ASPECTS OF *BRASSICA JUNCEA* MEAL TOXICITY: ALLYL ISOTHIOCYANATE RELEASE AND BIOASSAY

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ABSTRACT

Oilseed and oilseed meal extracted from members of *Brassicaceae* release broad-spectrum biocidal isothiocyanate when ground and exposed to moisture. The compounds are released when the seed enzyme myrosinase catalyzes the hydrolysis of glucosinolates producing glucose, sulfate, and pesticidal isothiocyanates. Allylisothiocyanate (AITC), the predominant isothiocyanate of *Brassica juncea,* has broad-spectrum biological activities against plants, animals and fungi. Knowledge of the concentration of AITC arising from a treatment with mustard and AITC toxicity to many target and non-target species is not known. Therefore, factors affecting AITC release and assays of mustard toxicity were conducted. The rate of AITC release from mustard meal was affected by temperature and pH. Current isothiocyanate extraction and quantification methods measure a change in the concentration of glucose (a predominant product of myrosinase-catalysed glucosinolate hydrolysis) to determine myrosinase activity. The objectives of this work were to study: 1) factors affecting myrosinase activity in mustard (*Brassica juncea*), 2) the effects of AITC on seed germination and 3) the toxicity of AITC and mustard meal.

Attempts were made to improve the Herb and Spice Method, the only available industrial method to measure total isothiocyanate production in mustard meal. The effects of a wide range of reaction temperatures (7 to 97°C) and incubation times (0 min to 2 h) on myrosinase-catalyzed conversion of sinigrin (a glucosinolate) to allyl isothiocynate (AITC) were studied. Significant inhibition of enzyme activity was observed at all temperatures over 57°C, and at 97°C no myrosinase activity was found. It was concluded that myrosinase-catalyzed conversion of sinigrin to AITC was a rapid process and detectable amounts of AITC could be found in samples in two min, and that higher temperatures inhibited myrosinase activity. The pH of the reaction mixture significantly affected myrosinase-catalyzed conversion of sinigrin to AITC. A change in pH did not affect the substrate, but severely affected the activity of myrosinase. Furthermore, other compounds viz., boric acid (H_3BO_3), succinic acid ($C_2H_4(COOH)_2$), calcium chloride ($CaCl_2$) and ethanol (C_2H_5OH), were explored for their ability to inhibit myrosinase activity. Calcium chloride and ethanol were particularly effective.

It was hypothesized that AITC might act as a plant growth promoter/regulator based on the fact that AITC and ethylene, a plant growth regulator, exhibit structural similarity ($R\text{-}CH{=}CH_2$, where R is $\text{-}CH_2SCN$ and -H in AITC and ethylene, respectively). Therefore, AITC might act as an ethylene analogue. Ethylene is known to promote seed germination and overcome seed dormancy in a dose- and species-dependent manner. Flax and tomato seeds were used as model systems to test the germination enhancing properties of AITC. It was concluded that AITC promoted flax and tomato seed germination and thus might be used for this application in agricultural practice.

An assay was developed for testing AITC toxicity in ground seed by exploring HSP70 expression in *Caenorhabditis elegans* as a marker of toxicity. *C. elegans* strain N2 was exposed to different concentrations (0 to 10 µM) of AITC for 2 h at room temperature. Western blotting with anti-HSP70 antibody showed a marked increase in the expression of *HSP70* protein in a dose-dependent manner. Assays of the expression of *HSP70A* mRNA by quantitative real time reverse transcriptase (RT) PCR revealed no significant change in the expression of *HSP70A* mRNA at low concentrations of AITC (< 0.1 µM). However, treatment with higher concentrations (>1 µM) resulted in four- to five -fold increase in expression of *HSP70A* mRNA over the control. To understand if mustard toxicity was due to AITC alone, or if other compounds in mustard ground seed affected *HSP70* transcript production, *C. elegans* was exposed to AITC or *Brassica juncea* cv. Arrid ground seed (Arrid is a mustard variety with a lower level of sinigrin (<3 µM per gram of seed), or both. ELISA revealed increased expression of HSP70 protein in *C. elegans* treated with AITC + ground seed, but the level of protein was less than that observed with AITC alone. These results indicated that mustard ground seed toxicity was contributed primarily by AITC, and that some ground seed components antagonized AITC toxicity in *C. elegans*.

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Akal Rachna K. Saini

Dedication

To my parents, for teaching me to do the best I can, my Husband and my cute daughter Amishi

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LIST OF ABBREVATIONS

ACN	Acetonitrile
AITC	Allyl isothiocyanate
ANDSA	7-aminonaphthalene-1, 3-disulfonic acid
AP-1	Activator protein 1
APS	Ammonium persulphate
BITC	Benzyl isothiocyanate
CE	Capillary Electrophoresis
CTAB	Cetyltrimethylammonium bromide
ERK	Extracellular signal-regulated kinase
ERN	Erucin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GLS	Glucosinolate
HPLC	High Pressure Liquid Chromatography
HPLC-MS	HPLC coupled with mass spectroscopy
HRP	Horseradish peroxidase
HSP	Heat shock protein
IBN	Iberin
ITC	Isothiocyanate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MECC	Micellar Electrokinetic Capillary Chromatography
NAC	N-acetylcysteine
NCRR	National Centre for Research Resources
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
PAGE	Polyacylamide gel electrophoresis
PBS	Phosphate buffered saline

PEITC	Phenethyl isothiocyanate
PVDF	Polyvinylidene difluoride
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SFN	Sulforaphane
TAE	Tris-acetate/EDTA
TBA	Tetrabutylammonium
TEMED	Tetramethylethylenediamine
TMAB	Tetramethylammonium bromide
TNF-α	Tumour necrosis factor-α

Chapter 1: Introduction

Canada is a major producer of condiment mustard seed. Mustard is the only oilseed crop adapted to the drier regions of the Canadian prairies and provides a suitable crop for rotation with wheat. Furthermore, mustard is resistant to many pests and diseases that affect canola and thus can be grown with fewer inputs. Covercropping with mustard has been tested recently for weed management (Haramoto and Gallandt, 2004). Seed producing crops from the plant family *Brassicaeae* are used for food and non-food applications. The oil produced from these crops has increasing demand in non-food applications such as industrial lubricants (Heath and Earle, 1995), biodiesel fuel (Kimber and McGregor, 1995) and biopesticides (Bones, and Rossiter, 1996; Brown and Morra, 2005). The protein-rich oilseed meal from canola may be used in animal feed (Sakorn, *et al.*, 1999) and human food (Hiron *et al.*, 2006).

In response to biotic challenges, mustard has evolved a broad spectrum of natural defence mechanisms, such as physical and chemical barriers. The glucosinolate (GLS) - myrosinase system referred to as 'The Mustard Bomb' is a primary chemical defence used by mustard species against a wide range of biotic challenges (Bones and Rossiter, 1996). Specific GLS (glucoraphanin, glucoerucin, gluconasturtiin, sinigrin, glucotropaeolin, glucoraphenin, glucoraphasatin, glucomoringin and glucobrassicin) are hydrolysed by the myrosinase enzyme (thioglucosidase) to produce an aglycone, which undergoes spontaneous non-enzymatic rearrangement to produce organic isothiocyanates (ITCs), thiocyanates, nitriles, epithionitriles, oxazolidinethiones, and organic cyanates (Fahey *et al.*, 2001; Mithen, 2001; Fenwick *et al.*, 1983; Chew, 1988). Many GLS products are of interest because of their broad spectrum of biological activities.

Mustard seed flour contains 28% to 36% protein. The presence of tocopherols in mustard contributes to its shelf life (Cahoon *et al.*, 2003). Mustard is widely known for its sharp taste and is an essential component of many dressings and sauces. The volatile oil in mustard inhibits the growth of certain yeasts, moulds and bacteria, enabling mustard to function as a natural preservative. GLS hydrolysis products have biocidal activity against a wide variety of organisms such as insects, plants, fungi and bacteria (Vaughn and Berhow, 2005; Ware, 2000). Certain ITCs interfere with iodine availability, and are responsible for morphological and physiological changes in the thyroid gland (Tripathi and Mishra, 2006). The fungicidal property of allyl isothiocynate (AITC) vapour against wild type and thiabendazole-resistant strains of *Penicillium expansum* has been demonstrated (Tunc *et al.*, 2007; Kiyoshi 2005; Mari *et al.*, 2002). The use of AITC produced from pure sinigrin or from *Brassica juncea* defatted meal may be an economically viable alternative to synthetic fungicides against *P. expansum*.

The relative toxicity of different GLS hydrolysis products is dependant both on the target organism and the chemical structure. Thus, it is important to determine toxicity of GLS hydrolysis products inorder to maximize the likelihood of effective pest suppression in pesticide applications (Sarwar *et al.*, 1998). These products possess potential biodegradable and biofriendly insect fumigant properties (Tsao *et al.*, 2002a) and nematocidal properties (Mitarai *et al.*, 1997). They may act on the insect respiratory system.

Specific ITCs are well-known cancer-inhibitory phytochemicals (Hu *et al.*, 2007; Thejass and Kuttan, 2007; Hwang and Lee 2006; Tang *et al.*, 2006; Jakubikova *et al.*, 2005; Smith *et al.*, 2004; Zhang, 2004; Myzak *et al.*, 2004; Thornalley, 2002). Allyl isothiocyanate (AITC), benzyl-ITC (BITC), phenethyl-ITC (PEITC), sulforaphane (SFN), erucin (ERN) and iberin (IBN) induce time- and dose-dependent G2/M arrest in leukemia cells (Jakubikova *et al.*, 2005). Mustard ITCs are mitotic inhibitors and/or apoptosis inducers. This activity suggests that they might be chemotherapeutic agents against cancer cells with multi-drug resistance phenotypes. Tang *et al.* (2006) recently demonstrated that naturally occurring ITCs, including AITC, BITC, PEITC and sulforaphane, strongly inhibited the growth of both human bladder and drug-resistant bladder cancer cell lines. Anti-proliferative mechanisms include causing the cleavage of

caspase-3, -8 and -9 in apoptosis induction and arresting cells in the cell cycle (S and G2/M phases). Sulforaphane [1-isothiocyanoto-4-(methylsulfinyl) butane], a degradation product of the GLS, glucoraphanin, is a potent inducer of detoxification enzymes, which are strongly correlated with the prevention of certain types of cancer (Brooks *et al.*, 2001; Matusheski *et al.*, 2001).

Due to the toxic nature of GLS degradation products, it is important to develop quick and efficient bioassays for screening traces of AITC in food products, animal feeds and oils. Stress caused by ITC toxicity could be used as a measure of toxicity.

Caenorhabditis elegans, a microscopic soil roundworm or nematode, is a model system for studying stress responses. They are readily transformed, small, transparent, and their genome has been sequenced (Leitz *et al.*, 2002; Candido and Jones, 1996; Stein et. al., 2001). The stress response in *C. elegans*, and most other organisms, is characterized by a rapid activation of heat shock genes and the synthesis and accumulation of heat shock proteins (HSPs). There is now extensive evidence in the literature that HSPs play important roles in tolerance to a variety of biotic and abiotic stresses (Vierling, 1991; Parsell and Lindquist, 1994; Hamilton and Coleman, 2001; Cranfield *et al.*, 2004). Thus, HSPs appear to be general stress proteins that are involved in maintaining cell function and stress survival or facilitating recovery from stress. Members of the 70 kDa heat shock protein (HSP70) family are ubiquitous in plants, animals and microorganisms and their structure and function are highly conserved among diverse organisms - from algae to mammals (Wu *et al.*, 1994). Some HSPs are constitutively expressed at low levels and are believed to act as molecular chaperones, proteins that assist proper protein folding, found in the cytosol and most cell organelles (Guy and Li, 1998; Parsell and Lindquist, 1994). HSP70 is involved in preventing protein aggregation and in refolding of denatured proteins produced in response to cellular stress. Furthermore, HSP70 is involved in regulating the heat shock response and other stresses through mitogen-activated protein kinase (MAPK) signaling (Hirt, 2000; Suri *et al.*, 2007). Thus, it is a hypothesis of this thesis that HSP70 protein may be used as an indicator of stress induced by AITC. DNA promoter sequences for HSP70 in *C. elegans* have been characterized (Snutch *et al.*, 1988).

The present study was aimed at inhibiting myrosinase activity in mustard seed meal, establishing the biological properties of AITC, and understanding its toxicity aspects using *C. elegans* as a model system.

The objectives of this research were as follows:

Objective 1. To study the factors affecting myrosinase activity.

Hypothesis: Myrosinase is known to convert sinigrin to AITC in the presence of water, and this leads to toxicity in mustard seed meal. Therefore, inhibiting myrosinase activity might be helpful in reducing AITC toxicity. As the activity of an enzyme depends upon reaction conditions such as temperature and pH, efforts were made to inhibit or reduce myrosinase activity by altering such reaction conditions.

Objective 2. To investigate the biological activity of allyl isothiocyanate.

Hypothesis: Structurally, AITC might act as an ethylene analogue due to a common CH_2=CH-R motif. Ethylene is known to promote seed germination in a dose-dependent manner (Kepczynski *et al.*, 1997). Therefore, it was hypothesized that AITC might promote seed germination.

Objective 3. To identify molecular indicators of AITC-induced stress in *C. elegans*.

Hypothesis: The anti-fungal and anti-bacterial properties of AITC are well established. However, to date, no literature is available to support its toxicity or stress inducing ability in living organisms. Efforts were made to establish such properties of AITC in *C. elegans*, an experimental model for this study. HSPs are synthesized under stressful conditions. Many HSPs are involved in the process of protein renaturation, folding and activation in a cell. Members of the *HSP70s* family play a major protective role against stresses (Cranfield *et al.*, 2004; Suri and Dhindsa, 2007) and are among the most

conserved molecules in the phylogeny. Different isoforms of *HSP70* are localized in different cell organelles, and among these isoforms, *HSP70A* is known to be expressed in the cytoplasm. Therefore, this protein was selected for further investigations. Bioassays were developed to quantify stress responses and to provide insight into the mechanism of AITC action.

Chapter 2: Literature Review

2.1 Mustard

Mustard is an annual herb that belongs to the division Magnoliophyta, class Magnoliopsida, order Brassicales and family Brassicaceae. Three types of mustard are used as a source of seed viz., yellow mustard (*Sinapis alba*), brown or oriental mustard (*Brassica juncea*) and black mustard (*B. nigra*). *B. nigra* possess a very strong and distinctive flavour. Saskatchewan accounts for nearly 90 per cent of Canadian mustard production. Members of the Brassicaceae family are characterized by the presence of an enzyme, myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147, formerly 3.2.3.1) that hydrolyzes glucosinolates to form an aglucone and D-glucose. The aglucone is unstable and spontaneously decomposes into nitriles, thiocynates, isothiocynates, oxazolindine-2-thiones or indoles, depending on the side chain, pH, presence of iron ions and proteins such as epithiospecifier proteins. Some of these hydrolysis products contribute to the characteristic flavours and odours of *Brassica* vegetables (McNaughton and Marks, 2003).

Mustard is rich in protein. Rapeseed is closely related to mustard and proteins of the two species are similar. Recently, rapeseed protein has been shown to have better emulsification capacity than that of whole egg (Yoshie-Stark *et al.*, 2007). Therefore, it could be used as a replacement for animal proteins. Yoshie-Stark *et al.* (2007) also showed health benefits of these proteins such as inhibition of angiotensin I converting enzyme which is beneficial for patients suffering from hypertension. These proteins also have been shown to have bile-acid-binding and free-radical-scavenging activities.

2.2 Glucosinolates

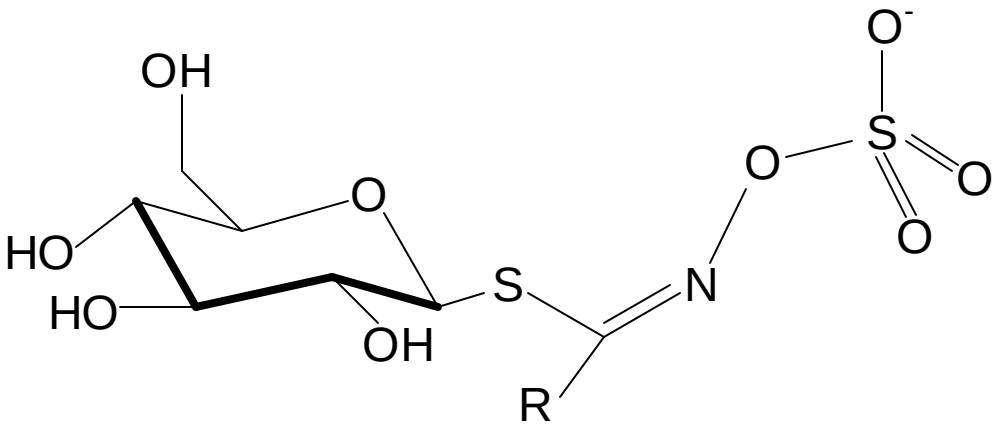
Glucosinolates (GLS) are a class of secondary metabolites that contain sulfur, nitrogen and a group derived from glucose. They are naturally occurring ß-D-thioglucosides N-hydroxysulphates found in fifteen families of dicotyledonous plants including Brassicaceae and related families of the order Capparales. These fifteen families are the Akaniaceae, Bataceae, Brassicaceae, Bretschneideraceae, Capparaceae, Caricaceae, Euphorbiaceae, Gyrostemonaceae, Limnanthaceae, Moringaceae, Pentadiplantdraceae, Resedaceae, Salvodoraceae, Tropaeolaceae and Tovariaceae (Rodman *et al.*, 1996, Fahey *et al*. 2001). By 2000, about 120 GLS had been identified (Rask *et al.*, 2000). GLS types and abundance in plant species are highly variable. For example, the main GLS in radish seed (*Raphanus sativus*) is 4-methylsulphinyl-3-butenyl glucosinolate, while mustard seed (*B. juncea*) predominantly contains propenyl glucosinolate. Cabbage seed (*B. oleracea*) contains mainly propenyl and 2-hydroxy-3-butenyl glucosinolate; rapeseed (*B. napus*) contains 4 major glucosinolates: 2-hydroxy-3-butenyl, 3-butenyl, 4-pentenyl, and 2-hydroxy-4-pentenyl. GLS are found in all parts of the plant and up to fifteen different GLS have been found in the same plant. Generally, levels are high in the seed i.e., up to ten per cent of the dry weight, whereas the levels in the leaf, stem and root are approximately ten times lower. The concentration varies within plants of single species as this depends on the tissue type, physiological age, plant health and nutrient availability (Brown and Morra, 2005). Plants use substances derived from GLS as natural pesticides and as a defense against herbivores. These substances are also responsible for the bitter or sharp taste of many common foods such as mustard, radish, horseradish, cress, cabbage, Brussels sprouts, kohlrabi, kale, cauliflower, broccoli, turnip and rapeseed.

