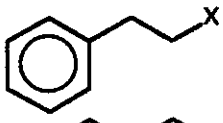
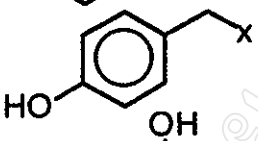
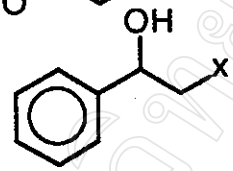
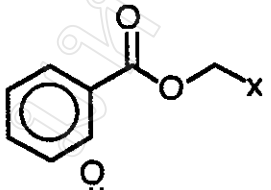
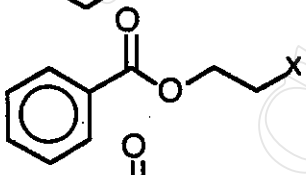
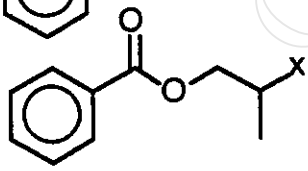
Side chain group	Scientific name	Common name
Aliphatic ketone		
	4-Oxopentylglucosinolate	-
	4-Oxoheptylglucosinolate	Glucocapangulin
	5-Oxoheptylglucosinolate	Gluconorcappasalin
	5-Oxooctylglucosinolate	Glucocappasalin
Alkylthioalkyl		
	2-(Methylthio)ethylglucosinonate	Glucoviorylin
	3-(Methylthio)propylglucosinolate	Glucoiberverin
	4-(Methylthio)butylglucosinolate	Glucoerucin
	3-(Methylsulfinyl)propylglucosinolate	Glucoiberin
	4-(Methylsulfinyl)butylglucosinolate	Glucoraphanin
Olefin		
	3-Propenylglucosinolate	Sinigrin
	2-Methyl-2-propenylglucosinolate	-
	1-Pentenylglucosinolate	Glucobrassicanapin
	4-Pentenylglucosinolate	-
	5-Hexenylglucosinolate	-

Table 1.2 Aromatic glucosinolates (derived from reference 3)

Side chain group	Scientific name	Common name
Aromatic		
	Phenylglucosinolate	-
	Benzylglucosinolate	Glucotropaeolin
	2-Phenylethylglucosinolate	Gluconasturtiin
	4-Hydroxybenzylglucosinolate	Glucosinalbin
	2(R)-Hydroxy-2-phenylethylglucosinolate	Glucobarbarin
Benzoyl		
	Benzoyloxymethylglucosinolate	-
	2-(Benzoyl)ethylglucosinolate	-
	2-Benzoyloxy-1-methylethylglucosinolate	-

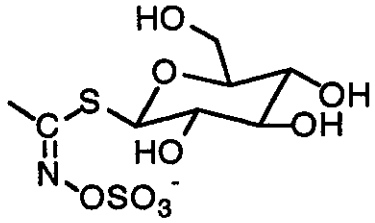
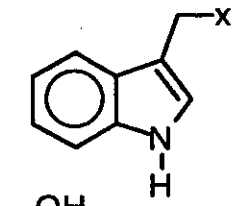
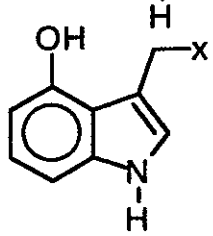
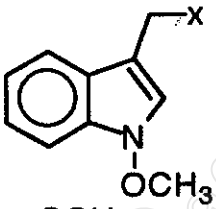
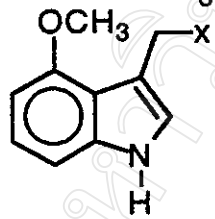
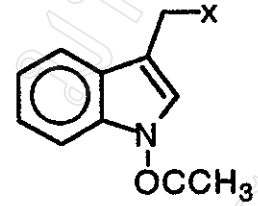
X = 

Table 1.3 Indole glucosinolates (derived from reference 3)

Side chain group	Scientific name	Common name
	Indol-3-ylmethylglucosinolate	Glucobrassicin
	4-Hydroxyindol-3-ylmethylglucosinolate	4-Hydroxyglucobrassicin
	1-Methoxyindol-3-methylglucosinolate	Neoglucobrassicin
	4-Methoxyindol-3-ylmethylglucosinolate	4-Methoxyglucobrassicin
	1-Acetyl-indol-3-ylmethylglucosinolate	1-Acetylglucobrassicin

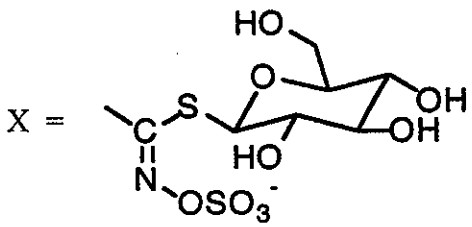
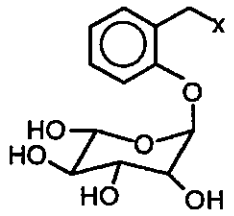
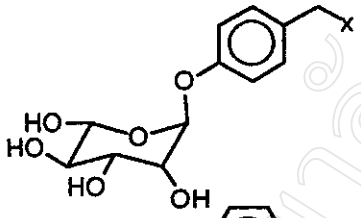
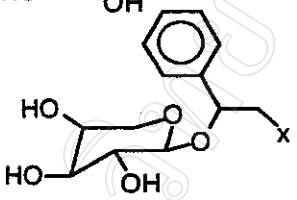
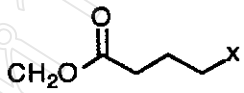
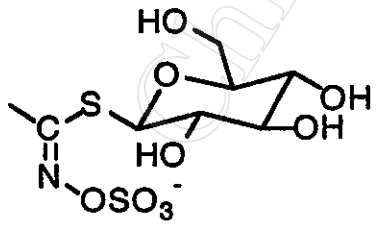
X = 

Table 1.4 Glycated glucosinolates (derived from reference 3)

Side chain group	Scientific name
	2-(α-L-Rhamnosopyranosyloxy)benzylglucosinolate
	4-(α-L-Rhamnosopyranosyloxy)benzylglucosinolate
	2-(α-L-Arabinopyranosyloxy)-2-phenylethylglucosinolate
	3-Methoxycarbonylpropylglucosinolate

X = 

After breeding over between those species, new hybrids have been generated [18]. Examples of these are *Brassica juncea*, *Brassica napus* and *Sinapis alba*. Therefore, each species comprises of different varieties (sub-species) which are illustrated in Table 1.5.

Glucosinolates are usually present in all parts of plants. Generally, levels in seeds are relatively high (upto 10 % of dried weight), whereas, the levels in leaf, stem and root are approximately ten times lower [3]. Distribution of glucosinolates among these plants seems to be species specific [3, 5]. For example, sinigrin (allylglucosinolate) is a main or probably an only glucosinolate existed in brown mustard seeds (*Brassica juncea*) [45]. Meanwhile, white mustard seeds (*Sinapis alba*) contain two major glucosinolates, sinalbin (p-hydroxybenzylglucosinolate) and sinigrin in a ratio of 7 : 3 [43]. However, at least 6 glucosinolate species are present in seeds of *Brassica oleracea* var. gemmifera [47]. Glucosinolate constituents in some *Brassica* species are shown in Table 1.6.

Brassica oilseeds have established as valuable in proteins [23, 25]. However, they contain significant amounts of anti-nutritional polyunsaturated fatty acids, especially erucic acid [23], and also the anti-nutritional glucosinolates [32]. In order to improve the acceptability of these oilseed meals as animal feeds, plant breeders have attempted to generate new breeds low content of erucic acid and also glucosinolates. 'Single-low variety' is defined as erucic acid composition in fixed-oil of such variety is less than 2 % [4, 48]. In the case of 'double-low variety', such oilseeds should contain low erucic acid as precedingly described, and the total glucosinolate content must not exceed 30 μmol/g meal [4].

Table 1.5 Some varieties of *Brassica* vegetables [18].