In the past, successions of reviews have addressed the biology and chemistry of GLS (Fahey *et al.*, 2001; Halkier and Gershenzon, 2006). GLS are grouped into a number of chemical classes on the basis of structural similarities. The most extensively studied GLS are the aliphatic, methylthioalkyl, aromatic and heterocyclic (e.g., indole) glucosinolates.

2.2.1 Chemical structure

GLS are water-soluble anions and belong to the glucosides. Every GLS contains a central carbon atom which is bound via a sulfur atom to the glycone group, and via a nitrogen atom to a sulfonated oxime group (Figures 2-1 and 2-2). In addition, the central carbon is bonded to a side group; different GLS have different side groups. The structure is composed of a thioglucosidase link to the carbon of sulphonate oxide. The sulphate group and the R group are present in the anti-stereochemical configuration. The structure of the R- group may be aliphatic, cyclic or heterocyclic (Kimber and McGregor, 1995). The properties of the R-group may vary from lipophilic to hydrophilic (Inolates, 2005). The approximately 120 described GLS share a chemical structure consisting of a ß-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, plus a variable R group derived from one of eight amino acids (Fahey *et al.*, 2001). GLS can be classified by their precursor amino acid and the types of modification to the R group. Compounds derived from Alanine *(Ala),* Leucine *(Leu),* Isoleucine *(Ile),* Methionine *(Met),* or Valine (*Val)* are called aliphatic GLS, those derived from Phenylalanine (*Phe)* or Tyrosine (*Tyr)* are called aromatic GLS, and those derived from Tryptophan (*Trp)* are called indole glucosinolates. The R groups of most glucosinolates are extensively modified from these precursor amino acids, with methionine undergoing an especially wide range of transformations (Fahey *et al.*, 2001). Most of the R groups are elongated by one or more methylene moieties. Both elongated and non-elongated R groups are subject to a wide variety of transformations, including hydroxylation, O-methylation, desaturation, glycosylation, and acylation.

When GLS were first discovered they were named after the plants in which they were found. With the discovery of more GLS, a semi-systematic system for their naming arose, based on the structure of the side chain. Table 2.1 shows common names for some GLS and indicates their side chain. The name of the side chain followed by the word "glucosinolate" gives the semi-systematic name. The suffix "ate" indicates the anionic nature of GLS.



Glycone **Sulfonated oxime**

Figure 2-1. Structure of a GLS.

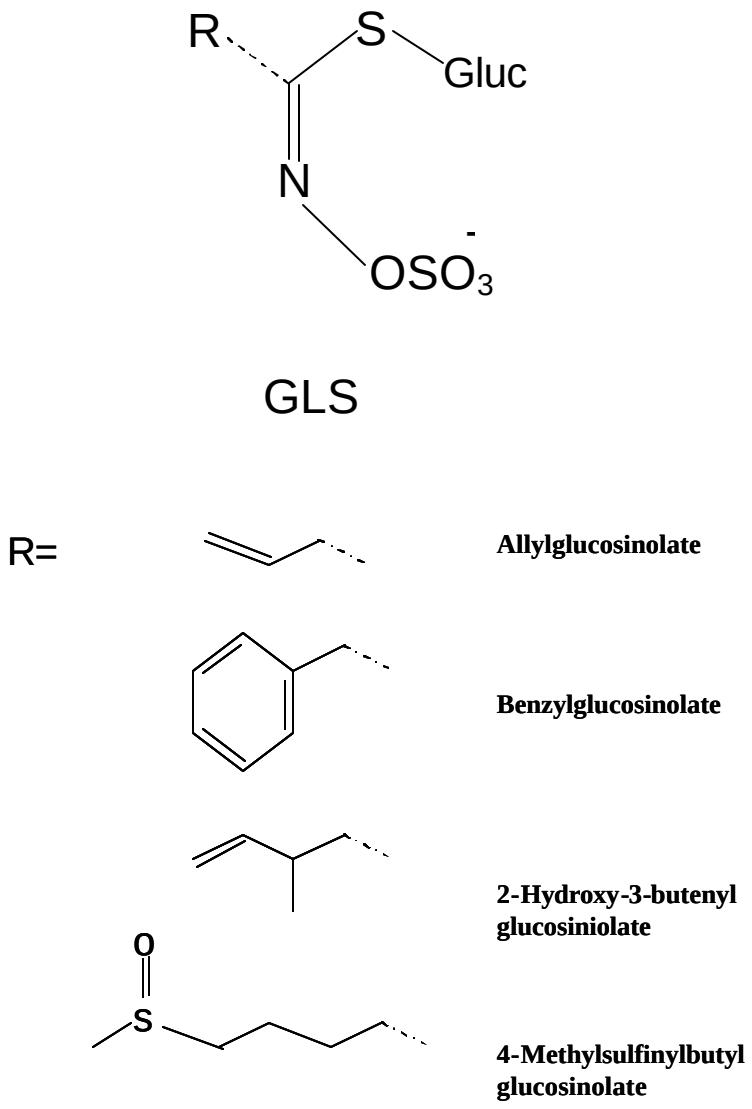


Figure 2-2. Chemical structure of GLS.

Table 2-1. Trivial names and the side chains of some GLS.

Trival name(s)	Side chain	Type
Gluconasturtin	2-Phenethyl	Cyclic
Glucobrassicin	3-indoly methyl	Heterocyclic
Progoitrin, epiprogoitrin	2-hydroxy-3-butenyl	Aliphatic
Sinigrin	2-propenyl	Aliphatic
(Gluco)sinalbin	p-Hydroxybenzyl	Cyclic

2.2.2 Biosynthesis

The formation of GLS can be conveniently divided into three separate phases. First, certain aliphatic and aromatic amino acids are elongated by inserting methylene groups into their side chains. Second, the amino acid moiety itself, whether elongated or not, is metabolically reconfigured to give the core structure of the glucosinolate. Third, the initially formed GLS is modified by various secondary transformations (Wittstock and Halkier, 2002; Figure 2-3).

2.2.3 Hydrolysis

Plants accumulating GLS always possess a thioglucoside glucohydrolase activity known as myrosinase, which hydrolyzes GLS to D-glucose and allelochemicals (Rask *et al.*, 2000). The products are glucose and an unstable aglycone that can rearrange to form isothiocyanates, nitriles and other products. GLS hydrolysis in intact plants appears to be hindered by the spatial separation of GLS and myrosinase or the inactivation of myrosinase, but these components mix together upon tissue damage, leading to the rapid formation of glucosinolate hydrolysis products. Myrosinase is present in myrosin cells and is separated from the GLS pool. Most of the biological activities of GLS are attributed to the actions of their hydrolysis products (Wittstock and Halkier, 2002).

GLS are degraded upon plant damage to a variety of hydrolysis products that are likely responsible for much of the biological activities of this class of compounds. The process begins with myrosinase-catalyzed hydrolysis of the thioglucoside linkage, leading to the formation of glucose and an unstable aglycone (Bones and Rossiter, 1996; Rask *et al.*, 2000). Depending on the structure of the side chain and the presence of additional proteins and cofactors, the aglycone then rearranges to form different products, including ITCs, oxazolidine- 2-thiones, nitriles, epithionitriles, and thiocyanates (Figure 2-4).

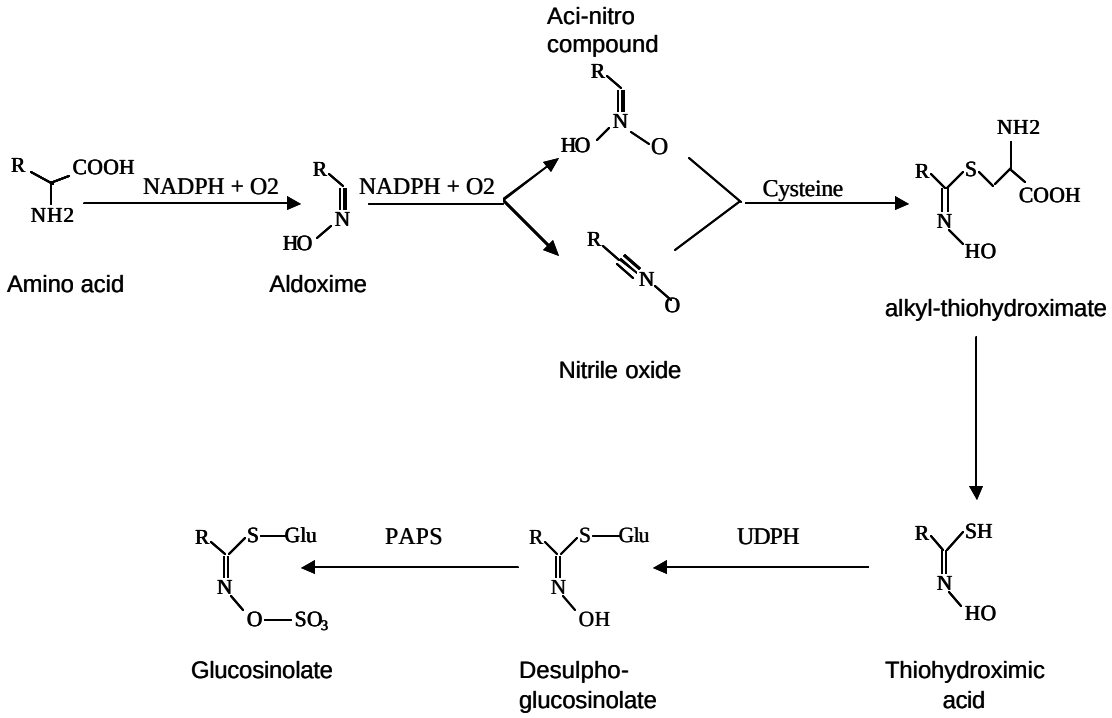


Figure 2-3. Biosynthesis of GLS.

The most common GLS hydrolysis products in many species are ITCs, which are formed from the aglycone by a Lossen rearrangement involving the migration of the side chain from the oxime carbon to the adjacent nitrogen. When the GLS side chain bears a hydroxyl group at C-2, the ITCs formed are unstable and cyclize to oxazolidine-2-thiones, a class of substances known to cause goiter. In other plants, a major percentage of GLS hydrolysis products are nitriles (Coles, 1976; Lambrix *et al.,* 2001). The formation of nitriles *in vitro* is favored at a pH of less than 3 or in the presence of Fe^{2+} ions (Galletti *et al.,* 2001; Gil and MacLeod, 1980). However, protein factors, such as the epithiospecifier protein (ESP), may be involved in nitrile formation *in vivo*, (Bernardi *et al.,* 2000; Foo *et al.,* 2000; MacLeod and Rossiter, 1985; Tookey 1973). When the glucosinolate side chain has a terminal double bond, ESP promotes the reaction of the sulfur atom of the thioglucoside linkage with the double bond to form a thirane ring, giving an epithionitrile. The process occurs only in the presence of myrosinase, and ESP is not known to have any catalytic abilities. The recent isolation of an *Arabidopsis* gene encoding an ESP showed that this protein not only promotes the formation of epithionitriles, but also the formation of simple nitriles from a large variety of GLS (Lambrix *et al.,* 2001). Other hydrolysis products include ITCs, which are formed from only three GLS: benzyl-, allyl-, and 4-methylsulfinylbutyl-glucosinolate (Figure 2-4), all of which form stable side-chain cations. Like nitrile formation, thiocyanate production is also associated with specific protein factors (Hasapis and MacLeod, 1982), but these have not yet been identified. The hydrolysis of indole glucosinolates is somewhat different from that of the other GLS types, because the initially formed ITCs are unstable at neutral or slightly acidic pH, and are converted to further metabolites, including indole-methanols, ascorbic acid conjugates, and oligomeric mixtures (Agerbirk *et al.*, 1998; Buskov *et al.*, 2000; Latxague *et al.*, 1991).

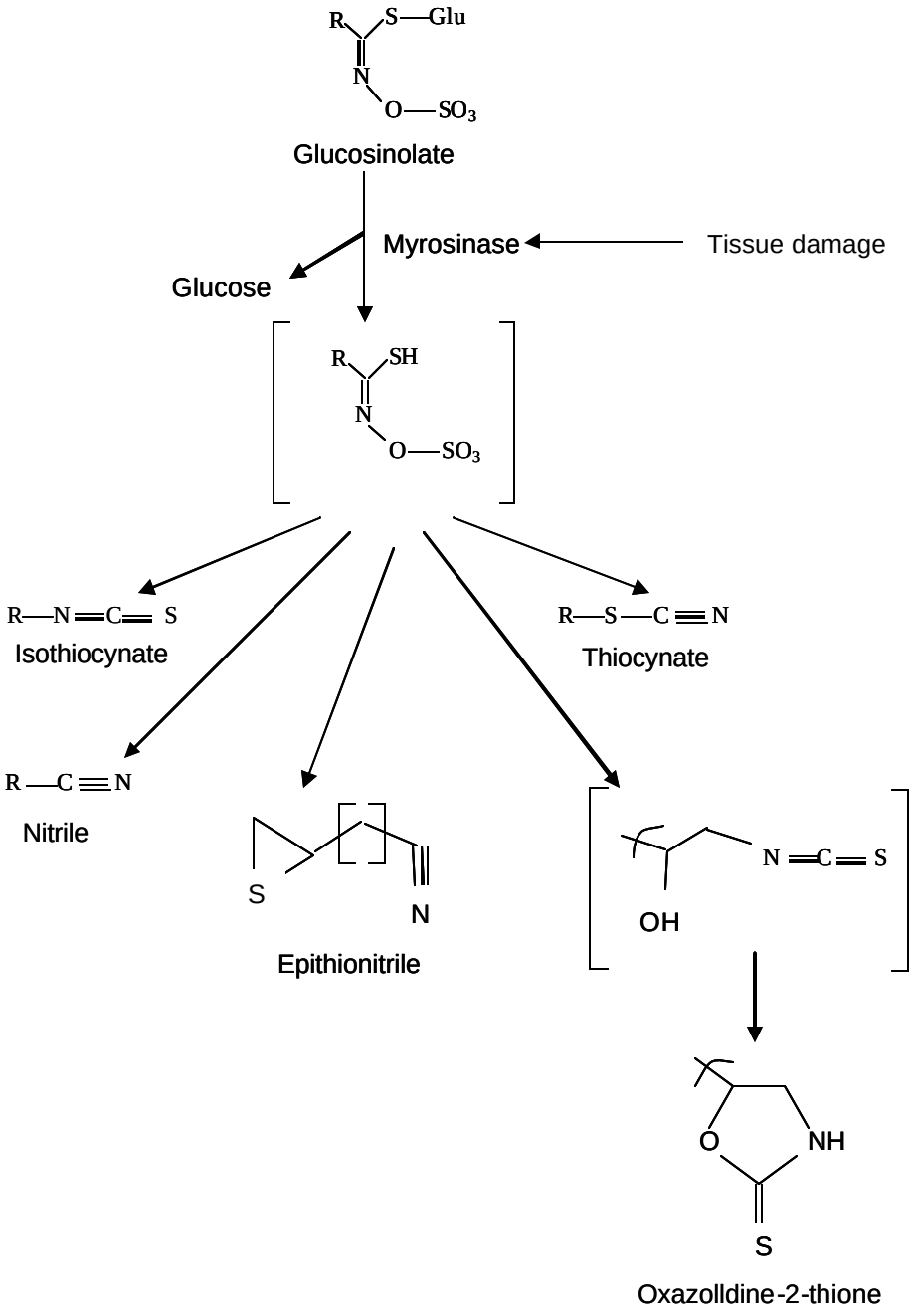


Figure 2-4. Outline of GLS hydrolysis. Brackets indicate unstable intermediates.

GLS hydrolysis products play important roles in plant defense systems against pests, fungi and bacterial infections (Manici *et al.*, 1997; Vaughn *et al.*, 2005, 2006; Vaughn and Berhow, 2005; Ware, 2000). Certain GLS breakdown products are volatile and, therefore, good candidates for insect fumigants. Propenyl isothiocynate released from the GLS sinigrin in *B. nigra, B. carinata* and *B. juncea,* is an effective fumigant for suppression of the growth of soil-borne fungal pathogens (Manici *et al.*, 1997; Rosa and Rodrigues, 1999). Although they are insecticidal, the mode of action of such natural products is not clear.

2.2.4 Extraction

Due to the hardy nature of myrosinase, the extraction of GLS is a complex procedure. Several methods of GLS extraction have been adopted, including denaturation of myrosinase by boiling or inactivation at -20ºC or by solvent extraction. Extracts for HPLC/GC are prepared in boiling water (Rangkadilok *et al.*, 2001; Matusheski and Jeffery, 2001; Szmigielska *et al.*, 2000) or by autoclaving samples (Jen *et al.*, 2001) or in boiling methanol (Zrybko *et al.*, 1997; Verkerk *et al.*, 2002), or in 50% acetonitrile (ACN; Tsao *et al.*, 2002b). After extraction, debris is removed either by filtration or centrifugation. The clarified extract is then concentrated and used for HPLC analysis. Other researchers have used liquid nitrogen to arrest myrosinase activity for storage of samples, and before analysis, frozen samples are added directly to boiling water or methanol (Karcher *et al.*, 1999). Any of the methods of extraction described above can be used, as the basic purpose is to inactivate myrosinase while extracting GLS from plant samples.

2.2.5 Purification and desulfation

After extraction of GLS, they are further purified as described by Szmigielska *et al.* (2000). In this method, GLS from the extract are bound to an anion exchange membrane. Initially, anion exchange membranes were prepared for use by shaking three times with saturated sodium bicarbonate solution (1 h each time). Membranes were then thoroughly washed and stored in distilled water for further use. The total exchange capacity of the membranes was 1.1mequiv/g (0.04mequiv/cm^2). GLS may also be

purified using a DEAE Sephadex A-25 anion exchange column (Oerlemans *et al.*, 2006). The column is washed with 1 mL Millipore water, loaded with 2 mL of the GLS extract, and then washed twice with 1 mL of 20 mM sodium acetate solution. Sulphatase enzyme is added to the column to cleave the sulfate by incubation overnight at room temperature. Finally, GLS are eluted with reverse-osmosis-purified water (3 x 0.5 mL).

2.2.6 Detoxification

Tripathi and Mishra (2007) reviewed the recent development of various treatment methods to reduce GLS content in mustard meal to minimize GLS -associated deleterious effects. Most of these methods involved hydrolysis or decomposition of GLS before consumption by humans or animals. Some of these methods are as follows.

1. Microwaving

Microwave irradiation at 2450 MHz for 2.5 min is known to inactivate myrosinase and decompose GLS (Verkerk and Dekker, 2004).

2. Metal solutions

Treatment of meal with copper sulphate solution inactivates ITC in meals (Das and Singhal, 2001, 2005) due to rearrangement reactions and the production of amines such as allylamine or thiourea (Rouzaud *et al.*, 2003).

3. Fermentation

The fermentation of mustard meal using *Rhizopus oligosporus* and *Aspergillus* sp. (meal: water ratio of 1:3, 25ºC, under aerobic conditions, 10 days) inactivated myrosinase and reduced total GLS (Vig and Walia, 2001). The complete degradation of GLS occurred after 60 h of fermentation at 30ºC (Rakariyatham and Sakorn, 2002). Decomposition of GLS was greater with longer fermentation periods. The reduction in GLS and their metabolites during fermentation may be due to the utilization of glucose and sulphur moieties of these compounds by microbial enzymes.

4. Heat treatment

Wet heating is more effective than dry heating (Burel *et al.*, 2000; Tripathi *et al.*, 2001; Leming *et al.*, 2004) for decomposing GLS.

2.2.7 Methods of GLS determination

Several methods have been used for the quantification of GLS. These are as follows.

1. High Pressure Liquid Chromatography (HPLC)
2. Gas chromatography (GC)
3. Capillary Electrophoresis (CE)
4. Determining total GLS by capillary electrophoresis via enzymatically released gluconic acid labeled with 7-aminonaphthalene-1,3-disulfonic acid (ANDSA)
5. Nuclear Magnetic Resonance (NMR)
6. Near IR spectroscopy
7. X-ray fluorescence

Some of the most commonly used methods are described below.

2.2.7.1 High Pressure Liquid Chromatography (HPLC)

HPLC has been used as an efficient analytical tool for accurate quantification of many metabolites. HPLC methods take advantage of the sophistication and automation of the instrument, along with its precision and accuracy. Reverse-phase HPLC has generally been the method of choice in recent years because it has the versatility of analyzing GLS in both intact and desulfonated forms. Spinks *et al.* (1984) developed the reverse-phase HPLC method for quantitative analysis of desulphoglucosinolates which is the most widely used method today. This method utilizes an on-column enzymatic desulfation treatment of plant extracts followed by HPLC detection of the resultant desulfoglucosinolates. Adaptation of the sulfohydrolase desulfation method as HPLC method, although the most widely used method for GLS separation, is still subject to difficulties in interpretation because of the effect of pH, time and enzyme activation on the desulfation products (Spinks *et al.*, 1984; Sang *et al.*, 1984). Typically, this method uses response factors determined with purified desulfosinigrin and uses desulfobenzyl glucosinolate as an internal standard. Correspondence of GLS retention times, and

comparison to standardized rapeseed extracts, are typically used to validate the chromatographic profile. Unfortunately, the biological activity of these molecules is compromised by the removal of the sulfate. After desulfation, they can no longer serve as substrates for myrosinase and thus their cognate ITCs are not available for bioassay or for direct measurement by cyclocondensation, a key tool in the study of the pharmacokinetics, pharmacodynamics and bioactivity of these compounds.

Therefore, several authors have made attempts to improve the HPLC method for GLS analysis. Zrybko *et al.* (1997) developed an HPLC method for separation and quantification of GLS in mustard using a Phenomenex 5 µm ODS column (40ºC) with the mobile phase water: methanol, with an ion pairing reagent consisting of 0.15% triethylamine and 0.18% formic acid. The mobile phase gradient was 100% water for 10 min, which was then increased to 100% methanol over the next 60 min. Using this method, the retention time for sinigrin detected at 235 nm was 10.5 min. Sinigrin was detected at 235 nm because the contribution to the UV absorption at this wavelength arises predominately from the GLS group and not from the R groups (Helboe *et al.*, 1980; Björkqvist and Hase, 1988). Later, Szmigielska *et al.* (2000) reported another HPLC method for separation and quantification of naturally occurring GLS from canola. They separated GLS on a Supelcosil LC-18 column using methanol and 0.1 N ammonium acetate (NH_4OAc) (3:97; v/v) as the mobile phase. In this way, detection was proportional to the molar concentrations of the GLS.

Jen *et al.* (2001) further improved the HPLC method for GLS analysis. They separated GLS with a reverse phase C-18 column using ACN-water (20:80; v/v) containing 0.02 M tetrabutylammonium (TBA) at pH 7.0. The retention time for sinigrin detected at 227 nm was 8.8 min. Rangkadilok *et al.* (2001) also used a C-18 reverse phase column. The column was saturated with 5 mM tetramethylammonium bromide (TMAB) before loading the sample and TMAB (5 mM) was used as the mobile phase. Sinigrin was detected in 8.84 min at 230 nm. This method is simple, efficient and quick.

The method developed by Tsao *et al.* (2002b) is the most commonly used method in industry. They used a Sphereclone ODS-2 column and the mobile phase was composed of 0.025 M ammonium acetate (NH_4OAc, pH 6.75) and ACN. The mobile phase was run isocratically for 2 min, then linearly increased to 50% NH_4OAc (0.025 M,

pH 6.75) and 50% ACN in 0.5 min, held for 7.5 min, and then brought back to 99% NH_4OAc (0.025 M, pH 6.75) and 1% ACN at 12 min. Sinigrin (228 nm) and AITC (242 nm) were detected as early as 2.8 min and 9.8 min, respectively.

The HPLC method for determining desulfonated GLS provides a simple means of determining the GLS profile (Verker *et al.*, 2002; Oerlemans *et al.*, 2006). Oerlemans *et al.* (2006) separated de-sulfonated GLS using a C-18 reverse phase column. Elution of desulfonated GLS was performed by a gradient system of water and ACN/water (20:80; v/v). Sinigrin was detected at 229 nm and was eluted as early as 4.8 min. Recently, Trenerry *et al.* (2005) used a C-18 column and a mobile phase of 0.005 M TMAB dissolved in 2% methanol/water for the separation of glucoraphanin in broccoli. The chromatograms were monitored at 230 and 270 nm.

Ion exchange and desulfonation methods are more sensitive and time consuming, and sometimes can lead to loss of GLS. Because the separated compounds are not suitable for physiological or biological studies, the use of such methods as a preparative technique is limited to specific purposes (Betz and Fox, 1994). Reverse phase HPLC methods for direct analysis of intact and non-derivatized GLS are advantageous.

Due to the importance of GLS and their hydrolysis products, there is a need for a quick, economic, efficient and simple HPLC method for detection of GLS in various sample types. The total GLS content is generally measured by adding up all of the individual GLS (Linsinger *et al.*, 2001). HPLC coupled with mass spectroscopy (HPLC-MS) also has shown promise.

2.2.7.2 Gas chromatography (GC)

Gas chromatography is useful for the separation and quantification of GLS, provided the GLS of interest is volatile after derivatization. Brown *et al.*, (1994) extracted GLS from plant tissues with methanol. The extract was placed on a DEAE Sephadex A-25 column that was first treated with 6 M imidazole in 5 M formic acid and rinsed with water. Other compounds were separated from the sample by rinsing with 67% methanol and water. GLS were desulfated overnight, removed from the columns using 60% methanol, and silylated in acetone. Silylated derivatives were separated and identified using a GC equipped with a quadrupole mass selective detector (MSD).

GCMSD operating conditions were: injector 260°C; interface 320°C; initial oven temperature 130°C for 1 min, ramped 15°C/min to 320°C and held for 10 min; purge (splitless injection) 0.5 min; helium flow of 1.14 mL/min; emission at 50 µA; repeller at 30 V; and scan range of *m*/*z* 25-470. The column coating was 5% phenyl-substituted methylpolysiloxane (HP-5MS; 30 m, 0.25 mm inside diameter, and 0.250-µm film).

Gas chromatography chemical ionization mass spectrometry has been found to possess advantages over gas chromatography electron impact mass spectrometry for the structural elucidation of GLS, separated as the volatile per-trimethylsilyldesulpho derivates (Eagles *et al.*, 2005). The technique demonstrates the versatility of mass spectrometry in GLS identification.

2.2.7.3 **Capillary Electrophoresis**

The separation of GLS and related desulfoglucosinolates has also been achieved by Micellar Electrokinetic Capillary Chromatography (MECC). MECC is a highly efficient separation technique that is complementary to HPLC and GC and is especially suited to the analysis of low to medium molecular weight ionic compounds. MECC separations are often faster and more cost effective than corresponding HPLC or GC procedures. MECC was introduced by Terabe *et al.* (1984) and has become one of the most widely used capillary electrophoresis (CE) methods due to the ability to separate both charged and neutral analytes. MECC separates compounds by using a surfactant as part of the separation buffer. This surfactant forms a micelle and neutral analytes separate based on their affinity for the micelle and their hydrophobicity. The amount of surfactant present in the system needs to be above the critical micelle concentration to allow for the micelles to form. Most MECC systems for GLS analysis are based on the cationic surfactant cetyltrimethylammonium bromide (CTAB) (Karcher and Rassi, 1999; Michaelsen *et al.*, 1992; Morin *et al.*, 2005). The surfactant coats the wall of the bare fused silica capillary and covers the silica with a layer of positive charges. The net result of this adsorption is the reversal of the direction of the electro-osmotic flow. The CTAB micelle is advantageous because it forms an ion-pair with the negatively charged sulfate group of the intact GLS, thus imparting strong analyte-micelle interactions. Michaelsen *et al.* (1992) successfully used CTAB to separate eleven intact GLS. The optimum

separation conditions were 18 mM borate, 30 mM phosphate, 50 mM CTAB at pH 7.0 with an applied voltage of 20 KV. Under these conditions, the CTAB micelle migrates against the electro-osmatic flow, whereas the negatively charged glucosinolate migrates with the electro-osmatic flow. Thus, stronger the ion-pair formation and hydrophobic interactions of a given GLS with the CTAB micelle, longer the migration time of that GLS. Bjergegaard *et al.* (1995) also separated a number of desulfoglucosinolates using the cationic surfactant sodium cholate. Paugman *et al.* (1995) used sodium dodecyl sulfate (SDS) as the surfactant to separate glucobrassicin and methoxyglucobrassicin, with tetramethylammonium hydroxide as the ion-pair reagent and methanol as an organic modifier. Bringmann *et al.* (2005) recently developed a capillary zone electrophoresis method for the analysis of GLS from *Arabidopsis thaliana*. The electrolyte used with the fused-silica capillaries was a 100 mM ammonium acetate buffer (pH 5.4) containing 30% ACN. The pH of the buffer was adjusted with acetic acid. The sample was loaded by applying a pressure injection of 50 mbar (=0.725 psi) for 10 sec on the anodic side and detected at 225 nm. Electrophoresis was conducted at 30^{o}C and 25 KV. Optimization of CE is required for rapid separation of GLS. Research is needed to identify possibly more GLS in mustard and to develop a quick, efficient and economic method of determining these GLS. The use of capillary electrophoresis is potentially the most efficient and fastest method for determination of GLS.

A selective and sensitive method for the determination of total GLS in plants by capillary electrophoresis-laser-induced fluorescence (CE-LIF) detection was developed by Karcher and Rassi (1999). The method was based on the enzymatic release of glucose from GLS in the presence of myrosinase. The released glucose was converted to gluconic acid by glucose oxidase. The resulting gluconic acid was then labeled selectively with the fluorescent tag 7-aminonaphthalene-1,3-disulfonic acid (ANDSA). Peak area resulting from gluconic acid-ANDSA was derived from the free glucose in the sample. This allowed quantitation of the total GLS in the sample. The peak area was normalized to the internal standard, N-acetlyneuraminic acid derivatized with ANDSA. Detection of GLS was performed at 228 nm. For Laser Induced Fluorescence detection of the ANDSA derivatives, a fluorescence emission bandpass filter of 380 ± 2 nm was

used. A 360 nm cut-off filter was used to reject the laser beam. Sodium phosphate (50 mM, pH 3.0) was used as an electrolyte.

2.2.7.4 Nuclear Magnetic Resonance (NMR)

Prestera *et al.* (1996) identified and isolated GLS by using mass spectrometery and NMR spectroscopy. Proton NMR spectra were obtained in D_2O at 600 MHz. Ammonium salts of GLS were dissolved in D_2O and dried in a vacuum centrifuge, and then dissolved in 625 µL of D_2O at a concentration of 1 to 10 mM and one-dimensional (1-D) proton spectra were obtained in high quality NMR tubes. Proton resonance assignments were initially based on the covalent structure of the R group as well as on the analysis of the coupling patterns in the 1-D proton NMR spectra.

2.3 Myrosinase

2.3.1 Occurrence

Myrosinases (Thioglucoside glucohydrolase; EC 3.2.3.1) are typical enzymes of the Brassicaceae family. Although the myrosinase- GLS system is always present in the organs of Brassicaceae in various arrangements and concentrations, it is only activated following tissue damage. It plays a defensive role against pathogens in general (Bones and Roostier, 1996; Chew, 1998; Louda and Mole, 1991; Rosa *et al,*. 1997). Myrosinase and GLS were first discovered in mustard seed by Bussy (1840). Myrosinase activity always appears to be accompanied by one or more GLS. GLS occur in all Brassicaceae (Cruciferae) species and have also been found in Akaniaceae, Bataceae, Bretschneideraceae, Capparaceae, Caricaceae, Drypetes (Euphorbiaceae), Gyrostemonaceae, Limnanthaceae, Moringaceae, Pentadiplantdraceae, Resedaceae, Salvodoraceae, Tovariaceae and Tropaeolaceae (Rodman, 1991). Enzymes with myrosinase activity have also been found in the fungi *Aspergillus sydowi* (Reese *et al.,* 1958; Othsuru *et al.,* 1969) and *Aspergillus niger* (Ohtsuru *et al.,* 1973), in the intestinal bacteria *Enterobacter cloacae* (Tani *et al.,* 1974) and *Paracolobactrum aerogenoides* (Oginsky *et al.,* 1965), in mammalian tissues (Goodman *et al.,* 1959) and in the cruciferous aphids *Brevicoryne brassicae* and *Lipaphis erisimi* (MacGibbon and Beuzenberg, 1978). The amount of myrosinase activity found in seed from cultivars of

Sinapis alba L., *Brassica campestris* L. and *Brassica napus* L. has been examined by Henderson and McEwen (1972), Bjørkman and Lønnerdal (1973) and Bones (1990). Myrosinase activity was found to be about ten times higher in *S. alba* than in *B. campestris* and the activity in *B. napus* was slightly higher than in *B. campestris* (Bones, 1990).

2.3.2. Distribution

The distribution of myrosinase isoenzymes appears to be both organ-specific and species-specific. Electrophoretic examination of isoenzymes from many plants, organs and tissues demonstrates that the pattern may vary with species, organ and age of the plant (MacGibbon and Allison, 1970; Henderson and McEwen, 1972; Buchwaldt *et al.*, 1986). Little is known about the physiological reasons for this difference. It has been postulated that the particular isoenzymes correspond to endogenous conditions found in that plant, or to conditions found in the target organism, or to particular GLS that dominate the profile of that tissue. It is not known if electrophoretic separation is separating isoenzymes with distinct amino acid sequences.

A systematic analysis of the variation in myrosinase activity in plants at different developmental stages and organs has been reported. Bones (1990) examined the myrosinase activity at different developmental stages and in different plant parts throughout the life cycle. The reported activities in different tissues varied, but all tested organs/tissues contained some myrosinase activity. Myrosinase activity could also be detected in callus cultures and in *in vitro* cultured plants (Bones, 1990). Except for the roots of fully grown plants where a high activity was observed, other organs of mature plants normally contained low myrosinase activity.