Species	Variety	Common name
Brassica oleracea	acephala	kale
	albogloba	Chinese kale
	botrytis	broccoli, cauliflower
	capitata	cabbage
	gemmifera	sprouts, Brussels sprouts
	gongylodes	kohrabi
	italica	Italian broccoli
	tronchuda	Portuguese kale
Brassica campestris	oleifera	turnip rape
	rapifera	turnip
	chinensis	Chinese mustard
	pekinensis	Chinese cabbage
	nipposinica	Japanese mustard
	dichotoma	Indian mustard
	trolocavalis	sarson
	ruvo	rovo kale
	septiceps	Italian kale
Brassica napus	-	rape, oilseed rape
	napobrassica	swede, Swedish turnip
	pabularia	early kale
Brassica juncea	-	brown mustard, Indian mustard, leaf mustard
Brassica nigra	-	black mustard
Sinapis alba	-	canola, white mustard

Table 1.6 Glucosinolate constituents in some *Brassica* crops

Sample	Species and variety	Glucosinolate constituent	Reference
seeds	*B. oleracea* var. gemmifera	sinigrin, progoitrin, gluconapin, glucotropaeolin, glucoerucin and neoglucobrassicin	[52]
seeds	*S. alba*	sinigrin and sinalbin	[43]
seeds	*B. napus*	gluconapin, glucobrassicanapin, progoitrin, gluconapoleiferin and gluconasturtiin	[53]
roots	*B. campestris* var. rapifera	glucococchkearin, gluconapin, progoitrin, glucoerucin, glucobrassicanapin, glucobrassicin, gluconapoleiferin, glucobeteroin and gluconasturtiin	[52]

1.2.4) Methods for determination of glucosinolates

In order to quantify the amounts of glucosinolates in native samples, it is necessary to destroy endogeneous myrosinase activity contained in such samples, prior to extraction of the glucosinolates. In case of full-fat samples such as oilseeds or oilseed meals, the samples should be defatted properly before extraction [49]. Extracting procedure usually performs with methanolic-water or boiling water [45, 49,

50]. Some impurities contained in crude extracts should be eliminated by means of anion-exchange chromatography or charcoal adsorption [50, 51].

a) Determination of total glucosinolates by thymol-sulfuric acid method [49]

The assayed principle is based on the determination of thioglucose released after glucosinolate hydrolysis in strong acid solution (80 % sulfuric acid). Colored complex forming between thioglucose and thymol will be measured an absorbance at 505 nm.

b) Determination of total glucosinolates by glucose released assay

In order to quantify the amounts of total glucosinolates, appropriate activity of myrosinase (usually from *Sinapis alba* seeds) are incorporated into the reaction mixtures, to liberate glucose from glucosinolates. Then, the amounts of glucose released could be measured by several conventional assays *e.g.* glucose oxidase method [51, 54], coupled-enzyme method [55] or dinitrosalicylic acid method [24].

c) Determination of individual glucosinolate by spectrophotometric assay [56]

This method is developed to determine pure glucosinolate concentration by monitoring an absorbance at λ_{max} of such glucosinolate. For quantification of pure sinigrin, it is usually performed spectrophotometrically at 227 nm ($\varepsilon_{227\ nm}$ of sinigrin = 7546 $M^{-1}cm^{-1}$) [56]. In case of progoitrin, an extinction coefficient of 6968 $M^{-1}cm^{-1}$ is used instead [28].

d) Determination of individual glucosinolate by gas chromatography [53, 57-59]

For GC analysis of glucosinolates, sulfate moiety should be eliminated out from the intact glucosinolates. This is usually employed with sulfatase hydrolysis (traditionally from *Helix pomatia*). Then, the desulfoglucosinolates are derivatized

with trimethylsilane, prior to being analyzed by gas chromatography. Limitation of the method has been mentioned for detection of indole glucosinolates.

e) Determination of individual glucosinolate by high performance liquid chromatography [60]

This method is suitable for the determination of all glucosinolate species. It is usually determined in forms of desulfoglucosinolates. Operation of the assayed system is time-consuming and requires experiences.

1.2.5) Compartmentalization of myrosinase and glucosinolates in plant tissues

In plant tissues, glucosinolates and their degradative enzyme were reported to be localized in different compartments. Glucosinolates were stored exclusively within vacuoles of non-specific cells of horseradish root tissues (*Armoracia rusticana*) [61, 62]. Subcellular localization of myrosinase of *Raphanus sativus* seed tissues revealed that the enzyme was appeared within specific myrosin cells [63]. Normally, the relative area occupied by myrosin cells is in the range of 4.5-6.5 % [19]. Employing an immunocytochemical technique, the myrosinase in radicles of *Brassica napus* seedlings was verified to be associated with a tonoplast-like membrane, surrounding protein bodies/vacuoles called "myrosin grains" of myrosin cells [7]. A "mustard oil bomb" model was proposed for the organization of the myrosinase-glucosinolate system [61]. Upon mechanical damage, pest or microorganism attack or tissue disintegration, the stored glucosinolates are exposed to their degradative enzyme, myrosinase. Then, glucosinolate hydrolysis occurs.

1.2.6) Glucosinolate Degradation

Glucosinolate degradation by myrosinase from various plant sources have been extensively studied. This degradative reaction has hypothesized to be processed with the two-step mechanism [17]. Firstly, the enzyme hydrolytically cleaves the thioglucoside linkage, yielding D-glucose and an unstable thiohydroxymate-O-sulfonate intermediate. Secondly, the intermediate undergoes non-enzymatic rearrangement to be any reaction products, depending on such factors as substrates, pH or availability of ferrous ion and epithiospecifier protein (ESP).

a) Nitrile and isothiocyanate formation

Rearrangement of the thiohydroxymate-O-sulfonate intermediate to nitrile or isothiocyanate is pH dependent. Nitrile is usually produced at acidic pH *via* protonation. In such reaction, an elemental sulfur is eliminated from the intermediate, giving the nitrile [4, 9-11, 13, 17]. While isothiocyanate formation is occured at neutral condition by mean of Lossen rearrangement [4, 9-11, 13]. The proposed mechanism for nitrile and isothiocyanate formation is shown in Figure 1.2. In case of progoitrin, rearrangement at neutral pH results in cyclization of the aliphatic side chain which then forms 2-oxazolidinethione, a potent anti-thyroid factor [17].

b) Thiocyanate formation

Evidences revealed that only three glucosinolates, sinigrin, benzylglucosinolate and 4-(methylthio)butylglucosinolate are capable of producing thiocyanate [4]. The mechanism of thiocyanate formation has been proposed to occur *via* Z-E isomerization, as shown in Figure 1.3 [12, 17]. E-isomer of such intermediates possess stable cations which then undergoes E-aglycone rearrangement to form thiocyanate. However, the absolute mechanism has not yet been elucidated.