Bones (1990) used enzymatic assays and ultrastructural observations of calli to examine the correlation between myrosinase and myrosin cells. A lack of observable myrosin cells by light microscopy and transmission electron microscopy was associated with low myrosinase activity.

2.3.3 Genetics

Myrosinase is not properly identified as a single enzyme, but rather as a family or group of similar enzymes. Multiple forms of the enzyme exist, both among species and within a single plant (Bones and Slupphaug, 1989; Falk *et al.*, 1995a; Lenman *et al.*, 1993a; Xue *et al.*, 1993), and all perform a similar function (Björkman, 1976). Although their genetic sequences are similar to those of other β-glycosidases (Lenman *et al.*, 1993b), mostly myrosinases are specific toward GLS (Durham and Poulton, 1990). These enzymes cleave the sulfur-glucose bond regardless of either the enzyme or substrate source. However, the particular enzyme and GLS substrate do influence reaction kinetics (Bones, 1990).

2.3.4 Purification

Myrosinase from white mustard (*Sinapis alba*) has been purified as early as in 1986 (Palmieri *et al.*, 1986). They used single step affinity chromatography on Con A-Sepharose for the isolation of myrosinase. The binding capacity of Con A-Sepharose was 6.6 mg/mL gel bed which corresponds to 150,000 U/mL of chromatographic bed. Moreover, the enzyme bound to Con A-Sepharose remained active towards GLS.

2.4 Allyl isothiocynate (AITC)

Allyl isothiocynate (AITC, CH_2=CH-CH_2-N=C=S) is a hydrolysis product of GLS which contributes to the peculiar odour of mustard oil. It is a colourless to pale yellow, volatile, highly flammable liquid with a molecular weight of 99.15 g. It is slightly soluble in water (0.1 mg/mL) and is soluble in organic solvents. AITC possess various biochemical and physiological properties (Keum *et al.*, 2005), which are discussed below.

2.4.1 A potent anti-microbial agent

Mustard seed flour contains 28% to 36% protein. The presence of tocopherols in mustard contributes to its shelf life (Cahoon *et al.*, 2003; Winther and Nielsen, 2006). Mustard is widely known for its sharp taste and it is an essential component of dressings and sauces. The oil in mustard inhibits the growth of certain yeasts, moulds and bacteria.

enabling mustard to function as a natural preservative. AITC vapour significantly reduced the growth of *Penicillium expansum*, the causal agent of blue mould on pears (Mari *et al.*, 2002). Therefore, AITC may have potential as an economically viable alternative to synthetic fungicides.

GLS hydrolysis products may have biocidal activity against a wide variety of organisms such as insects, plants, fungi and bacteria (Tunc *et al.*, 2007; Vaughn and Berhow, 2005; Ware, 2000); some may have human health benefits. ITCs are among the most potent products and are suspected to be the major inhibitors of microbial activity. Benzyl ITC is sometimes used as an antibiotic to treat infections of the respiratory and urinary tract (Mennicke *et al.*, 1988). Bacteriostatic, bactericidal, and fungicidal effects of many ITCs are well documented (Brown and Morra, 2005). The fungicidal property of AITC vapour against wild type and thiabendazole-resistant strains of *Penicillium expansum* has been demonstrated (Tunc *et al.*, 2007; Kiyoshi 2005; Mari *et al.*, 2002). Bacteriocidal effects of mustard flour against *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* have also been documented (Rhee *et al.*, 2003). The use of AITC produced from pure sinigrin or from defatted *Brassica juncea* meal may be an economically viable alternative to synthetic fungicides against *P. expansum*. AITC has been shown to be effective against cheese-related fungi such as *Penicillium commune, P. roqueforti* and *Aspergillus flavus* (Winther and Nielsen, 2006), but the incorporation of AITC in the packing material contributed to unacceptable mustard flavour in cheese.

2.4.2 Herbicidal activity

GLS-containing plants have a reputation for inhibiting the growth of nearby weeds and other crops such as wild oat (*Avena sterilis*), wheat and pea (Jones, 1992). Stands of wild *B. nigra* appeared to inhibit germination of annual grasses, and broccoli (*B. oleracea*) residues amended to soil reduced germination and growth of lettuce for 10-21d (Patrick *et al.*, 1963). *Sinapis alba* seed meal applied to the soil is also known to suppress weed emergence (Ascard and Jonasson, 1991; Johansson, 1992; Johansson and Ascard, 1994; Oleszek *et al.*, 1994). For example, mustard meal or “cake” applied in rows between cole crops with the intent of attracting enemies of the cabbage root fly also reduced numbers of several annual weeds (Ascard and Jonasson, 1991).

2.4.3 As a biofumigant

AITC has been shown to have toxic effects on insects and other invertebrates (Brown and Morra, 2005). Noble *et al.* (2002) demonstrated larval mortality by AITC. The relative toxicity of different GLS hydrolysis products such as ITCs and nitriles is important in maximizing the likelihood of effective pest suppression in pesticide applications (Sarwar *et al.*, 1998). ITCs have been used as biofumigants for the control of soil-borne pests. These products possess potential biodegradable and bio-friendly insect fumigant (Tsao *et al.*, 2002a) and nematocidal properties (Mitarai *et al.*, 1997). They may act on the insect respiratory system in their mode of action. Leaching of ITCs and their degradation in soil has also been studied (Gimsing *et al.*, 2005, 2006 and 2007). The rates of ITC formation and degradation are critical to the toxicity and leaching of GLS and ITCs in soil. Degradation of GLS is much faster in a clay soil (half-life, 3.5-6.8 h) than in a sandy soil (half-life, 9.2-15.5 h). AITC is the most toxic compound formed from allyl GLS (sinigrin) hydrolysis and is possibly most important for biofumigation (Noble *et al.*, 2002). Additionally, ITCs can be lost by volatilization. However, for fumigants, volatilization is significant for spreading in soil. Therefore, volatilization can be a concern for health and environmental reasons.

2.4.4 Nematocidal effects

Nematocidal effects of GLS hydrolysis products on the sugar beet cyst nematode *Heterodera schachii* were shown (Lazzeri *et al.*, 1993). AITC, gluconapin, glucotropeolin and glucodehydroerucin had strong nematocidal effects, whereas GLS alone, glucoraphenin and sinalbin had no toxic effect. The potato cyst nematode (*Globodera rostochiensis*) has been controlled by the use of GLS hydrolysis products (Buskov *et al.*, 2002). They reported 100% mortality by adding active myrosinase to phenethylglucosinolate, benzyl-enylglucosinolate and prop-2-enylglucosinolate.

2.4.5 Anti-cancer properties

The ITCs are known to have cancer-preventive properties (Zhang, 2004; Myzak *et al.*, 2004). Anti-cancer properties of glucosinolates have also been reported (Hu *et al.*, 2007; Thejass and Kuttan, 2007; Hwang and Lee, 2006; Tang *et al.*, 2006; Jakubikova *et al.*, 2005; Smith *et al.*, 2004; Zhang, 2004; Myzak *et al.*, 2004; Thornalley, 2002). For example, suplforaphane [1-isothiocyanoto-4-(methylsulfinyl) butane], a degradation product of the glucosinolate, glucoraphanin, is a potent inducer of phase II detoxification enzymes, which are strongly correlated with the prevention of certain types of cancer (Brooks *et al.*, 2001; Matusheski *et al.*, 2001). Allyl isothiocynate (AITC), benzyl-ITC (BITC), phenethyl-ITC (PEITC), sulforaphane (SFN), erucin (ERN) and iberin (IBN) induce time- and dose-dependant G[2]/M arrest in HL60 cells (Jakubikova *et al.*, 2005). Mustard ITCs are mitotic inhibitors and/or apoptosis inductors, which suggest that they could be chemotherapeutic agents in cells with multidrug resistance phenotypes. Dietary isothiocyanates inhibit the growth of human bladder carcinoma cells (Tang and Zhang, 2004). Tang *et al.* (2006) recently demonstrated that naturally occurring ITCs, including AITC, BITC, PEITC and SFN, potently inhibited the growth of cells in human bladder cancers and drug-resistant bladder cancer cells. They also demonstrated a mechanism for the anti-proliferative property of AITC. The AITC breaks down caspase-3, -8 and -9 which are known inducers of apoptosis and cell arrest, thereby inhibiting apoptosis. Suplforaphane [1-isothiocyanoto-4-(methylsulfinyl) butane], a degradation product of the GLS, glucoraphanin, is a potent inducer of detoxification enzymes, which are strongly correlated with the prevention of certain types of cancer (Brooks *et al.*, 2001; Matusheski *et al.*, 2001).

2.4.6 Toxicity of other GLS hydrolysis products

Other than AITC, gluconapin, glucotropeolin and glucodehydroerucin have been shown to possess strong nematocidal properties against sugar beet cyst nematode *Heterodera schachii* (Lazzeri *et al.*, 1993). GLS-derived nitriles have the ability to increase the phase 2 detoxification enzyme glutathione S-transferase, quinine reductase and glutathione in the mouse (Tanii *et al.*, 2005). Maximum potency was observed in

lung and stomach, which is of interest in light of epidemiological studies demonstrating an inverse association between *Brassica* intake and the incidence of lung and stomach cancers.

2.4.7 Time release of AITC

The beneficial effects of AITC were discussed above. However, the application of AITC in food systems is limited due to its volatility and strong odour which affect the taste of food (Chacon *et al.*, 2006). Its application in food packaging systems is also limited by its existence as an oil form and its volatility. Recently, AITC coupled with α- and β-cyclodextrin has shown potential in the food industry (Li *et al.*, 2007). A controlled release of AITC from the complexes was achieved, which is of great benefit in masking the strong odour, prolonging the antimicrobial time and enhancing the antimicrobial effect of AITC. AITC release was accelerated by increased relative humidity and the release rate of AITC from the α-cyclodextrin-AITC complex was much slower than that from the β-cyclodextrin-AITC complex. This opens many avenues of research with target to the mafucture of storage bags for increased shelf life of fruits and vegetables.

2.4.8 Extraction

Jogdeo *et al.* (2000) demonstrated a steam distillation method for recovery of AITC from mustard meal by using Amberlite XAD-4 adsorbent lining in the condenser. Using a pure AITC standard, recovery was 95% using this steam distillation method. A solvent extraction method for AITC was described by Padukka *et al.* (2000), and was also used by Li *et al.* (2007) and other researchers. Mustard meal was mixed with distilled water and hexane (5:7, v/v) and heated at 85ºC for 20 min using an upright glass condenser. This extraction process was repeated four times.

2.4.9 Quantification

Various methods of AITC quantification have been developed. Some of these methods are described as follows.

2.4.9.1 HPLC

Reverse phase HPLC methods have been explored for the analysis of ITCs (Mathaus and Fiebig, 1996). Later, Jogdeo *et al*. (2000) developed an HPLC method for quantification of AITC using a stationary phase of Lichrospher 100 PR-18. The detector was set at 245 nm and methanol was used as solvent (Jogdeo *et al.*, 2000). In successive years, methods were further improved for better resolution, recovery and detection. Tsao *et al*. (2002b) developed a reverse phase HPLC method for direct and simultaneous detection of sinigrin and AITC in mustard samples. The detection limit for AITC was 0.1µg/mL. A Sphereclone ODS-2 column was employed and the detector was set at 242 nm for AITC and 228 nm for sinigrin. Compounds were separated using a step gradient of mobile phase composed of 0.025 M ammonium acetate (pH 6.75) and acetonitrile (AcN).

2.4.9.2 Colorimetric assay

Recently, a colorimeteric method based on the colour reaction between allyl isothiourea and potassium ferricyanide in dilute acetic acid was developed for the determination of AITC in mustard meal (Mukhopadhya and Bhattacharyya, 2006). The colour developed by this reaction was measured at 600 nm. This method requires 3 h for analysis and can detect AITC in the range of 2.5 to 7.5 µg. In the method described by Li *et al.* (2007), the absorbance of a hexane extract was measured at 248 nm.

2.4.9.3 Ion-pair electrospray mass spectroscopy

Mellon *et al.* (2002) developed a voltage electrospray LC/MS method to analyze GLS in plant extracts. This method has been shown to be effective for all the GLS by many workers (Bennett *et al.*, 2004). A significant concentration of 4-hydroxy-3-indolylmethylglucosinolate was found in the majority of *Brassica* species.

2.4.10 Thermal degradation

It has been shown that AITC is unstable and gradually degrades into other compounds having a garlic-like odour. AITC in aqueous solution, when heated at 100ºC for 1 h, disintegrates into N,N-diallylthiourea, a major degradation product (Chen and

Ho, 1998); other products obtained were diallyl sulphide, diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide, allyl thiocyanate, 3H-1,2-dithiolene, 2-vinyl-4H-1,3-dithiin, 4H-1,2,3-trithiin and 5-methyl-1,2,3,4-tetrathiane.

2.4.11 Heat shock proteins as markers of stress

Heat shock proteins (HSP) play important roles in the tolerance of living organisms to a variety of biotic and abiotic insults (Vierling, 1991; Parsell and Lindquist, 1994; Hamilton and Coleman, 2001). Thus, HSPs appear to be general stress proteins that are involved in the maintenance of proper cell function and, thereby, help in cell survival by facilitating recovery from stress (Vierling, 1991; Parsell and Lindquist, 1994; Downs and Heckathorn, 1998; Guy and Li, 1998; Heckathorn *et al.*, 1998). Most commonly, HSP70, HSP110, HSP52 and small HSPs (for example, HSP27), play vital roles in protecting cells in stressful conditions. Members of the 70 KDa heat shock protein (HSP70) family are ubiquitous in plants, animals and microorganisms. Their structure and function are highly conserved among diverse organisms, from algae to mammals (Wu *et al.*, 1994). HSP70 is involved in preventing protein aggregation and degradation in response to cellular stress caused by an insult. Therefore, HSP70 can be used as a marker of AITC toxicity.

2.5 Research needs

To improve knowledge of AITC production from mustard meal and the biological activity of whole mustard meal, it is important to develop rapid analytical methods. AITC is released by the action of myrosinase on sinigrin and thus it is also important to study the enzymatic production of AITC. This requires the development of methods to inhibit myrosinase activity in mustard meal, while allowing accurate measurement of AITC. It is also important to investigate whether AITC possesses any plant growth regulation properties. Research is needed to develop bioassays for screening AITC bioactivity and toxicity.

Chapter 3: Materials and Methods

3.1 Biological materials

Germination trials were conducted with flax (*Linum usitatissimum* cv. Vimy) seed which was obtained from Dr. Gordon Rowland, Crop Development Centre, University of Saskatchewan, Saskatoon, SK, and tomato (*Solanum lycopersicum* cv. Roma) seed purchased from Early's Home and Garden Centre, Saskatoon, SK. *Caenorhabditis elegans* strain N2 used in the toxicity study was provided by *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health (NIH), the National Centre for Research Resources (NCRR), Bethesda, MD. *Brassica juncea* cv. Arrid seed was obtained from Dr. Kevin Falk, Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon, SK. Seed was produced on plots near Saskatoon in 2006.

3.2 Measurement of AITC in seed meal

Allyl isothiocynate (AITC) in seed meal was quantified using a modified American Spice Trade Association method where AITC is recovered by steam distillation (Figure 3-1). *B. juncea* seed (5g) was ground in a coffee grinder and then extracted in 100 mL of deionized water containing a few drops of anti-foam (Sigma-Aldrich Canada Ltd., Oakville, ON). This mixture was incubated at 37ºC for 2 h with continuous stirring. After adding ethanol (95%, 20 mL) and a few boiling chips, the mixture was distilled in the dark by covering the distillation flask with aluminium foil. Sixty millilitres of distilled solution was collected in a flask containing 10 mL of 33.5%

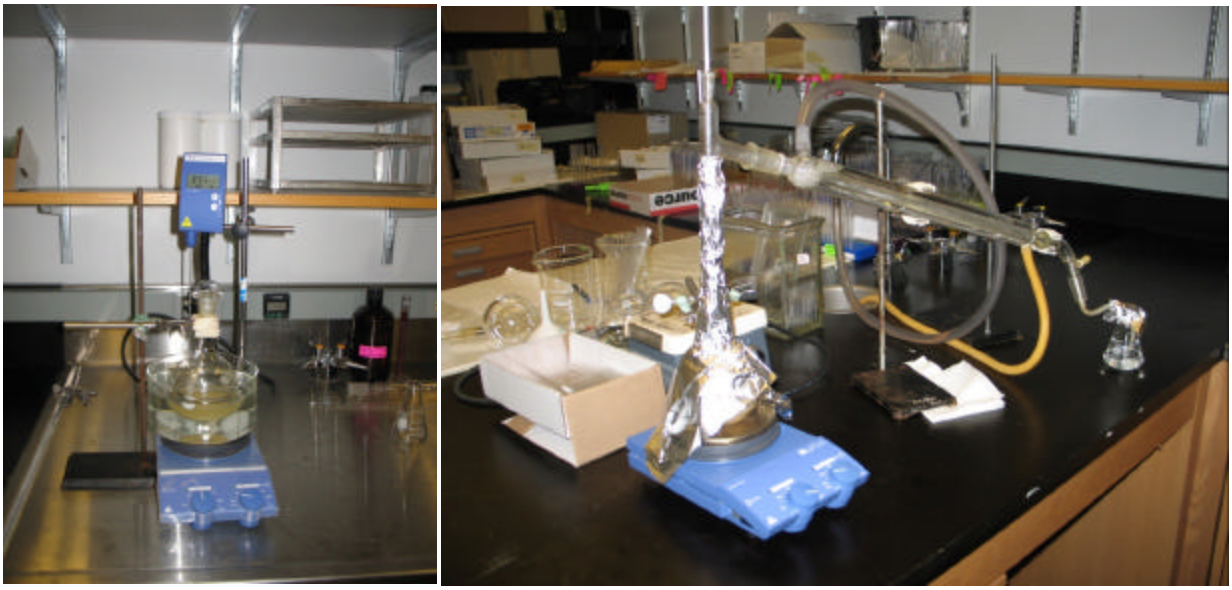


Figure 3-1. Apparatus used for quantification of volatile oil in mustard meal.

ammonium hydroxide solution. To this mixture, 20mL of 0.1N silver nitrate (Sigma-Aldrich) was added and the final volume was adjusted to 100 mL with distilled water before incubating overnight in the dark by covering the flask with aluminium foil. This resulted in the formation of a black precipitate which was removed by filtration with Whatman grade No. 4 filter paper (GE Health Care, Piscataway, NJ). Two titrations were performed, each using 50 mL of this filtrate. Fifty millilitres of the filtrate was acidified with 5 mL of concentrated nitric acid (analytical grade, Sigma-Aldrich) and was titrated with 0.1N ammonium thiocyanate (analytical grade, Sigma-Aldrich) after adding 5 mL of 8% $FeNH_4(SO_4)_2.12H_2O$ indicator (Sigma-Aldrich). Per cent volatile oil was calculated using the formula below:

$$\text{Volatile oil (\%)} = \frac{[(\text{mL} \times \text{N})\ AgNO_3 - 2(\text{mL} \times \text{N})\ NH_4SCN] \times 0.04958 \times 100}{\text{Weight of sample (g)}}$$

3.3 Kinetics of myrosinase

To understand the kinetics of myrosinase activity in *Brassica juncea* seed, 5 g ground seed was extracted in water at different temperatures (7 to 97ºC) for various times (0-5 min at intervals of 30 seconds and 5, 15, 30, 60 and 120 min). Myrosinase activity was determined as percent volatile oil released using the method described in section 3.2.

3.4 Inhibition of myrosinase activity

Efforts were made to inhibit myrosinase activity by altering reaction conditions (pH and incubation temperature). The pH of the reaction mixture was changed by adding sodium hydroxide (NaOH), hydrochloric acid (HCl), acetic acid (CH_3COOH), succinic acid ($C_2H_4(COOH)_2$) or boric acid (H_3BO_3). The pH of the reaction mixture was varied from 1.5 to 13. Further, the effects of calcium chloride ($CaCl_2$) and ethanol (C_2H_5OH) were also studied. Myrosinase activity was determined as percent volatile oil released using the method described in section 3.2. Data presented are averages of four replicates.

3.5 Seed germination

Effects of AITC, IAA and Ethephon (2-Chloroethyl-dioxido-oxophosphorane) on the germination of flaxseed and tomato seed were studied. Flaxseed and tomato seed were treated with different concentrations (0, 0.01, 0.05, 0.1, 0.5 and 1 mM) of AITC, IAA and Ethephon and incubated in the dark. Observations were recorded from day 0 to day 6. Data presented are averages of three replicates of 20 seeds per plate. Each study was replicated twice.

3.6 Maintainance of *C. elegans* cultures

C. elegans N2 strain was grown in sterile plates (10 mm; VWR, Edmonton, AB) on 10% bacteriological agar (Sigma-Aldrich) layered with 1 mL of autoclaved Baker's yeast (1%, w/v). Cultures were incubated in the dark at room temperature and sub-cultured to fresh plates every 15 days. For chemical treatments, two-week-old nematodes from three plates were pooled in a 10 mL sterile tube. Movements of nematodes were examined 30-60 min after each treatment under a microscope at 10X magnification.

3.7 Chemical treatment of nematodes

To study the toxicity or stress effects of AITC, *C. elegans* N2 strain was used as a model system. *C. elegans* was treated with different concentrations (0.001-10 µM) of AITC (Sigma-Aldrich) and incubated in the dark at room temperature for 2 h. Controls were untreated organisms maintained under the same incubation conditions. After treatment and incubation, cultures were centrifuged at 400 x *g* for 10 min at 4°C, and the pellet was quickly rinsed with sterile distilled water and centrifuged at 400 x *g* for 10 min at 4°C. After removing the supernatant, the pellet was stored at -80°C until analyzed further.

3.8 Sequence alignment and phylogenetic study

The GenBank data base was searched to identify sequences of isoforms of HSP70. Amino acid sequences of different isoforms of HSP70 in *C. elegans* viz., HSP70A (GenBank Accession No. M18540), HSP70C (GenBank Accession No.

T15513), HSP70D (GenBank Accession No. P20163) and HSP70F (GenBank Accession No. P11141) were aligned using the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk). The phylogenetic tree was generated using the same software to determine the proximity of these HSPs in *C. elegans.*

3.9 Protein extraction

C. elegans samples stored at -80°C were ground in liquid nitrogen using a sterile mortar and pestle. Ground tissues were lysed in 200 µL of freshly prepared lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride (NaCl), 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL antipain, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 7.5% polyvinylpolypyrrolidone). Extracts were kept on ice and mixed for one minute using a vortex mixture (Mandel Scientific, Guelph, ON) at maximum speed. Lysed tissues were maintained on ice for 30 min with occasional mixing for 30 seconds. The lysate was centrifuged to remove cell debris, and the cell-free lysate was divided into aliquots of 50 µL and stored immediately at -80°C until further use, at which time aliquots were thawed on ice and used within an hour. Aliquots were not reused after thawing.

3.10 Protein quantification

Protein was quantified using the Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA) with albumin from bovine serum (BSA, Sigma-Aldrich) as a standard. Standard 96-well microplates (BD Falcon, Mississuaga, ON) were used in this assay. A standard BSA stock solution of 400 µg/mL concentration was made in sterile distilled water and was diluted in sterile water so as to generate concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL. Unused BSA standards were stored at -20ºC. Standard and sample solutions (10 µL) were added to separate microplate wells and 200 µL of diluted dye reagent was added to each well. After mixing using a multi-channel micropipette, the microplate was incubated at room temperature for 5 min and the optical density of each well was measured using a microplate reader (NOVOstar BMG Labtech Inc., Durham, NC) set to 595 nm absorbance and with the aid of NOVO Star version 1.20-0 software. The average of the duplicate readings for each standard

was used to generate a standard curve by plotting the mean absorbance on the x-axis, and a best fit curve through the points was made using Microsoft Excel (Microsoft Canada Co., Mississauaga, ON). The regression equation and the r^2 value of the slope were generated using this program.

3.11 Qualitative analysis of HSP70 proteins by Western blotting

The level of expression of HSP70 was determined in extracts of control or variously-treated *C. elegans*. Proteins (20µg) from each sample were separated on 12% polyacrylamide gels (BioRad Laboratories) and blotted onto polyvinylidene difluoride (PVDF; BioRad Laboratories) membranes using the apparatus shown in Figure 3-2. Phosphate buffer saline (PBS) was prepared by dissolving NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g) and KH_2PO_4 (0.24 g) in 1 L of distilled water, and the pH was adjusted to 7.4. This buffer was sterilized using a 0.2-micron filter and stored in an autoclaved glass bottle at 4ºC. To prevent non-specific binding, the Hybond ECL PVDF membrane (Amersham Biosciences UK Ltd., Bucks, UK; Product No. RPN1010D) was blocked with 5% skim milk in 0.1% Tween-20 in PBS buffer (PBST; pH 7.4) for 1 h at room temperature and incubated overnight with goat anti-human HSP70 antibody (AbCam, Cambridge, MA) diluted to 1:5,000 with PBST. Unbound antibody was removed by vigorous washings with PBS + 0.2% Tween-20 followed by PBS (pH 7.4). This was followed by 1 h exposure to horseradish peroxidase (HRP)-labeled rabbit anti-goat secondary antibody (1:5,000, v/v; AbCam). Following washings as described above, the antigen-antibody complexes were detected by chemiluminescence (GE Health Care, Piscataway, NJ) and exposed on X-ray films (GE Health Care). Detailed protocols are described below:

Protein gels:

The BioRad Mini gel system (BioRad Laboratories) was used for SDS polyacrylamide gel electrophoresis (PAGE). Glass plates were cleaned with ethanol and the glass assembly was set up on the gel casting system according to the manufacturer's instructions. The compositions of the resolving and stacking gels are provided below:

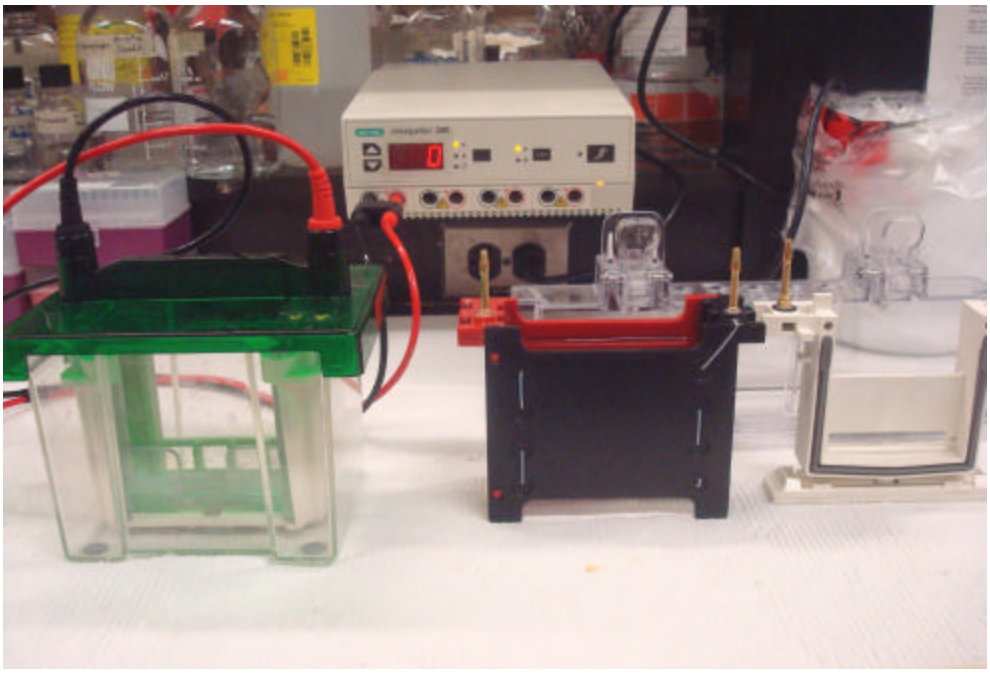


Figure 3-2. Apparatus used for polyacrylamide gel electrophoresis and Western blotting.

Resolving gel (for 20 mL): 5 mL 40% acrylamide (BioRad Laboratories), 5 mL 1.5 M Tris pH 8.8, 200 µL 10% (w/v) sodium dodecyl sulfate (SDS), 10 µL N,N,N',N'-tetramethylethylenediamine (TEMED), 100 µL 10% (w/v) ammonium persulphate (APS) and 9.7 mL sterilized distilled water.

Stacking gel (For 20 mL): 1 mL 40% acrylamide (BioRad), 2.5 mL 0.5 M Tris pH 6.8, 100 µL 10% (w/v) SDS, 10 µL TEMED, 50 µL 10% (w/v) APS and 6.4 mL sterilized distilled water.

Electrophoresis buffer:

Electrophoresis buffer (10 X) was made by dissolving 30.3 g/L Tris base, 144 g/L glycine and 10 g/L SDS in distilled water. The buffer was sterilized by autoclaving (121ºC for 20 min) and stored at room temperature.

Western blotting:

The blotting cassette was placed in blotting buffer to ensure that the pads were completely saturated with buffer. In sequential order, starting from the side facing the positive electrode, a sandwich of pads, filter paper, gel, PVDF membrane, filter paper and pads was assembled. Air bubbles were avoided in preparing the sandwich assembly. The assembled cassette was closed and placed in the electro-blotting tank (BioRad Laboratories). Charge-mediated transfer of proteins was performed at 100 volts for 60 min. After transfer, the cassette was disassembled and the PVDF membrane was removed, trimmed and blocked with blocking buffer (5% (w/v) skim milk in 1X PBST) at room temperature for 60 min. Following three washes with PBS, each for 10 min, goat anti-human HSP70 monoclonal antibody (AbCam) was added at a 1:1000 (v/v) dilution as recommended by the manufacturer. The membrane was incubated overnight at 4ºC with gentle rocking. Unbound antibody was removed by washing three times for 30 min with PBST. This was followed by three rinses x 10 min with PBS. The membrane was incubated with rabbit anti-goat HRP secondary antibody (1:5000, v/v; AbCam) for 60 min and excess unbound antibody was removed by washing with PBST

and PBS as described above. The chemical compositions of the various buffers and solutions mentioned above are shown below:

Blotting buffer: Blotting buffer was made by dissolving 3g/L Tris base, 14.4 g/L glycine and 10% (v/v) methanol in ice-chilled, sterile, distilled water to a final volume of 1 L. This buffer was prepared fresh and stored on ice until used.

10 X Phosphate buffer saline (PBS): PBS buffer (10X) stock was prepared by dissolving 87 g/L NaCl, 22.5 g/L $Na_2HPO_4.2H_2O$ and 2 g/L KH_2PO_4 in distilled water. The pH of the buffer was adjusted with 1 M KOH and then the buffer was autoclaved. PBS buffer was stored at 4ºC. 1X working concentration of the buffer was made by diluting with sterile distilled water prior to use.

PBS-Tween (PBST): Tween-20 (0.25%, v/v) was added to PBS buffer.

Immunofluoroscence detection:

After washing the membrane with PBS, bands were detected using an ECL Western blotting immunofluoroscence detection kit (GE Health Care UK Ltd., Bockinghamshire, UK, Product No. RPN2106) according to the manufacturer's instructions. The membrane was exposed to high performance chemiluminescence 5x7″ X-ray films (GE Health Care, Product No. 28906835) in a dark room and developed.

3.12 Sequence alignments

Based on the information provided by AbCam, antibody peptide was aligned with the HSP70A peptide of *C. elegans* (GenBank accession No. M18540) using web based ClustalW version 1.83 (http://www.ebi.ac.uk). This antibody was raised against a synthetic peptide corresponding to residues near the C terminus of human HSP70 (GenBank accession No. NM_005346). The significance of this work was to show specificity of the commercial HSP70 antibody with its complementary sequences in *C. elegans.*

3.13 Quantification of HSP70 by an Enzyme-Linked Immunosorbant Assay (ELISA)

Enzyme-Linked Immunosorbant Assay is a quantitative sandwich immunoassay. Inducible HSP70 was quantified using a HSP70 ELISA Kit (Stressgen Biotechnologies, Ann Arbor, MI; Product No. EKS-700) according to the manufacturer's instructions. Briefly, a standard curve was made using 0.78-50 ng/mL recombinant human HSP70 and absorbance was measured at 450 nm using a microplate reader (NOVOstar BMG Labtech Inc., Durham, NC). Equal amounts of cell lysate (20 µg total proteins) were loaded into wells coated with an immobilized mouse monoclonal antibody for inducible HSP70 and detected with a rabbit anti-human HSP70 polyclonal antibody. The rabbit polyclonal antibody was subsequently bound by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody. The assay was performed with tetramethylbenzidine (TMB) substrate that resulted in development of a blue colour. The colour development was stopped with acid stop solution which converted the endpoint colour to yellow. The absorbance was read at 450 nm. The HSP70 concentrations of the samples were quantified by interpolating absorbance readings from the standard curve.

3.14 RNA isolation

Total RNA was isolated from *C. elegans* by using the RNeasy mini kit (Qiagen, Mississauga, ON) followed by treatment with RNase-free DNase (Qiagen) following the manufacturer's instructions. Nematodes were disrupted in Buffer RLT® and homogenized by vigorously mixing for 2 min with a vortex mixer at maximum speed following incubation on ice for 20 min. Ethanol was added to the lysate, creating conditions that promoted selective binding of RNA to the RNeasy column. The sample was then applied to the RNeasy Mini spin column, and incubated for 2 min at room temperature before removal of contaminants that were washed away during centrifugation. Total RNA bound to the membrane, and was eluted in 25 µL of RNase-free water. All binding, washing and elution steps were performed by centrifugation at 9000 x *g*, 4ºC, for 1 min.

3.15 Formaldehyde-Agarose Gel Electrophoresis

Integrity of RNA was confirmed by agarose gel electrophoresis. Agarose gels (1%, w/v) were prepared in 1X TAE buffer (2 M Tris base, 5.71% v/v glacial acetic acid and 0.05 M EDTA, pH 8.0) by boiling for one minute. After the gel cooled to 50ºC, formaldehyde (1.2%, v/v) was added maintain single strand nature of RNA. Ethidium bromide (1 µg) was added to 30 mL of agarose gel and the gel was cast in a gel casting tray cleaned with hydrogen peroxide.

One volume of 5X loading buffer was added to 4 volumes of RNA sample (for example, 10 µL of loading buffer and 40 µL of RNA) and mixed thoroughly by pipetting several times. This mixture was incubated at 65°C for 3 to 5 min, chilled on ice, and loaded onto the equilibrated Formaldehyde Agarose gel.

Agarose gel was electrophoresed in 1X formaldehyde gel running buffer at 100 volts for about 20 min using a mini gel electrophoresis system (GibcoBRL, Burlington, ON). A stock of 50X solution of TAE buffer was prepared and was diluted to 1X using sterile distilled water. Gels were viewed under a UV transilluminator.