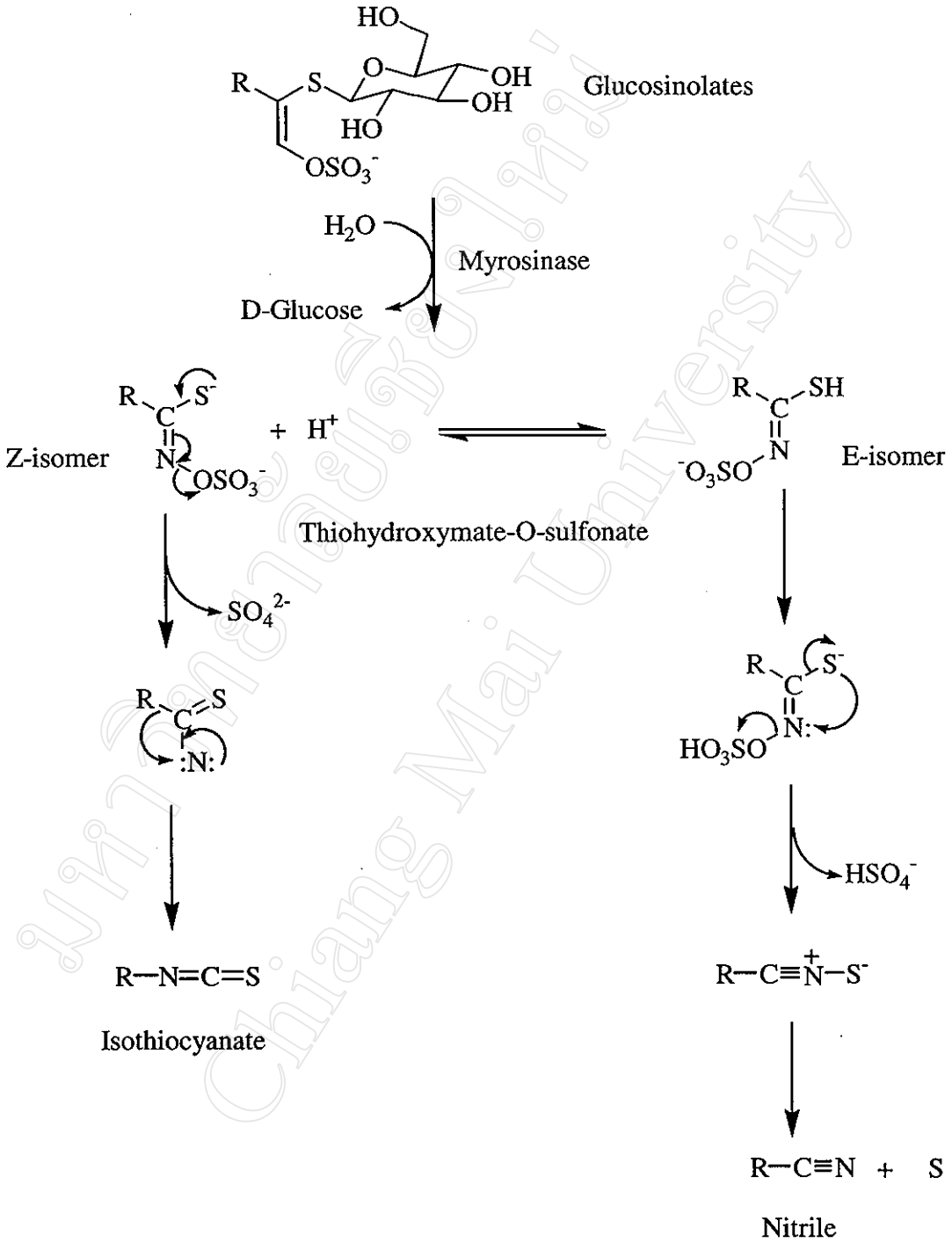


Figure 1.2 Mechanism of nitrile and isothiocyanate formation from glucosinolate degradation (derived from reference 4, 16)

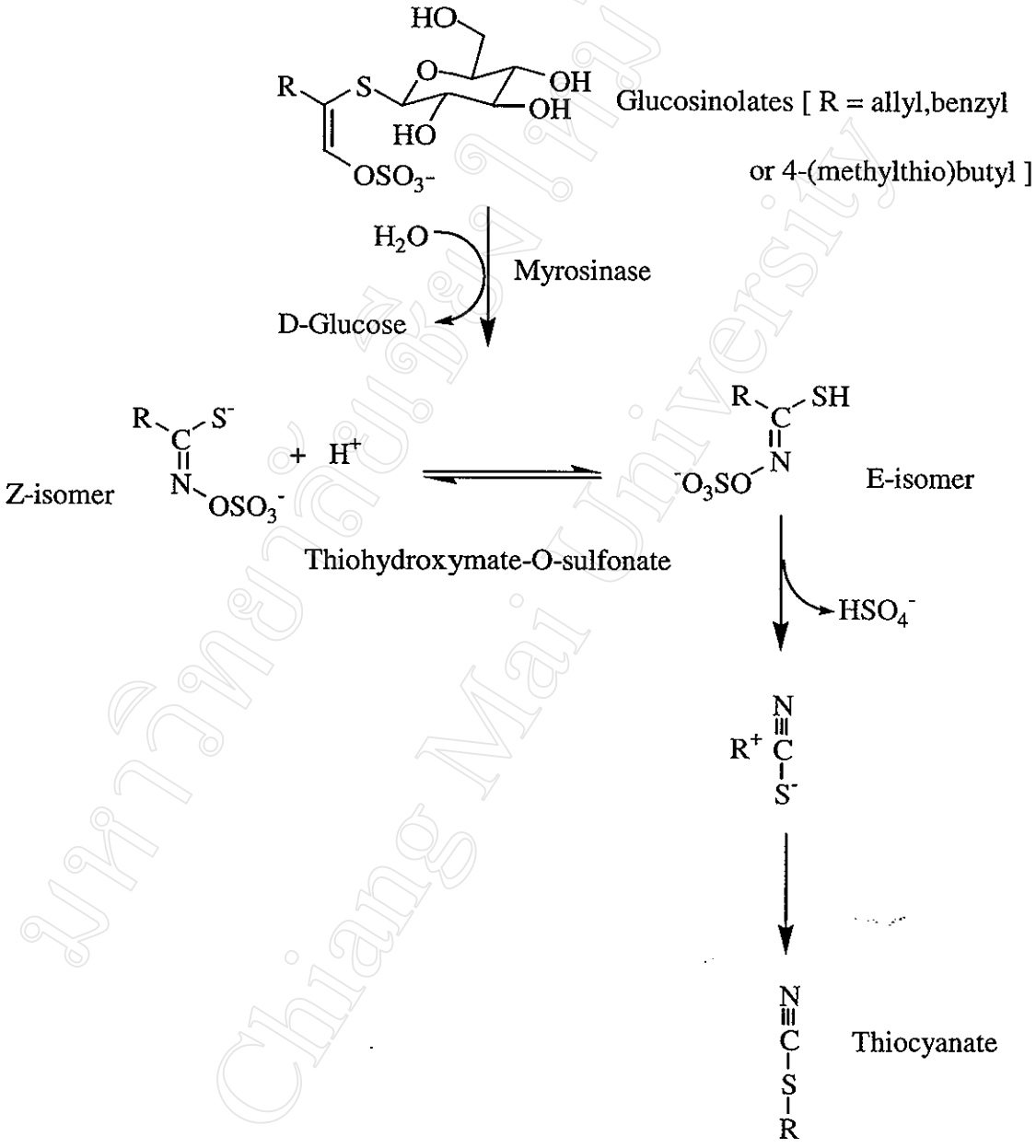


Figure 1.3 Mechanism of thiocyanate formation from glucosinolate degradation (derived from reference 4, 16).

c) Cyanoepithioalkane formation

Cyanoepithioalkane (epithionitrile) is exclusively produced from alkenylglucosinolates such as sinigrin, gluconapin, glucobrassicanapin and progoitrin with the presence of ferrous ion and epithiospecifier protein (ESP), as shown in Figure 1.4 [4, 15, 64]. ESP is a small protein (30-40 kDa) present in several species of *Cruciferaes* [65-66]. ESP functions as a sulfur migrating factor, having ferrous ion as an essential cofactor.

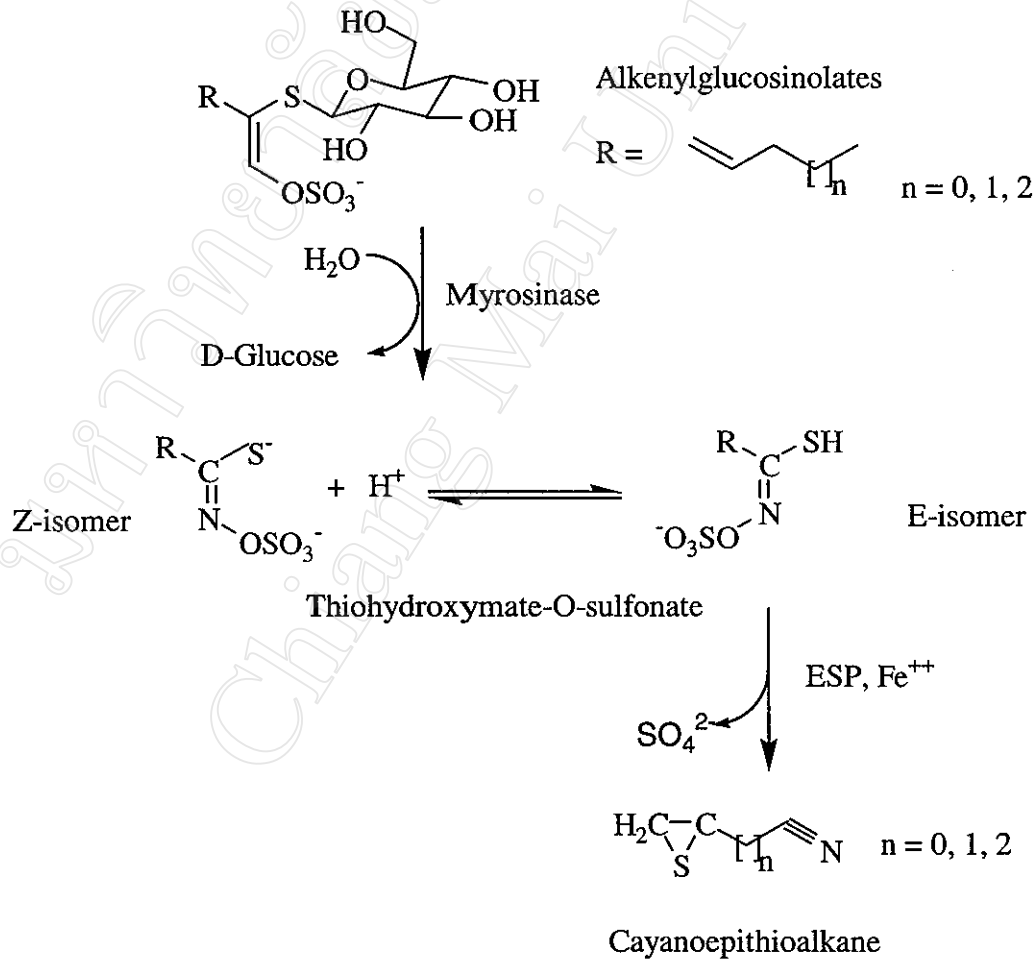


Figure 1.4 Mechanism of cyanoepithioalkane formation from glucosinolate degradation (derived from reference 4, 16).