<u>10X formaldehyde agarose gel buffer:</u>
200 mM 3-[N-morpholino] propanesulfonic acid (MOPS) (free acid)
50 mM sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

<u>1X formaldehyde agarose gel running buffer:</u>
100 mL 10X formaldehyde agarose gel buffer
20 mL 37% (12.3 M) formaldehyde
880 mL RNase-free water

<u>5X RNA loading buffer:</u>
16 µL saturated aqueous bromophenol blue solution
80 µL 500 mM EDTA, pH 8.0
720 µL 37% (12.3 M) formaldehyde

2 mL 100% glycerol

3084 µL formamide

4 mL 10X formaldehyde agarose gel buffer

RNase-free water to 10 mL

Stability: Approximately 3 months at 4°C

3.16 Quantification of RNA

RNA samples were diluted in RNase-free water and the concentration of RNA was quantified by Nano drop spectrophotometer (Thermo Fisher Scientific, Ottawan ON) at 260 nm. An absorbance of 1 unit at 260 nm corresponded to 40 µg RNA/mL. To check the purity of the RNA, the ratio between the absorbance values at 260 nm (for RNA) and 280 nm (for protein contamination) was calculated. Cuvettes were washed with 10% hydrogen peroxide following several rinses with sterile distilled water.

Concentration of RNA (µg/mL) = 40 µg/mL x A_{260nm} x dilution factor

Total amount of RNA = concentration x volume of sample in mL

3.17 cDNA synthesis

mRNA was reverse transcribed at 42ºC for 40 min using the QuantiTect reverse transcription kit (Qiagen) and a mixture of universal oligo dT and random primers according to the manufacturer's instructions. The RNA template was incubated with 1X genomic DNA wipeout buffer for 2 min at 42ºC. Quantiscript reverse transcriptase (with RNase inhibitor), 1X Quantiscript RT buffer and the RT primer mixture were added to the RNA. The mixture was incubated at 42ºC for 40 min to synthesize cDNA. Reverse transcriptase was inactivated by heating the reaction mixture at 95ºC for 3 min. The cDNA was stored at -20ºC until used.

3.18 Determination of *HSP70* transcript levels by semi-quantitative reverse transcriptase PCR (RT-PCR)

In order to determine the changes in HSP70 mRNA levels during AITC exposure, reverse transcriptase PCR (RT-PCR) was performed on the total RNA extracted from *C. elegans* subjected to AITC for different times. The SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen, Burlington, ON) was used for semi-quantitative analysis of the expression of *HSP70A* (Gen-Bank Accession No. M18540) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; GenBank Accession No. X04818). The reactions were performed using the following primer pairs; 5'-ATGAGTAAGCATAACGCTGTT-3' and 5'-ACAGTGTTATGTGGGTTCATG-3' for *HSP70A* and 5'-AACCATGAGAAGTACGAC-3' and 5'-CTGTCTTCTGGGTTGCGG-3' for *GAPDH*. Each reaction was performed using 100 ng of total RNA and reverse transcribed at 55°C for 30 min. After initial denaturation of the cDNA at 94°C for 2 min, 30 amplification cycles (94°C for 15 sec, 57°C for 30 sec, and 68°C for 1 min) were performed, followed by a final extension at 68°C for 5 min. Two units of Platinum Taq DNA polymerase were substituted for the SuperScript III RT/Platinum *Taq* mixture in the reaction. A negative control reaction consisted of all of the components of the reaction mixture except RNA. Reactions were amplified and PCR products were subjected to electrophoresis on a 1% 1X Tris-acetate/EDTA (TAE) agarose gel stained with ethidium bromide and imaged under UV light. The intensity of the amplified bands was analyzed by densitometry of the photograph and all results were normalized to *GAPDH*.

3.19 Electrophoresis of DNA in agarose gels

To resolve the products of PCR, 1% (w/v) agarose gel in 1X TAE buffer was prepared containing 1 µL of ethidium bromide in 30 mL agarose. Agarose gel was subjected to electrophoresis conditions of 1X TAE buffer at 100 volts for about 20 min using a mini gel electrophoresis system (Invitrogen Canada Inc.). A stock of 50 X solution of TAE buffer (2 M Tris base, 5.71% v/v glacial acetic acid and 0.05 M EDTA, pH 8.0) was prepared and was diluted to 1X using sterile distilled water.

3.20 Determination of *HSP70* transcript levels by quantitative real time RT-PCR (qRT-PCR)

Complementary DNA (cDNA) was used for qRTPCR analysis for the expression of *HSP70A* (GenBank Accession No. M18540) gene using the QuantiFast SYBR Green PCR kit (Qiagen; Product No. f204054). *GAPDH* (GenBank Accession No. X04818) was used as the reference housekeeping gene. The reactions were performed using the primer pairs 5'-ATGAGTAAGCATAACGCTGTT-3' and 5'-ACAGTGTTATGTGGGTTCATG-3' for *HSP70A* and 5'-AACCATGAGAAGTACGAC-3' and 5'-CTGTCTTCTGGGTTGCGG-3' for *GAPDH*. A negative control reaction consisted of all of the components of the reaction mixture except the RNA. Real-Time PCR analysis was performed using a LightCycler (Stratagene, La Jolla, CA, Figure 3-3) according to the manufacturer's instructions. The cDNA was denatured at 95°C for 5 min. This was followed by amplification of the target DNA through 45 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 seconds and elongation at 60°C for 30 seconds. Relative expression levels were calculated after correction for expression of *GAPDH*.

3.21 Excision and elution of DNA from agarose gels

To confirm if the correct gene was being amplified by real time qRT-PCR, the amplified product was sequenced. The PCR product was separated on 0.8% low melting agarose. The appropriate band was excised with a sterile scalpel blade while observing with a UV transilluminator. The DNA was eluted and purified from the agarose gel using the QIAprep gel extraction kit (Qiagen) following the manufacturer's instructions. Briefly, 1 volume of gel was added to three volumes of buffer QG and incubated at 50ºC for about 5 to 10 min on a dry hot plate to completely dissolve the gel. The sample was mixed using a vortex mixer (Mandel Scientific) set at maximum speed to further enhance solubility. DNA was precipitated by adding an equal volume of isopropanol. Briefly, 1 volume of gel was diluted with three volumes of buffer QG and incubated at 50ºC for about 5 to 10 min on a dry hot plate to completely dissolve the gel. The sample was mixed using a vortex mixer (Mandel Scientific) set at maximum speed to further enhance solubility. DNA was precipitated by adding an equal volume of isopropanol.

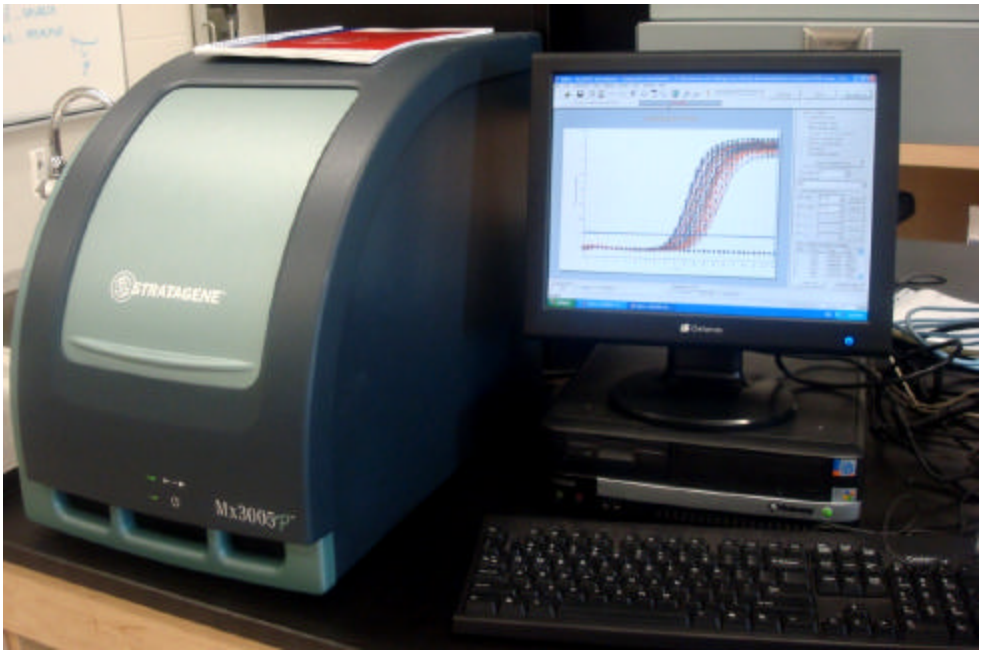


Figure 3-3. Real time PCR machine used for the study.

The contents of the tube were loaded onto a QIAquick spin column and centrifuged at maximum speed for 1 min. QIAquick columns were washed with 500 µL of Buffer QG to remove traces of agarose. Columns were then washed with 500 µL of PE buffer to remove salts, and columns were air dried by spinning for 1 min. The column was then placed in a sterile microfuge tube and the purified DNA was eluted using 50 µL of 10 mM Tris.HCl, pH 8.5. Samples were stored at -20ºC until further use.

3.22 Gene sequencing

Purified DNA was sequenced using an automated gene sequencer. The sequencing PCR reaction mixture (10µL) contained 10 ng of the PCR-amplified product, 2 µL terminator ready reaction mixture, 1X sequencing buffer (80 mM Tris.HCl pH 8.0 and 2 mM $MgCl_2$) and 0.32 pM of primers used to amplify the product. Amplifications were performed in a thermocycler which was programmed for 25 cycles of denaturation at 94ºC for 15 sec, annealing at 56ºC for 5 sec and extension at 60ºC for 2 min. Amplified DNA was precipitated using 95% non-denatured ethanol at room temperature. Before sequencing, the DNA pellet was dissolved in 12µL of template suspension reagent, denatured at 95ºC for 2 min and loaded onto an Automatic DNA sequencer (Applied Biosystems, Foster City, CA).

3.23 Statistical analysis of the data

Data were analyzed using SigmaStat version 3.1 (Systat Software Inc., Chicago, IL). Groups were compared by one-way or two-way analysis of variance (ANOVA) and differences were considered significant at $P<0.05$.

Chapter 4: Results

4.1 Factors affecting myrosinase activity

4.1.1 Kinetics of myrosinase

Myrosinase is known to release allyl isothiocynate (AITC) by acting on sinigrin in the presence of water. AITC contributes to the toxicity of mustard meal. Therefore, inhibiting myrosinase activity might be helpful in reducing AITC toxicity. Attempts were made to improve the "Herb and Spice Method", the only available industrial method to measure total AITC production in mustard meal. This method is imprecise and time consuming (requires 2 h of extraction for the quantification of one sample). To develop an improved method for measuring AITC production in mustard powders, a study of the effect of temperature on the kinetics of myrosinase activity was conducted.

A variety of reaction temperatures (7 to 97ºC) and incubation times (30 sec to 2 h) were studied. The seed meal was continuously agitated at various temperatures for 30 sec to 2 h and total AITC was measured as % volatile oil. A large proportion of the AITC was released in 5 min of incubation at all temperatures below 87ºC (Figure 4-1). Significant inhibition of enzyme activity was observed at all temperatures over 57ºC, and at 97ºC no myrosinase activity was detected. Hence, it was concluded that: 1) myrosinase catalyzed conversion of sinigrin to AITC was a rapid process since detectable amounts of AITC could be found in samples as early as 5 min, and 2) higher temperatures inhibited myrosinase activity. Incubation time was reduced to less than 5 min in a subsequent investigation of enzyme kinetics. No AITC was found at zero incubation time. AITC yield increased with time and attained a maximum at 5 min. Thus, an incubation time of 5 min at 27ºC (Figures 4-2 and 4-3) was used for all subsequent studies.

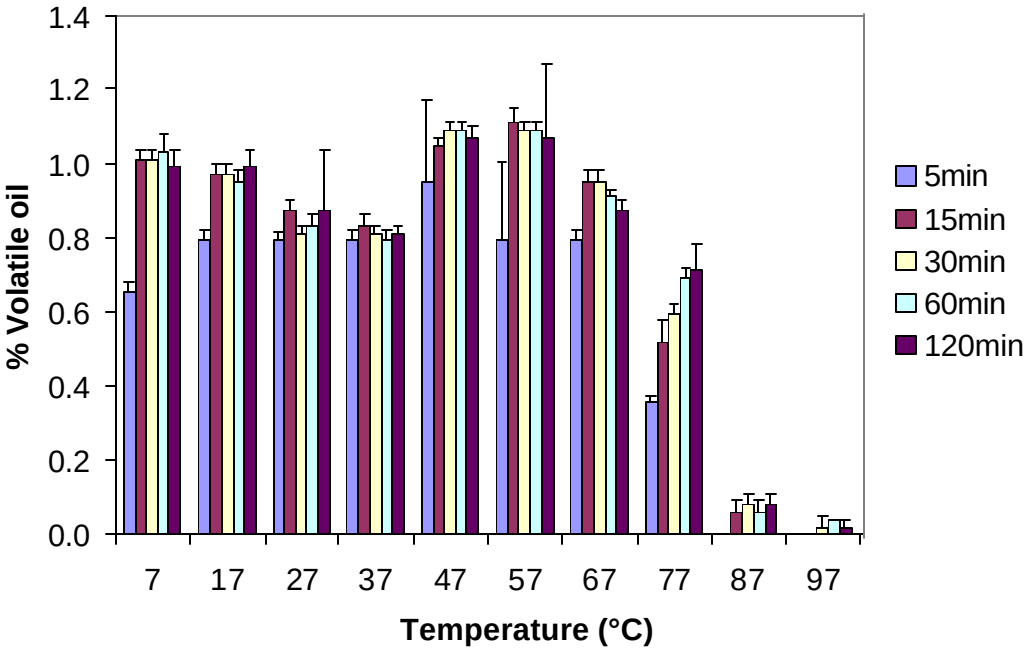


Figure 4-1. Effect of temperature and incubation time on the yield of AITC, which is a measure of myrosinase activity.

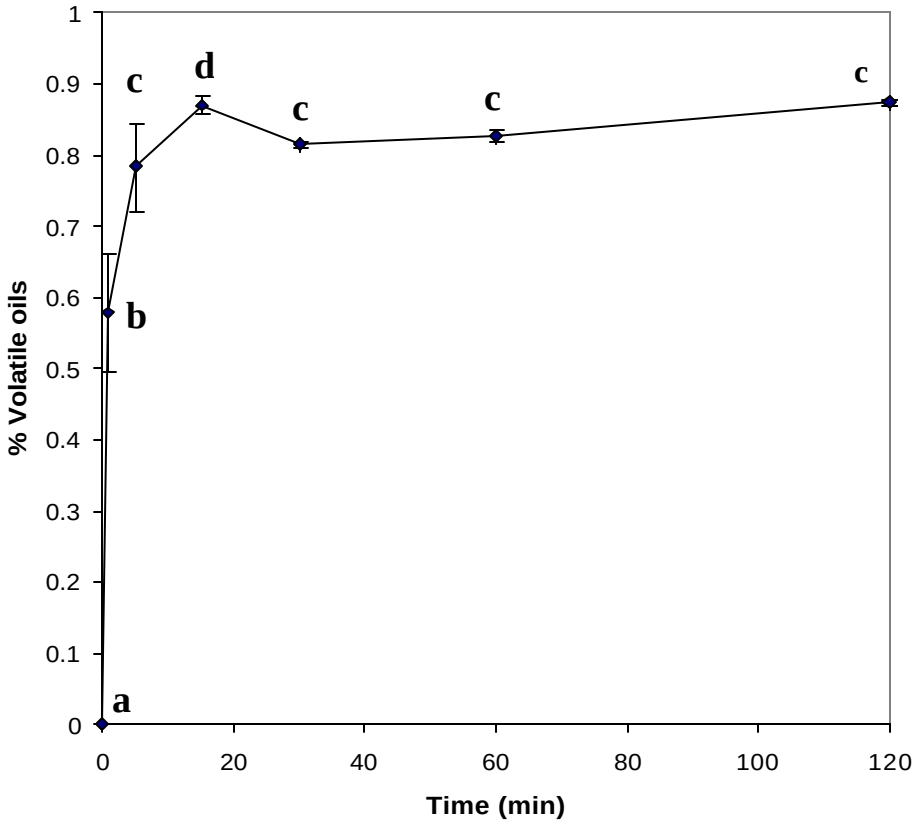


Figure 4-2. Myrosinase attains near-maximum activity in 5 min at 27°C. Data was analysed by one way ANOVA. Values marked with different letters are significantly different from each other ($P<0.05$).

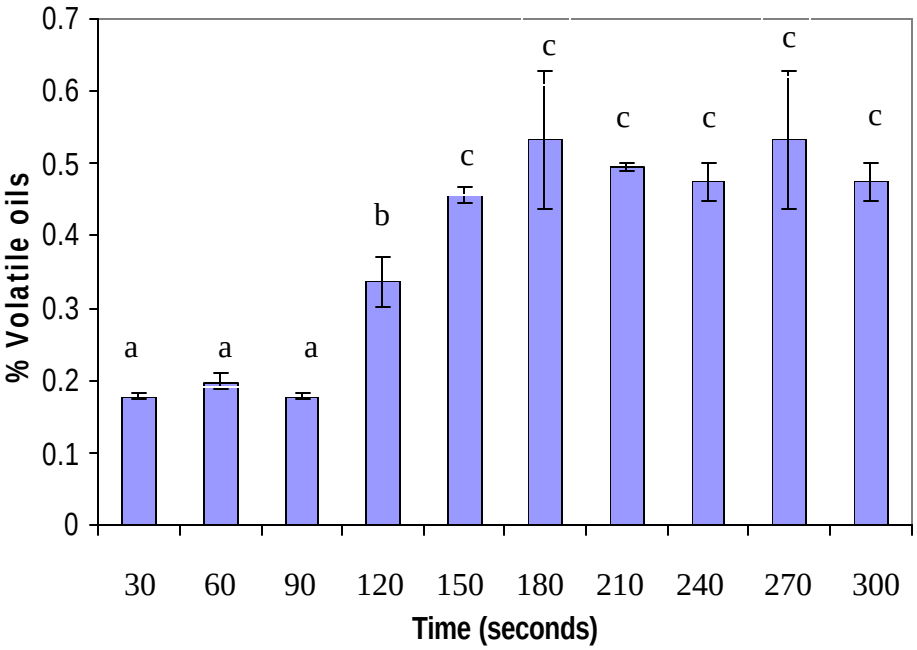


Figure 4-3. Myrosinase activity measured as % volatile oil at 27 ºC.