1.2.7) Glucosinolate metabolism in *Cruciferous* plants

Biological functions of glucosinolates and their degradation products in these plants seem to be diversed. In respect of this, myrosinase plays role in regulation of glucosinolate metabolism. Mechanical wounding and infestation by flea beetle (*Psylloides chrysocephala*) of oilseed rape (*Brassica napus*) correlated with significant decrease of aliphatic glucosinolate levels [67]. Similar effects were demonstrated by other pest pathogens such as *Alternaria brassicae* [68] and *Deroceras reticulatum* [69]. The aliphatic glucosinolates were suggested to play biological role as a defense mechanism against pest attack or microorganism infection, since their hydrolytic products were volatile and strongly pungent [4]. Indole glucosinolates were proposed to involve in growth regulation. Indole-3-acetonitrile, a degradation product of indol-3-methylglucosinolate has been stated as a precursor of indoleacetic acid, one of plant hormones [70]. Glucosinolates also play role in sulfur and nitrogen mobilization throughout life cycle of plants [48]. However, variation of substrate specificity of myrosinase isoenzymes indicates multifunctions of the myrosinase-glucosinolate system which are not yet completely understood.

1.2.8) Significance of glucosinolates

Glucosinolate resist to be hydrolyzed by O-glycosyl hydrolases. Replacing the O-glycosidic linkage with S-linkage of native glycosides will lead to formation of corresponding thioglycosides, traditionally known as substrate analogues. Thioglycosides are powerful for the study of catalytic mechanism and crystal structure of O-glycosyl hydrolases. The enzyme still remains binding ability towards substrate analogue but lack of hydrolyzing activity. For examples, methyl-4,4′-dithio-α-malto-

trioside, a substrate analogue of α-methylmaltotrioside, was synthesized to elucidate crystal structure of pancreatic α-amylase [71]. In order to synthesize a non-hydrolyzable substrate analogue for myrosinase, the thioglycosidic linkage should be modified. In this case, the S-glycosidic linkage was replaced with C-linkage. For example, C-glucotropaeolin was made as an analogue of the glucosinolate glucotropaeolin, as shown in Figure 1.5 [72]. Chemical modification at C_2 of the glucose moiety, resulted in generating of new derivatives *e.g.* 2′-deoxyglucotropaeolin and 2′-deoxy-2′-fluoroglucotropaeolin (Figure 1.5).

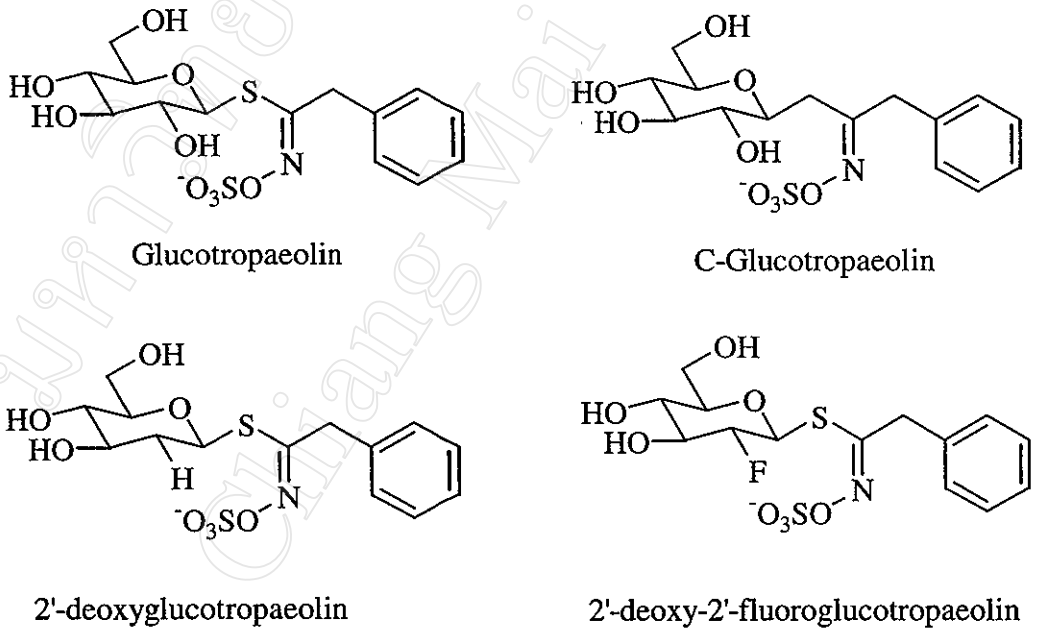


Figure 1.5 Chemical structures of glucotropaeolin and its derivatives

1.2.9) Significance of glucosinolate breakdown metabolites

a) Food flavoring

Metabolites generated from glucosinolate degradation exhibit distinct flavour. So that, in which some species are used as food flavouring. For example, allylisothiocyanate, a main component in mustard volatile from black or brown mustard seeds (*Brassica nigra* or *Brassica juncea*), has long been exerted as condiment in oriental menu and some food products [18, 73]. While a less hot taste p-hydroxybenzylisothiocyanate, an active ingredient in essential oil from white mustard seeds (*Sinapis alba*), is usually used in western menu [18].

b) Goitrogen

A number of reports have been established that glucosinolate breakdown compounds such as thiocyanate, isothiocyanate or nitrile were potentially anti-nutritional. These compounds compete the uptake of iodide ion into thyroid glands, causing hypothyroidism. Goitrin (vinyloxazolidine-2-thione), a hydrolytic product of progoitrin is named as the most potent anti-goitrogenic agent [74]. Progoitrin is usually present in different species of the genus *Brassica*, especially rapeseeds (*Brassica napus*) [68].

c) Anti-microbial activity

Isothiocyanates have known for their microbiostatic and microbiocidal properties against a wide range of microorganisms. Allylisothiocyanate (AIT) has been established to be one of the strongest anti-microbial substance [75]. However, other sinigrin hydrolytic products such as allylcyanide, allylthiocyanate and 1-cyanoepithiopropane do not [76]. In liquid medium, AIT has a minimum inhibitory concentration (MIC) of 50-500 ppm against bacteria and of 1-4 ppm against yeasts [73, 76]. Due to

its volatility at atmospheric condition, AIT is applied as gaseous phase in preservation of a variety of food products. Minimum inhibitory concentrations of vapourized AIT required for yeasts, bacteria and molds were in a range of 16-37, 16-62 and 34-110 ng/ml, respectively [77]. The mechanism of inhibition has been proposed *via* non-specific inactivation of metabolic enzymes through the covalent interaction with sulfhydryl-containing proteins [78]. However, only AIT from natural sources is permitted to be used as food prevervatives, while the synthetic AIT is not [73, 75].

d) Anti-neoplasm

Over the past 20 years, many researchers have found that fruits and vegetables contained numerous cancer chemoprotective agents. In particular of *Cruciferous* vegetables, glucosinolate metabolites generating by intestinal microflora are believed to possess anti-cancer properties [3, 26]. This was supported by the evidence that phenethylisothiocyanate, a hydrolytic product of gluconasturtiin, showed the potential to inhibit the induction of lung and esophageal cancer in both rat and mouse tumor models [79]. This inhibition effect was correlated with the reduction of carcinogen DNA-adducts and level of cytochrome P_{450} oxygenases. Sulforaphane, one of hydrolytic products of 4-(methylsufinyl)butylglucosinolate (glucoraphanin), was stated as the most potent anti-cancer compound. Mechanism of anti-carcinogenesis was established to elevate the levels of quinone reductase and glutathione transferase (phase 2 detoxifying enzymes) [80, 81]. Glucoiberin, cyanohydroxybutane and indole-3-carbinol were also reported for their property of cancer prevention but they are less active, comparing to that of the sulforaphane [26].

1.3) PLANT MYROSINASES

A term 'myrosyn', later known as myrosinase, was firstly defined by Bussy who discovered this enzyme in black mustard seeds (*Brassica nigra*) in 1890 [82]. The myrosyn protein involves in the hydrolysis of a mustard oil glycoside, 'acide myronique', later known as sinigrin, to produce mustard volatile oil [82]. Myrosinase (thioglucoside glucohydrolase EC 3.2.3.1 or β-thioglucosidase or glucosinolase or sinigrinase or mustard glycoside hydrolase) is defined as an enzyme responsible to hydrolyze β-thioglucosidic linkage of glucosinolates [3, 4]. Up to now, myrosinase is the only known glucosinolate-hydrolyzing enzyme [6]. The enzyme has been reported to be present in plants, microorganisms and also in some aphidses. After Bussy's exploitation, a number of works concerning isolation and characterization of myrosinase have been reported. Most works concentrated in *Cruciferous* plants and related families, mainly *Brassica* seeds and seedlings.

1.3.1) Occurrence of myrosinase isoenzymes

Myrosinases from various plant sources were stated to form as isoenzymes. These isoenzymes can be classified into two sub-groups, naturally-occurring and inducible enzymes [8]. The naturally-occurring type was established to occur as species specific. For examples, seven myrosinase isoenzymes were separated from *Sinapis alba* seeds by isoelectric focusing [83]. Whereas, only two isoenzymes appeared in *Brassica juncea* and *Brassica napus* seeds, as detected by the same technique. MacGibbon reported that the presence of myrosinase isoenzymes in *Rhodoeadales* were depended on whether samples were made from leaf, stem, root or seed [84]. In addition, expression of these isoenzymes was due to growth staging. The

77-kDa myrosinase prolypeptide was present in all parts of 10-days seedlings (*Brassica napus*). However, the 52-kDa polypeptide was present only in cotyledons and buds, but not in stem and root [8].

In case of the inducible myrosinases, sulfate ion was established as an inducing substance for enzyme expression [8]. The 68-kDa myrosinase polypeptide was formed exclusively in cotyledons, buds and stem of 10-days seedlings (*Brassica napus*) grown in the sulfate-supplemented medium. When plants grown up to 21 days, the 42- and 110-kDa polypeptides were consecutively expressed in buds and leaves, but not in other plants tissues. No expression of these myrosinase polypeptides was observed if the seedlings were cultured in sulfate-starved medium [8].

1.3.2) Isolation plant myrosinases

Isolation of myrosinase isoenzymes from rapeseeds (*Brassica napus*) was described by Lonnerdal and Janson [85]. Purification precedure included chromatography on Sephadex G-50, DEAE cellulose and Separose 6B column. Four isoenzymes designated A, B, C and D were obtained distinctively. In particular of the isoenzyme C, a native mass of 135 kDa was assessed both Sephadex G-200 gel-filtration and analytical ultracentrifugation. The subunit mass of 65 kDa was estimated by Sepharose 6B chromatography with the presence of 6 M guanidine hydrochloride. Native protein was analyzed to contain approximately 14 % carbohydrate. Re-separation of the isoenzyme C with isoelectric focusing revealed that three isoforms with pI values between 4.96 and 5.06 were distinctively separated.

In *Brassica napus* seedling, two isoenzymes were isolated by ammonium sulfate fractionation, followed by DEAE Sepharose, Mono Q and Superose chromato-

graphy, stepwiely [20]. The isoenzyme I was stated as a dimeric protein, comprising of a 75-kDa and a 62-kDa polypeptide subunit. Whereas, the isoenzyme II was a trimeric protein with an appearent molecular mass of 188 kDa. The native protein assemblied with 75-, 68- and 62-kDa subunits. The myrosinase I was heavily glycosylated, whereas, the myrosinase II was relatively less.

Myrosinase isoenzymes were isolated from white mustard seeds (*Sinapis alba*) by Sephadex G-50, DEAE cellulose and Sephadex G-200 chromatograghy, and isoelectric focusing, stepwisely [27]. Three myrosinase isoenzymes, designated A, B and C with pI values of 5.90, 5.45 and 5.08, respectively were distinctively obtained. The isoenzyme C formed as a main isoenzyme with a native molecular mass of 151 kDa, as estimated by Sephadex G-200 chromatography. The native protein comprised of two identical subunits whose subunits mass was 62 kDa each. Carbohydrate content of the purified enzyme was analyzed to be 18 % of the total molecular mass.

On the basis that plant myrosinases contained significant amounts of carbohydrate, affinity chromatography have been employed for enzyme purification. Highly purified myrosinase from *Sinapis alba* seeds was obtained by one-step separation on a Concanavalin A-Sepharose column [86]. The final enzyme preparation exhibited broad band on SDS-PAGE, corresponding to a molecular weight of 140 kDa. In such seeds, three isoenzymes were separated by Concanavalin A-Sepharose column followed by Sephacryl S-200 column [87]. Three isoenzymes, A, B and C were separated, which were corresponded to the results described by Bjorkman and Janson [88]. Re-separation of the isoenzyme C by chromatofocusing revealed that three isoforms with pI values between 5.05 and 5.15 were obtained. A native molecular mass of the isoenzyme C was assessed to be 135.1 kDa by analytical ultracentrifugation. However,

a molecular weight of 71.7 kDa was estimated by Sephacryl S-200 chromatograpy with the presence of 6 M guanidine hydrochloride. It was, therefore, suggested that the enzyme was a dimeric protein, comprising of two identical subunits.

In brown mustard seeds (*Brassica juncea*), four different isoenzymes were separatable by means of ammonium sulfate precipitation, and a combination of chromatographic separations on DEAE Sephadex A-50, CM Sephadex and Sephadex G-200 column, consecutively [54, 89]. Of which, three isoenzymes, designated F-IA, F-IIA and B exhibited the same pI value at 4.6 while the other isoenzyme, F-IIB was 4.8. Native molecular weights of the three isoenzymes were assessed to be the same (153 kDa), as estimated by Sephadex G-200 gel-filtration and analytical ultracentrifugation. However, a molecular weight of 125 kDa for the F-IIB was assessed by the same methods. From SDS-PAGE analysis, a subunit mass of 40 kDa was calculated for the three isoenzymes, whereas, the F-IIB was 30 kDa. Amino acid compositions of the three isoenzymes were very similar, while that of the F-IIB was rather different from the rest. This indicated that F-IA, F-IIA and B were the same protein, but having different degrees of glycosylation.

The myrosinase from *Wasabia japonica* was isolated by ammonium sulfate fractionation and chromatography on Sephacryl S-200, DEAE Sephadex, Sephacryl S-200, DEAE Sephadex and Sepharose 6B column, stepwisely [90]. A molecular mass of the native enzyme was estimated to be 580 kDa by Sephacryl S-200 chromatography. However, a molecular mass of approximately 45-47 kDa was calculated from SDS-PAGE, indicated that the enzyme comprised of 12 polypeptide subunits.

1.3.3) Physico-chemical properties of plant myrosinase

a) Temperature and pH optima

All isolated myrosinases from *Brassica* plants have been reported to be active at high temperatures. Of these, 75, 60, 60 and 55 °C were optimum temperatures for the enzymes from *Brassica napus* seedling [20], and seeds of *Brassica napus* [91], *Sinapis alba* [91] and *Brassica juncea* [89, 92], respectively. However, the activity of *Wasabia japonica* myrosinase was optimal at 37 °C [90].