4.1.2 Inhibition of myrosinase activity in mustard meal

To study reaction kinetics in response to temperature, pH and other factors, it was necessary to have an effective method of starting and stopping the reaction. It was known that pH could significantly alter the rate of myrosinase activity, and a study was conducted to determine the effect of pH on myrosinase kinetics.

Enzyme activity was determined at pH ranging from 1.5 to 13, and the results were compared with the control (water at pH 6.0). The reaction was conducted at 27°C for 5 min. An increase or decrease in pH resulted in a reduction in enzyme activity as compared to the control (Table 4-1), indicating that the enzyme had optimal near-neutral conditions. When the reaction pH was brought back to 6.0 in the mixture containing acetic acid or NaOH, further AITC was released due to residual enzyme activity, but the treatment initiated in the presence of HCl did not exhibit residual enzyme activity. Further, the addition of 1 g of ground *Sinapis alba* seed (a source of enzyme, but devoid of substrate i.e., sinigrin) to the neutralized treatments resulted in the release of AITC, presumably from unreacted sinigrin. Thus, it may be concluded that the change in pH did not affect the substrate but severely affected the activity of myrosinase.

Furthermore, different concentrations of HCl were used to inhibit the activity of myrosinase. Two milliliters of 2 N HCl per 100 mL reaction mixture (yielding a final HCl concentration of 0.04 N) significantly inhibited myrosinase activity compared to the control (Figure 4-4). Results were significant at $P<0.05$.

Other compounds viz., H_3BO_3, $C_2H_4(COOH)_2$, $CaCl_2$ and C_2H_5OH were explored for their ability to inhibit myrosinase activity. The addition of $C_2H_4(COOH)_2$, calcium chloride or ethanol significantly reduced myrosinase activity (Figure 4-5). One-way ANOVA revealed significant differences in all treatments ($P<0.05$). Boric acid slightly increased myrosinase activity.

Table 4-1. Effect of pH reaction on the yield of AITC.

Alkali/Acid	pH	% volatile oil	Neutralize to pH 6.0	Addition of 1 g powdered *Sinapis alba*
			% volatile oil	% volatile oil
Water (Control)	6.0	0.793	--	0.821
NaOH	13	0.079	0.158 (HCl)	0.872
CH_3COOH	3.0	0.039	0.178	1.150
HCl	1.5	0.118	0.079	1.189

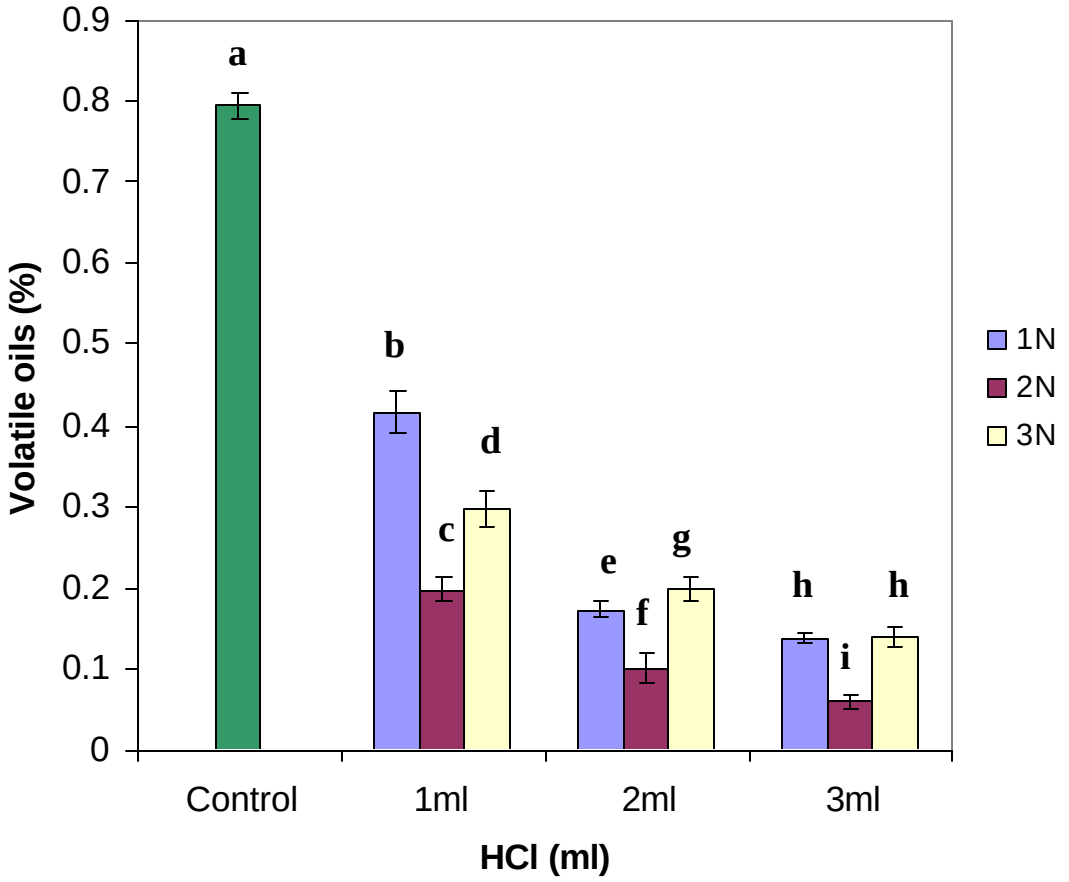


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Figure 4-4. Inhibition of myrosinase activity by HCl.

Data was analysed by two-way ANOVA. Values marked with different letters are significantly different from each other ($P<0.01$).

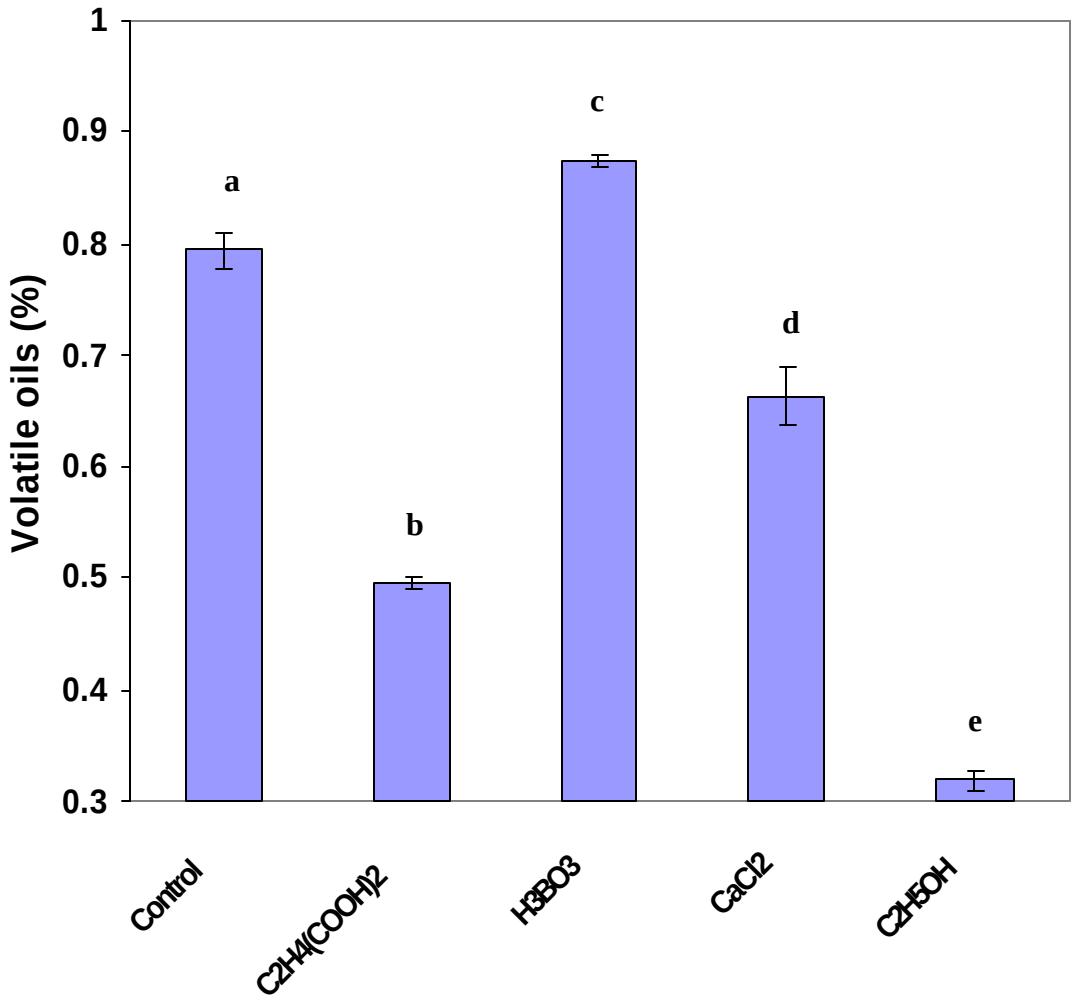

Figure 4-5. Effect of different compounds on myrosinase activity.

Values are the average of four replicates per treatment. Data were analysed by one-way ANOVA. Values marked with different letters were significantly different from each other ($P<0.05$).

4.2 Biological activity of AITC

In the present study, it was hypothesized that AITC might act as a plant growth promoter/regulator based on the structural similarity of AITC to the plant growth regulator, ethylene, exhibit structural similarity (Figure 4-6). Therefore, AITC might act as an ethylene analogue. AITC and ethylene share a common general formula which is R-CH=CH_2, where R is -CH_2SCN and -H in AITC and ethylene, respectively. Ethylene is a well known plant growth regulator known to promote seed germination and to overcome seed dormancy in a dose- and species-dependent manner (Esashi *et al.*,, 1988; Abeles *et al.*, 1992; Kepczynski *et al.*, 1997; Nascimento 2003). Therefore, it was hypothesized that AITC might promote seed germination. Flaxseed and tomato seed were used as model systems to test the germination promoting properties of AITC, along with Ethephon® and IAA.

4.2.1 Effect of AITC on the germination of flaxseed

The effects of different concentrations (0 to 1 mM) of AITC, IAA and Ethephon® were tested on the germination of flaxseed. Observations were taken every 24 h up to 6 days after treatment, and the data presented are averages of three replicates of 20 seeds per treatment. Higher concentrations (0.1 to 1 mM) of AITC showed stimulatory effects and promoted flaxseed germination (Figure 4-7). The effect of Ethephon® (0.5 mM) was comparable to the control; all other concentrations of Ethephon® tested inhibited flaxseed germination (Figure 4-8). All concentrations of IAA tested inhibited germination of flaxseed as compared to the control, and concentrations higher than 1 mM completely inhibited germination (Figure 4-9). IAA is known to inhibit lettuce seed germination (Khan and Tolbert, 1966).

Growth of seedlings in AITC was comparable to that of the control, whereas Ethephon® and IAA inhibited seedling growth (Figure 4-10). It may be concluded that AITC at a higher concentration (1 mM) promoted flaxseed germination.

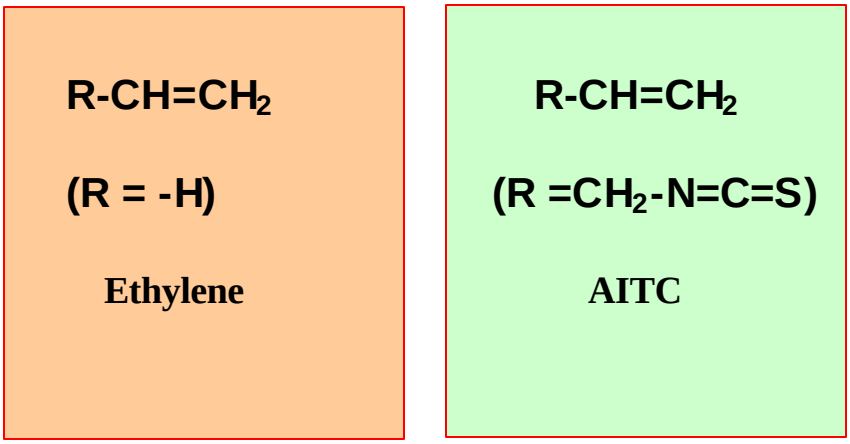

Figure 4-6. Formulas of AITC and ethylene.

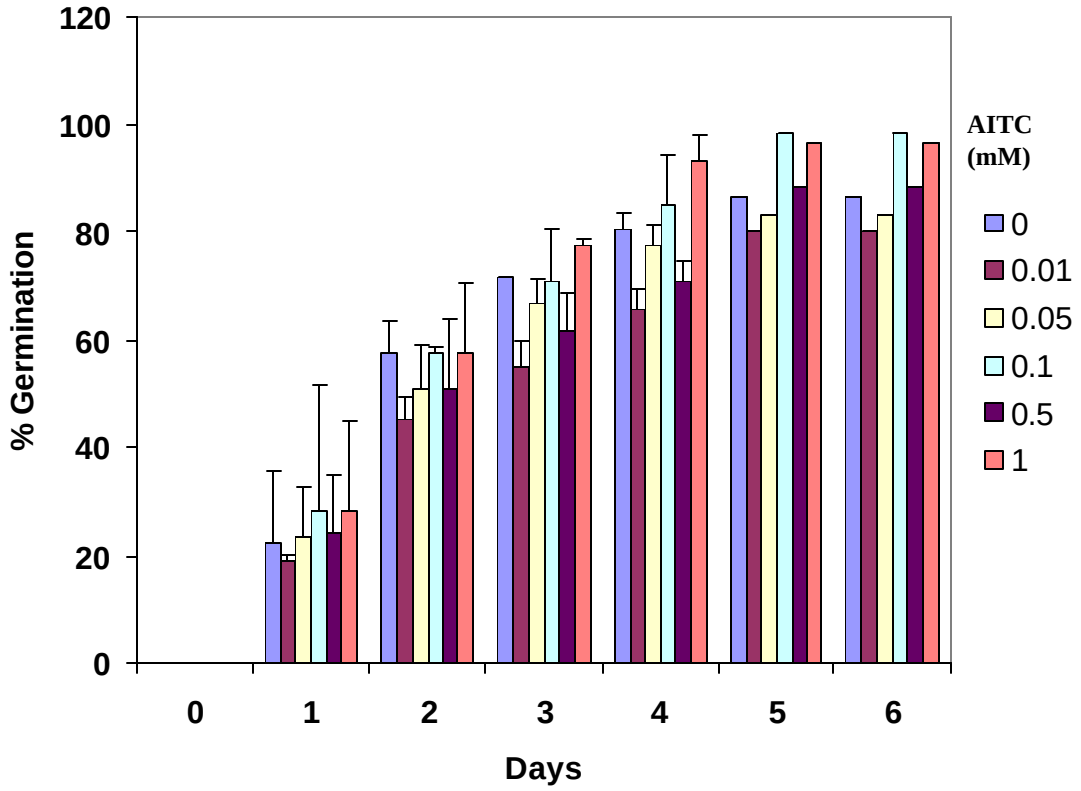

Figure 4-7. Effect of AITC on the germination of flaxseed.
Values are averages of six replicates per treatment.

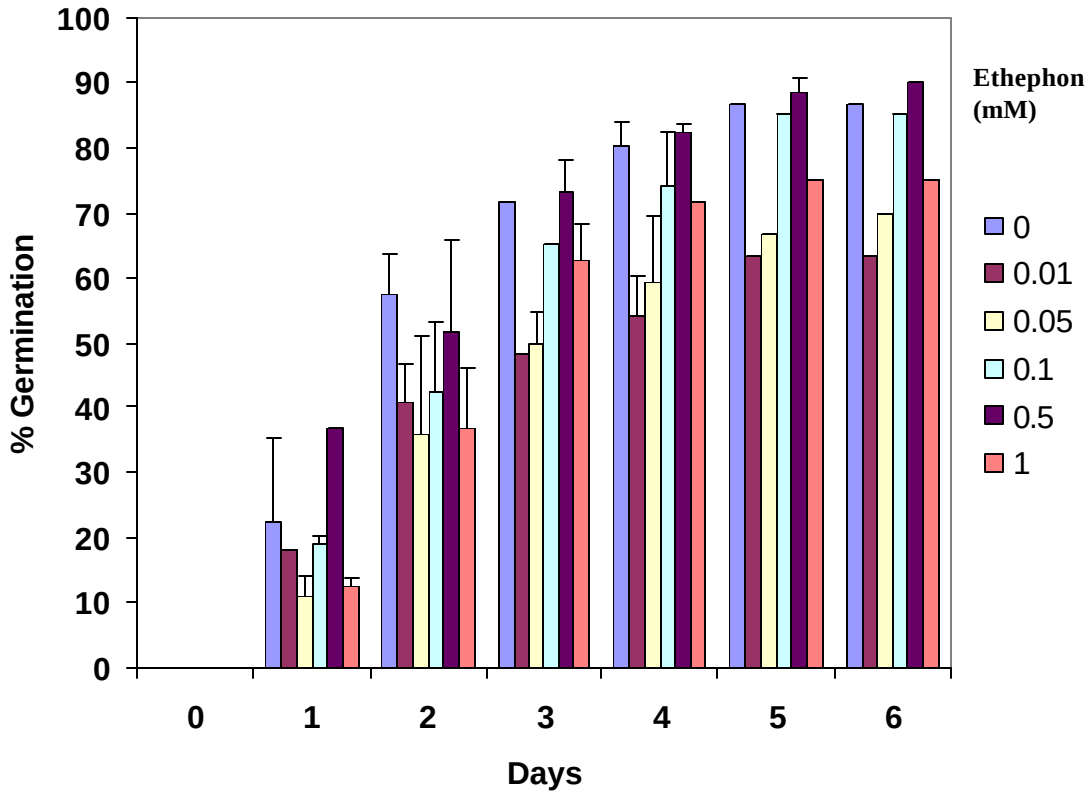

Figure 4-8. Effect of Ethephon® on the germination of flaxseed.
Values are averages of six replicates per treatment.