Plant myrosinases have been stated to function at a wide pH range from 3 to 10 [91, 92]. However, their optimum pH seemed to be acidic. Myrosinases from *Brassica napus* seedling [20], seeds of *Brassica napus* [91], *Sinapis alba* [91] and *Brassica juncea* [89, 92], and roots of *Wasabia japonica* [90] were reported to be mostly active at pH 5.4, 4.5, 5.8, 5.5 and 6.5, respectively.

b) Enzyme stability

Concerning enzyme stability, most plant myrosinases were considered as thermostable enzymes. The *Brassica juncea* myrosinase was stable up to 50 °C for 10 min with no significant decrease of enzyme activity [92, 93]. Whereas, the myrosinase from *Wasabia japonica* was stabilizable at 30 °C for 20 min [90]. Myrosinases from *Brassica juncea*, *Brassica napus* and *Sinapis alba* seeds were quite stable at a wide pH range from 4 to 9 [91-93]. Imidazole buffer, pH 6.0 was stated as the most suitable medium to preserve myrosinase activity [91]. For long-term preservation, enzyme activity could be totally retained up to 6 months if maintained in the imidazole buffer containing 0.02 % sodium azide, at 4 °C [91].

c) Kinetic constants and substrate specificity

Plant myrosinase exhibited high affinity for glucosinolates. K_m values for sinigrin of three myrosinase isoenzymes from *Brassica napus* seedling were reported to be 68, 71 and 72 μM, respectively [19]. In cases of isoenzyme A, B and C from *Sinapis alba* seeds, K_m values of 50, 60 and 30 μM, repectively, were stated [91]. While those values of 30, 180 and 47 μM were reported for the enzymes from seeds of *Brassica napus* [91], and *Brassica juncea* [54, 92, 94], and roots of *Wasabia japonica* [90], respectively. Myrosinases also demonstrated hydrolyzing activity towards p-nitrophenyl-β-D-glucoside (pNPG). K_m value of the *Brassica juncea* myrosinase for pNPG hydrolysis was found to be 2 mM [94].

Plant myrosinases show strong affinity towards glucosinolate substrates, but having low affinity for O-glycosides. For examples, the *Brassica juncea* myrosinase showed slight hydrolyzing activity for O-glycosides as salicin, pNPG and arbutin, while no activity detected for amygdalin, α-methylglucoside, β-methylglucoside and β-phenylglucoside [94]. Degree of substrate specificity seems to be isoenzyme-specific. For examples of myrosinases from *Brassica napus* seedling, the isoenzyme I showed higher affinity for aliphatic glucosinolates than for indole glucosinolates. Alternatively, the isoenzyme II exhibited stonger affinity for indole glucosinolates than for aliphatic glucosinolates [20].

Study of enzyme specificity towards a series of deoxyglucotropaeolins (d-GTL) revealed that enzyme activities (from *Sinapis alba* seeds) were decreased from native-GTL, 6d-GTL, 4d-GTL to 3d-GTL [95]. Whereas, no enzyme activity could be detected with 2d-GTL. This indicated that hydroxy group at the C_2 position of glucose

ring of glucosinolates played a role in binding between myrosinase and glucosinolates [95].

d) L-ascorbic acid on activation of myrosinase activity

L-ascorbic acid was stated as an activator for most myrosinases from plant sources. No activity of the *Wasabia japonica* myrosinase was appeared if L-ascorbic acid was omitted from the reaction mixture [90]. Anyhow, mode of activation was isoenzyme-specific. With 1 mM L-ascorbic acid supplement in the reaction mixture, activities were increased by 20, 50 and 40 fold for the *Sinapis alba* isoenzyme A, B and C, repectively [91]. In such cases, appearent K_m values of those isoenzymes were increased from 50, 60 and 30 μM to 400, 400 and 300 μM, respectively [91].

The β-thioglucosidase activity of *Brassica juncea* myrosinase was enhanced approximately 25 times with the presence of 1 mM L-ascorbic acid, whereas, the activity of pNPG hydrolysis was not affected [92]. L-Ascorbic acid directly affected optimum temperature of the enzyme. The optimum temperature of 35 °C for sinigrin-hydrolyzing activity of the *B. juncea* myrosinase was observed with L-ascorbic acid, comparing to 55 °C without L-ascorbic acid. However, no alteration on optimal temperature of pNPG-hydrolyzing activity was appeared. Spectroscopy analysis revealed that the enzyme was conformationally changed, due to the interaction with L-ascorbic acid [96].

e) Enzyme inhibition

In the study of enzyme inhibition, myrosinase activity from *Brassica juncea* seeds was strongly inhibited by fluorodinitrobenzene (FDNB) and trinitrobenzene-sulfonic acid (TNBS), suggesting that sulhydryl and histidyl residues were required for enzyme catalysis [89]. Salicin and D-glucose were stated as competitive inhibitors for

sinigrin-hydrolyzing activity with K_i values of 0.18 and 1.5 M, respectively [94]. In case of *Sinapis alba* myrosinase, methyl-β-D-glucoside was a competitive inhibitor against sinigrin-hydrolyzing activity with K_i value of 0.2 mM [91, 97]. Whereas, glucono-δ-lactone was a non-competitive inhibitor for the activity of sinigrin hydrolysis with K_i value of 120 mM [97].

Since glucotropaeolin has a greater affinity (K_m 75 μM) for *Sinapis alba* myrosinase than the traditional substrate, sinigrin (K_m 156 μM), several species of glucotropaeolin derivatives were synthesized for the study of enzyme inhibition [46]. 2′-Deoxyglucotropaeolin (2d-GTL) was named as a potent competitive inhibitor with K_i value of 1.9×10^{-5} M [95]. Subsequently, a hydrolyzable substrate, 2′-fluoro-2′-deoxyglucotropaeolin was introduced for the study of myrosinase inactivation and also crystal structure of the enzyme [98]. The inhibitor possesed a K_i value of 0.9 mM. In this case, a stable fluoroglucosyl-enzyme intermediate was then formed with a K_{react} of 0.015 h^{-1} (half-life 46 h). Reactivation of the intermediate could occur rapidly in the presence of sodium azide with K_{react} of 0.035 h^{-1}, corresponding to a half-life of 20 h [98].

1.3.4) Myrosinase-binding proteins

Myrosinase isoenzymes from *Brassica napus* seeds existed in association with some proteins, named myrosinase binding proteins (MBP). Two myrosinase binding proteins with molecular masses of 50 and 52 kDa, were found exclusively in those seeds [99]. While another type of MBP, called myrosinase binding protein-related proteins (MBPRP) was found in all parts of plants [63]. Biological functions of these

proteins have been proposed to response during tissue damage [99]. However, the absolute function is still to be elucidated.

1.3.5) Methods for activity assay of enzyme myrosinase

Several methods for determination of myrosinase activity have been developed which are based on either end-point or continuous-monitoring principle. Sinigrin (allylglucosinolate) is traditionally used as a substrate.

a) Spectrophotometric assay

This kinetic method is based on detection of the amounts of sinigrin degraded by monitoring the absorbance change with time at 227 nm (ε_{227} nm for sinigrin = 7546 $M^{-1}cm^{-1}$) [56]. Myrosinase activity is defined as the amounts of enzymable to hydrolyze 1 μmol of sinigrin per minute at specified conditions. This spectrophotometric method is suitable for kinetic studies of purified enzymes, but not for activity assay in crude enzyme extract, since some substances may interfere the absorbance at 227 nm. Limitation of the method is mentioned that the assayed system cannot operate at high substrate concentrations, due to absorbance limit. For the determination of β-glucosidase activity, the assay is employed using p-nitrophenyl-β-D-glucoside (pNPG) as a substrate [97].

b) Sulfate-released assay

This method was firstly designed for localization of myrosinase isoenzymes in gels after electrophoresis or isoelectric focusing [83, 84]. Sulfate released from glucosinolate hydrolysis will form an insoluble salt with barium ion which then being visualized as opaque bands. However, this assay principle was occasionally introduced for measuring enzyme activity [54].

c) Glucose-released assay

By this principle, myrosinase activity can be assessed by measuring the amounts of glucose liberated from sinigrin hydrolysis by means of several conventional methods such as dinitrosalicylic acid [24], glucose oxidase [51, 88] and coupled-enzyme assay (HK/G-6-PD) [55]. Despite the coupled-enzyme assay, it is an acceptable method for kinetic measurement of enzyme activity [55]. Myrosinase activity is expressed as the amounts of enzyme that catalyzes sinigrin to produce 1 μmol of glucose in 1 minute at specified conditions.

1.4) GLUCOSINOLATE-DEGRADING MICROORGANISMS AND MICROBIAL MYROSINASES

Over the past 40 years, a number of microorganisms including bacteria and fungi have been reported for their glucosinolate-degrading properties. Most works concentrated in the characterization of glucosinolate degradation by intact microbial cells. However, there were considerably less works focused on purification and characterization of microbial myrosinases, comparing to plant enzymes.

1.4.1) Screening of glucosinolate-degrading microorganisms

Glucosinolate-degrading microorganisms were screened from different sources. *P. aerogenoides* [33], *E. coli* [33] and *L. acidophilus* LEM220 [28] were isolated from fecal samples, whereas, *E. cloacae* no. 406 [34] was from sinigrin solution. Other species such as *B. subtilis* [33], *B. cereus* [33], *P. aeroginosa* [33], *P. vulgaris* [33], *A. aerogens* [33], *A. faecilis* [33], *S. epidermidis* [33], *L. agilis* R16 [45] and *A. niger* AKU3302 [100] were from culture collection. In case of the fungi *A.*

clavatus II-9 [43] and *F. oxysporum* @146 [43], they were isolated from *Brassica* crops. However, mushroom and petrochemically contaminated soil samples have also been reported as sources of such glucosinolate-degrading bacteria as *Streptomyces* sp., *Bacillus* sp. and *Staphylococcus* sp. [24].

Plate culture method was introduced for screening of glucosinolate-degrading microorganisms [24]. Sinigrin was an only sole of carbon source contained in the agar medium. Strains capable of growth in this selective medium were mentioned as the strains with glucosinolate-degrading property.

1.4.2) Assessment of glucosinolate degradative potential

To assess glucosinolate-degrading activity of interesting strains, liquid culture was usually employed. The strains should be pre-cultured in their suitable growth medium, prior to being grown further in the medium supplemented with glucosinolates [28, 33, 34, 45]. Pure sinigrin or progoitrin were traditionally used as model glucosinolate substrates. Glucosinolate degradation potential was assessed by measuring the amounts of sinigrin or progoitrin degraded by time. Several bacterial strains such as *P. aerogenoides* [33], *E. coli* [33], *L. agilis* R16 [45] and *L. acidophilus* LEM220 [28] were examined for their degradative potential by this method. For the assesment of degrading glucosinolate of two fungal strains, *A. clavatus* II-9 and *F. oxysporum* @146 was also achieved by the same concept [43].

1.4.3) Myrosinase in glucosinolate degradation *via* intact microbial cells

There are few reports describing glucosinolate degradation by intact microbial cells. The ability to degrade sinigrin of a variety of bacterial species such as

Streptomyces sp., *Bacillus* sp., *Staphylococcus* sp., *P. aerogenoides*, *E. coli*, *B. subtilis*, *B. cereus*, *P. aeroginosa*, *P. vulgaris*, *A. aerogens*, *A. faecilis* and *S. epidermidis* was shown to be unstable [24, 33, 101]. If cultivation was carried out further in sinigrin-free medium, the degradative potential for sinigrin was then irreversibly lost. Progoitrin-hydrolyzing activity was demonstrated in cell-free extract of *P. aerogenoides*, but not in that of *E. coli* [33]

Sinigrin degradation in liquid culture of *Lactobacillus agilis* R16 [45], *Aspergillus clavatus* II-9 [43] and *Fusarium oxysporum* @146 [43] resulted in production of the hydrolytic product allylisothiocyanate, allylcyanide and none, respectively. Myrosinase activity was exclusively detected in cell-free extract of the *Aspergillus*. Several investigators suggested that sinigrin degradation by the *Aspergillus* was an intracellular process, involving with myrosinase [43]. Whereas, none of enzyme activity was detected in cell-free extracts or culture filtrates of the *Lactobacillus* and *Fusarium*. However, the formation of allylisothiocyanate by the *Lactobacillus* indicated the action of myrosinase. It was suggested that the enzyme was extremely sensitive [45]. Myrosinase activities also involved in glucosinolate degradation by *E. cloacae* no. 406 [34], *A. niger* AKU3302 [100], *A. sydowi* IFO4284 [102, 103] and *A. sydowi* QM31c [14]. It was demonstrated that *A. sydowi* IFO4284 [102, 103] and *A. sydowi* QM31c [14] were only known extracellularly myrosinase-producing strains. For other glucosinolate-degrading strains, there was no information stating whether myrosinase was produced or not [24, 33].

1.4.4) Cultivation for myrosinase production

From most reports, cultivation to produce myrosinase was traditionally employed with two-step culture. Pre-culture was usually performed in normal growth medium. Then, microbial cells were harvested and grown further in induction medium. Due to the expensive price of sinigrin, materials containing glucosinolates such as mustard flour (*Brassica juncea* or *Sinapis alba*) were introduced to supplement as a part of induction medium [14, 34, 102, 104]. Therefore, derived medium from *Brassica* oilseed meal were stated as enriched nutrients which were used for cultivation of microorganisms, including bacteria, yeast and fungi [105, 106].

Myrosinase was demonstrated to be produced by *Paracolobactrum aerogenoides* [33]. The bacterium was pre-cultured in growth medium (nutrient broth containing 1 % glucose) at 37 °C for 18 h, prior to further growth in the induction medium (nutrient broth containing 0.025 % progoitrin) for an additional 3 days. Myrosinase activity was detected in cell-free extract obtained after sonication, not in cultured filtrates. The crude enzyme was mostly active in 0.12 M potassium phosphate buffer, pH 7.4 while it was less active in 0.12 M potassium phosphate buffer, pH 5.6, 0.03 M tris bufer, pH 7.4 and 0.03 M sodium acetate buffer, pH 5.6. It was found that the enzyme activity was not stimulated by L-ascorbic acid at a final concentration of 5 %.

Cultivation of *Enterobacter cloacae* no. 406 for myrosinase production was revealed by Tani *et al.* [34]. Pre-culture was carried out in the synthetic medium comprised 1 % meat extract, 1% yeast extract and 0.1 % potassium dihydrogen-phosphate, pH 7.0 at 28 °C for 16 h. Enzyme production was acheived in the induction medium comprised 0.2 % potassium dihydrogenphosphate, 0.1 % ammonium chloride,

0.1 % magnesium sulfate heptahydrates, 0.1 % sodium chloride and 0.3 % sinigrin at 28 °C for 48 h under reciprocal shaking. Myrosinase activity was present in cell-free extract obtained after sonication. Substitution of sinigrin with other inducers such as amygdalin, salicin, arbutin, α-methylglucoside, cellobiose, maltose, glucose, galactose, xylose, sucrose, mannose, mannitol and sorbitol, resulted in no myrosinase production. While, replacing 0.3 % sinigrin with 0.01 % sinigrin plus 6 % mustard extract (*Brassica juncea*), resulted in production of the same amounts of enzyme activity. However, mustard extract alone had poor-inducing ability on myrosinase production.

A cultivation for myrosinase production by *Aspergillus sydowi* IFO 4284 was reported by Ohtsuru *et al.* [102, 104]. The strain was pre-cultured in growth medium (5 % malt extract, 2 % sucrose, 0.2 % potassium dihydrogenphosphate, 0.1 % ammonium sulfate, 0.03 % sodium nitrate and 0.03 % manganese sulfate heptahydrate, pH 6.5) at 29 °C for 3 days. It was then grown further in the induction medium (0.1 % yeast extract, 0.2 % potassium dihydrogenphosphate, 0.1 % ammonium sulfate, 0.09 % sodium nitrate, 0.09 % manganese sulfate heptahydrate, 0.09 % glucose and 20 % mustard extract (*Brassica juncea*), pH 6.5) at 29 °C for 2 weeks under reciprocal shaking. During cultivation, pH of cultured broth was adjusted to be neutral by adding 1 N NaOH. In this case, myrosinase activity of 400 U was detected in 5-l cultured medium.

The capability of producing myrosinase by *Aspergillus sydowi* QM31c was demonstrated by Petroski *et al.* [14]. Pre-culture was employed by growth in synthetic medium (1 % starch, 0.25 % mustard flour (*Sinapis alba*), 0.01 % yeast extract, 0.1 % urea, 0.2 % potassium dihydrogenphosphate, 0.14 % ammonium sulfate, 0.03 % magnesium sulfate heptahydrate, 0.03 % calcium chloride, 0.05 mg % ferrous sulfate

heptahydrate, 0.15 mg % manganese sulfate monohydrate, 0.25 mg % zinc sulfate heptahydrate and 0.02 mg % cobalt chloride hexahydrate, pH 6.3) at 25 °C for 3 days. Enzyme production was also carried out in the same medium but sinigrin was aseptically added into the 24-h stage II culture, to a final concentration of 2 mg %. Myrosinase activity of 32 U was obtained from 330-ml cultured fitrates of the 72-h stage II culture.

Production of an intracellular myrosinase by *Aspergillus niger* AKU3302 was reported by Ohtsuru *et al.* [107]. Pre-culture of the fungus was performed in synthetic medium (5 % malt extract, 2 % sucrose, 0.2 % potassium dihydrogenphosphate, 0.1 % ammonium sulfate, 0.03 % sodium nitrate and 0.03 % magnesium sulfate heptahydrate, pH 6.5) at 29 °C for 48 h. Enzyme production stage was carried out in the induction medium (0.1 % yeast extract, 0.2 % potassium dihydrogenphosphate, 0.1 % ammonium sulfate, 0.03 % sodium nitrate, 0.03 % magnesium sulfate heptahydrate, 0.1 % glucose and 20 % mustard extract (*Brassica juncea*), pH 6.5 at 29 °C for 48 h. Approximate 4.5 U of myrosinase activity was detected in cell-free extract obtained after fungal cell disruption of 100-ml culture.When the mustard extract was substituted with various carbon sources *e.g.* malt extract, glucose, sucrose, L-ascorbic acid, cellobiose, amygdalin and tyramine, no myrosinase-inducing ability was observed. However, supplement with 0.1 % L-ascorbic acid in the induction medium, resulted in enhance myrosinase yield (8.8 U/100-ml cultured medium). Crude enzyme extract was very unstable, and was gradually inactivated during dialysis and salting out with ammonium sulfate. Fifty-two percents of enzyme activity were lost when the enzyme was maintained at 20 °C for 2 days. A combination of stabilizing agents,

mercaptoethanol (1 mM) and L-ascorbic acid (1 mM) was found to preserve the activity if stored at the preceding conditions

The capability of producing myrosinase by *Aspergillus clavatus* II-9 was described by Smiths *et al.* [43]. The fungus was pre-cultured in sinigrin-glucose medium (0.2 % sinigrin, 0.2 % glucose and 0.67 % yeast nitrogen base in 0.1 M potassium phosphate buffer, pH 5.6) at 25 °C. Fungal cells collected at late log phase were grown further in either sinigrin-glucose medium or sinigrin medium. Growth in the sinigrin-glucose medium revealed that the cells completely exhausted sinigrin, and then glucose. Sinigrin degradation in the sinigrin medium was complete within 12 h. In either cases, allylcyanide was the only hydrolytic product formed in cultured filtrates but its accumulation was found to be delayed. Myrosinase activity was present in cell-free extract. Although *Fusarium oxysporum* @146 demonstrated potentially for sinigrin degradation in either sinigrin and sinigrin-glucose medium. However, enzyme activity was not detected in cell-free extracts or cultured filtrates.

1.4.5) Isolation of microbial myrosinases

Isolation of myrosinase from *Aspergillus sydowi* IFO4284 was reported by Ohtsuru *et al.* [103]. This separation procedure included ammonium sulfate fractionation, followed by DEAE cellulose and DEAE Sephadex chromatography, stepwisely. Highly purified enzyme with a specific activity of 10.07 U/mg protein was obtained, comparing to the specific activity of 0.065 U/mg protein of crude enzyme.

An improved isolation of myrosinase from *A. sydowi* IFO4284 was described by Ohtsuru *et al.* [102]. Purification protocol was employed by ammonium sulfate precipitation and chromatographic separations on DEAE cellulose, DEAE Sephadex,

Sephadex G-200 and Sephadex G-100 combined G-150 column, stepwisely. The final enzyme purification exhibited one band on cellulose acetate electrophoresis. The purified myrosinase gave a specific activity of 19.4 U/mg protein when compared with 0.12 U/mg protein of crude enzyme.

An intracellular myrosinase from *Aspergillus niger* AKU3302 was isolated by Ohtsuru *et al.* [100]. The purification protocol included DEAE Sephadex chromatography and isoelectric focusing. Highly purified enzyme with a specific activity of 1.916 U/mg protein was obtained, comparing to 0.139 U/mg protein of crude enzyme extract.

1.4.6) Physico-chemical properties of isolated microbial myrosinases

There was no report on whether microbial myrosinases were isoenzymes or not. Anyway, all reported myrosinases from microbial sources possessed different physico-chemical properties from those plant enzymes. In term of stability, microbial myrosinases were stated as unstable enzyme. Comparing between bacterial and fungal enzymes, fungal enzymes were relatively more stable. Microbial enzymes exhibited optimum pH at neutral range from 6.8-7.6 [34, 102, 107], whereas, plant enzyme were acidic ranging from 5.5 to 6.5 [4, 20, 54, 87, 89, 91]. Temperature optima of microbial enzymes were low as about 28-34 °C [34, 107], except the *S. sydowi* enzyme was 37 °C [102]. While most plant myrosinases were optimally active between 55-70 °C [4, 20, 54, 87, 89, 91].

Plant enzymes showed high affinity for glucosinolates, but lower affinity for pNPG [4, 102, 107]. This was rather different from microbial enzymes. K_m values of

the *A. sydowi* and *A. niger* myrosinases for sinigrin were 3.6 and 3.3 mM, respectively. While, those values for pNPG were 0.1 and 1.5 mM, respectively [102, 107].

The *A. sydowi* myrosinase exhibited wide specificity towards sinigrin, pNPG, salicin, arbutin, cellobiose, β-methylglucoside and β-phenylglucoside [102]. This agreed with hydrolyzing activity of the *A. niger* myrosinase towards sinigrin, pNPG, amygdalin, arbutin and salicin [107]. A wide range of substrate specificity of microbial enzymes may lead to broader applications.

There is no information describing glucosinolate degradation by purified microbial myrosinases. The partially purified enzyme from *Aspergillus sydowi* QM31c was able to produce 1-cyanoepithiopropane from sinigrin hydrolysis with the presence of epithiospecifier protein (ESP) and ferrous ion [15]. However, allylisothiocyanate was formed instead if the ESP and ferrous ion were omitted. Myrosinase activity in cell-free extract of the *A. clavatus* II-9 was capable to produce allylisothiocyanate from the substrate sinigrin at pH 6.2, whereas, no liberation of allylcyanide was detected [43]. This was contrast to the result of sinigrin consumption by the intact fungal cells that allylcyanide was the only product formed.

1.5) RATIONALES AND PURPOSE OF THE STUDY

Myrosinases are a group of glucosinolate-hydrolyzing enzymes, existing in all glucosinolate containing plants of the order *Capparales*, and some bacterial and fungal species. Glucosinolate degradation leads to the production of D-glucose, sulfate and glucosinolate breakdown metabolites *e.g.* nitrile, thiocyanate, isothiocyanate or cyano-epithioalkane. These breakdown metabolites were stated as anti-nutritional substances. However, some isothiocyanate compounds have been introduced for use as food

flavouring, food preservatives and being possibly applied as therapeutic approach. Physico-chemical properties of myrosinases from various plant sources have been extensively studied. Mechanisms of glucosinolate degradation by isolated plant enzymes are mostly clarified.

Glucosinolate-degrading microorganisms are defined as the strains being capable of consuming glucosinolates, and most of them produce myrosinase. There are several possible applications of glucosinolate-degrading strains *e.g.* biological detoxification of materials containing glucosinolates or production of some therapeutic compounds. Enzyme from microbial sources may also use in the industrial production of mustard volatile oil. From most reports, glucosinolate degradations by intact microbial cells were varied from species to species, and being different from those plant enzymes. Upto now, researches concerning on microbial myrosinases were rare. In term of producibility and stability, fungal enzymes are advantageous over bacterial enzymes.

In this study, glucosinolate-degrading microorganisms will be screened from local area of northern Thailand, where mustard seed meal (*Brassica juncea*) has long been deposited. All isolated strains will be examined for glucosinolate degradative potential and myrosinase-producing ability. The selected fungal strain will be used for myrosinase production. Cultured conditions of producing myrosinase will be studied by using brown mustard seed meal (*Brassica juncea*) as a main component of cultured medium. Myrosinase will then be purified and studied enzyme characteristics.