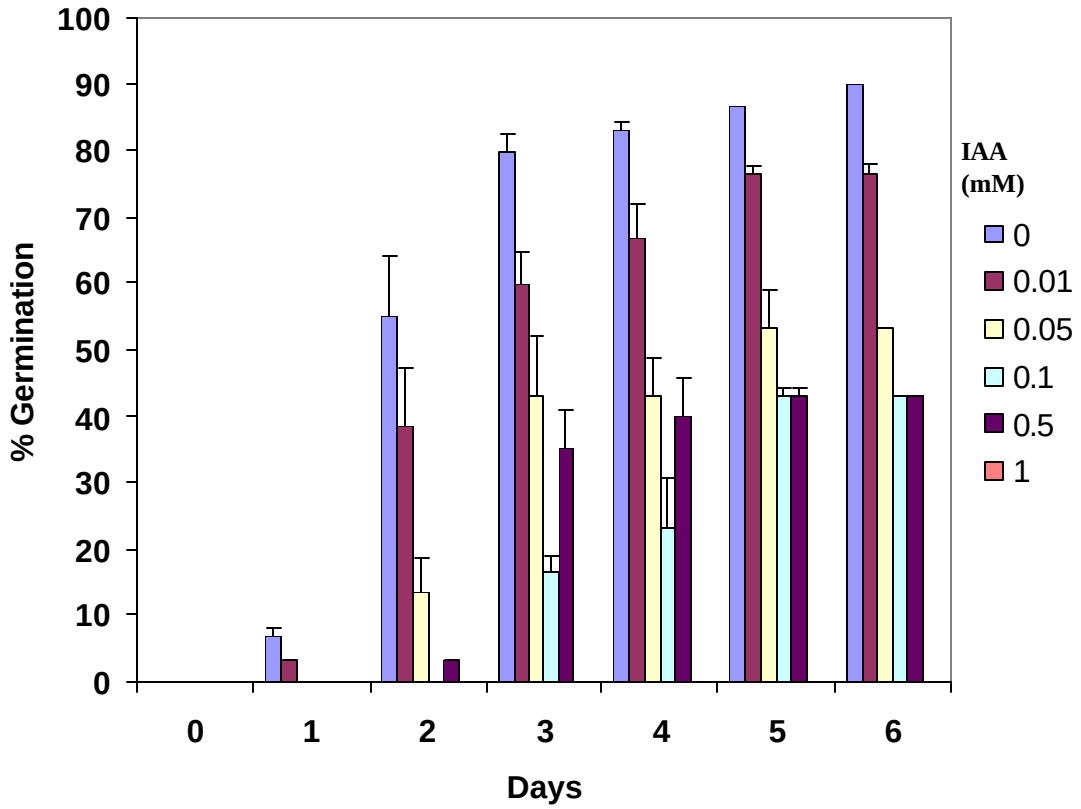

Figure 4-9. Effect of IAA on the germination of flaxseed.
Values are averages of six replicates per treatment.

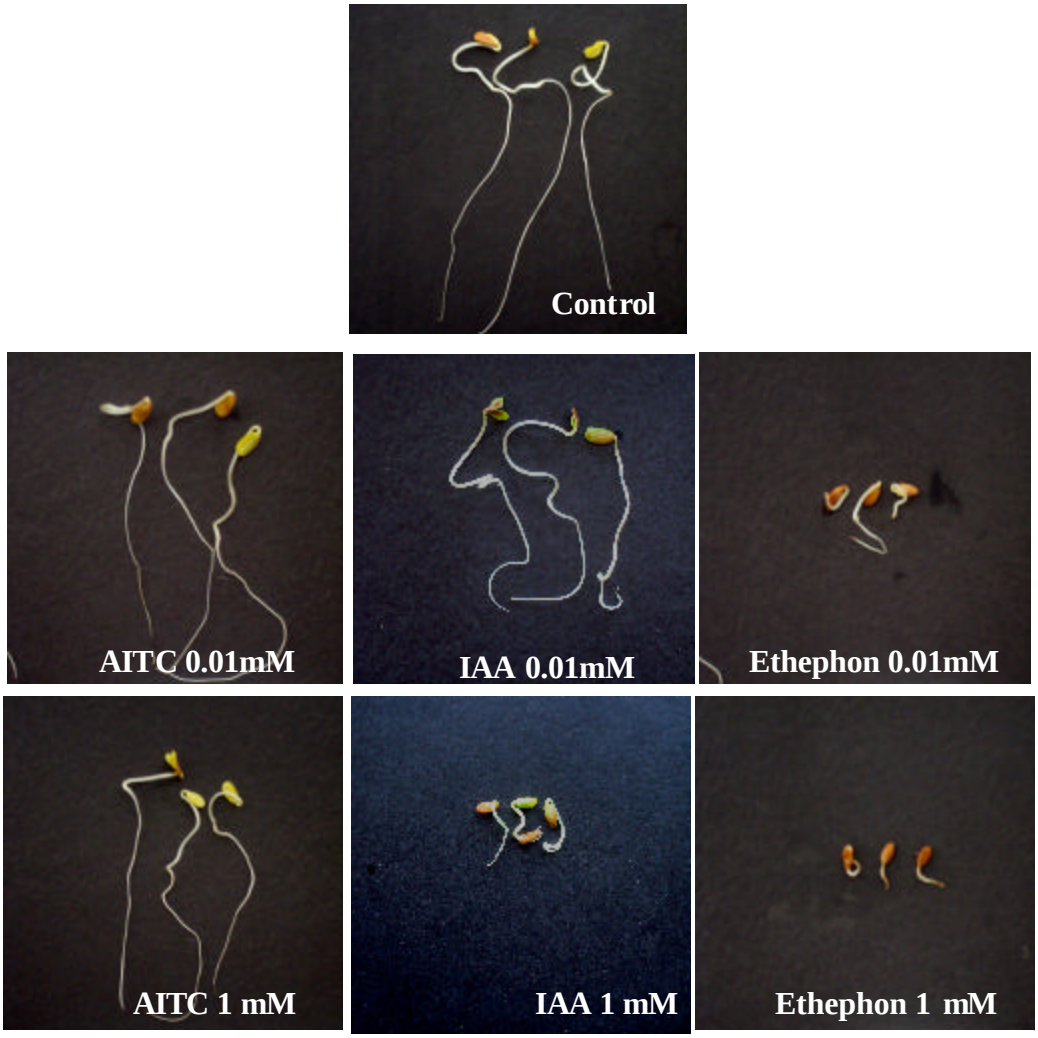

Figure 4-10. Ten-days-old flaxseedlings grown in different concentrations of IAA, Ethephon® and AITC.

4.2.2 Effect of AITC on the germination of tomato seed

To further evaluate the effect of AITC on seed germination, the effects of different concentrations (0 to 1 mM) of AITC, IAA and Ethephon® on the germination of tomato seed were tested. Observations were taken from 0h to 6 days after treatment, and data presented are averages of three replicates of 20 seeds per treatment. Concentrations of AITC higher that 0.1 mM enhanced seed germination and lower concentrations were comparable to the control (Figure 4-11). Most concentrations of Ethephon® tested had an inhibitory effect on tomato seed germination (Figure 4-12). Unlike with flaxseed, lower concentrations (0.01 to 0.1 mM) of IAA enhanced tomato seed germination (Figure 4-13). Growth of seedlings was inhibited by Ethephon® and IAA as compared to AITC and the control (Figure 4-14). Therefore, it was concluded that AITC enhanced seed germination and thus might have use in agricultural practice.

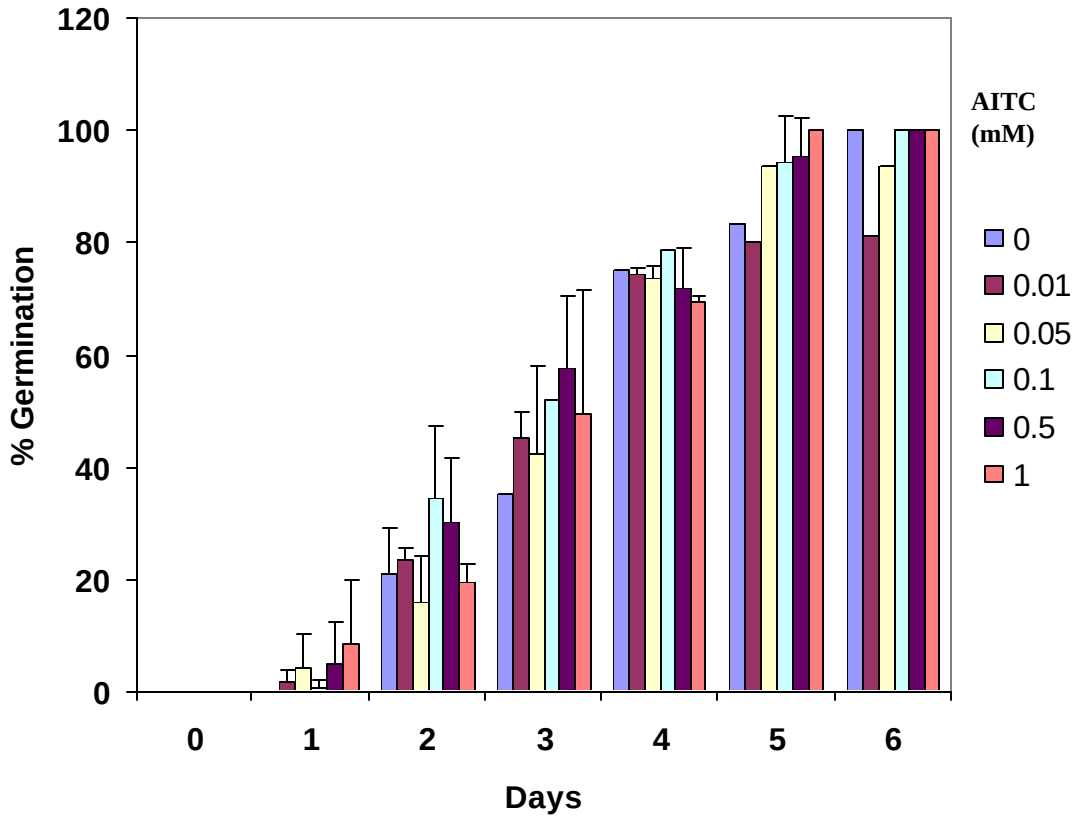

Figure 4-11. Effect of AITC on the germination of tomato seed.
Values are the average of six replicates per treatment.

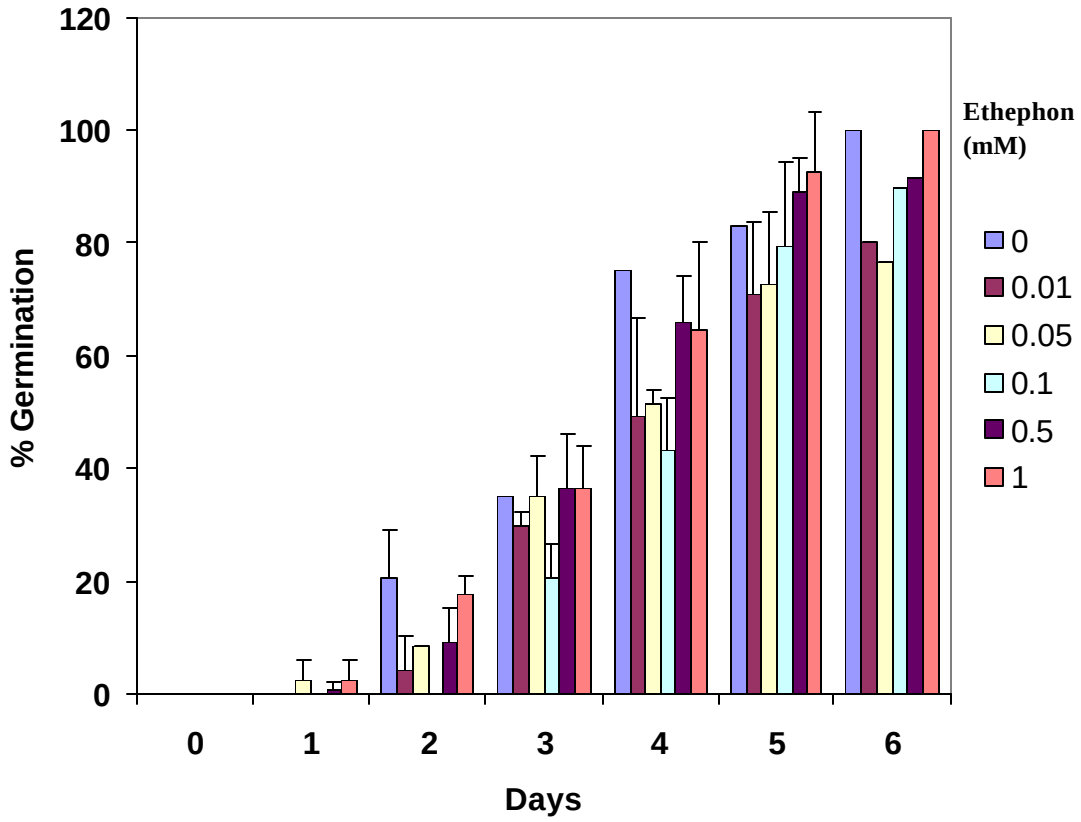

Figure 4-12. Effect of Ethephon® on the germination of tomato seed.
Values are averages of six replicates per treatment.

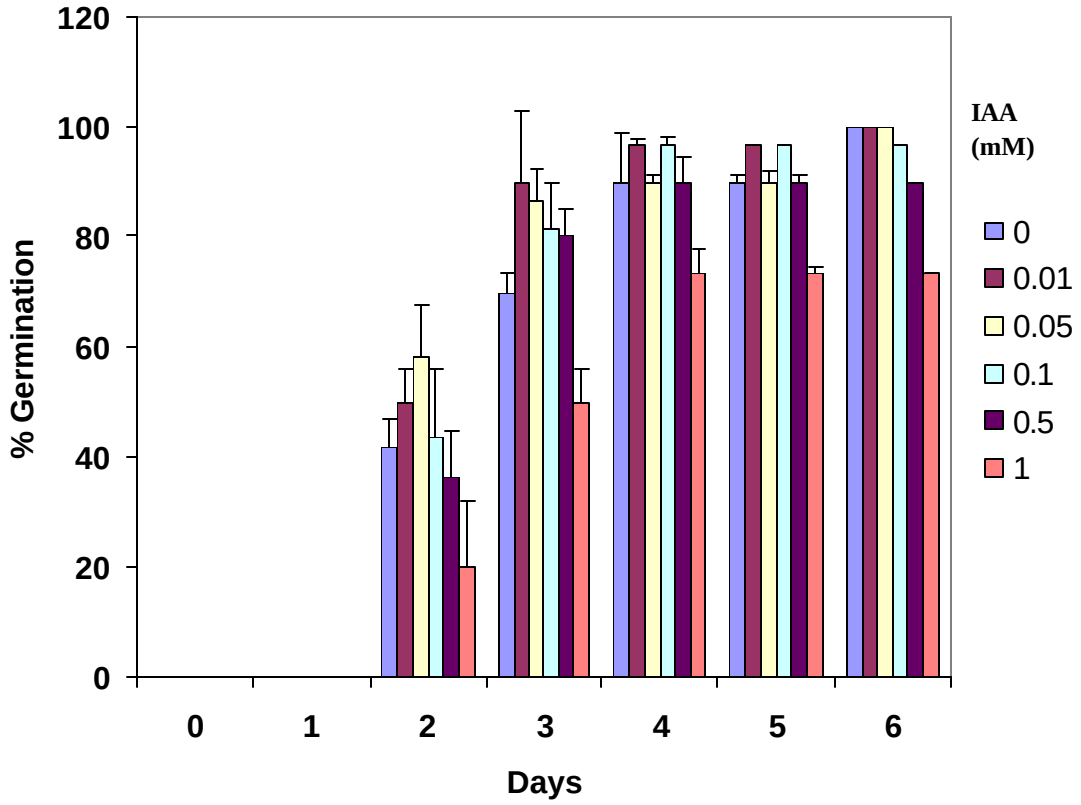

Figure 4-13. Effect of IAA on the germination of tomato seed.
Values are averages of six replicates per treatment.

Figure 4-14. Ten-days-old tomato seedlings grown in different concentrations of IAA, Ethephon® and AITC.

4.3 HSP70 as an indicator of stress/toxicity in *C. elegans*

Heat shock proteins play important roles in the tolerance of living organisms to a variety of biotic and abiotic stresses (Vierling, 1991; Parsell and Lindquist, 1994; Hamilton and Coleman, 2001). Thus, HSPs appear to be general stress proteins that are involved in maintenance of proper cell function, and thereby help in cell survival during stress by facilitating recovery from stress (Vierling, 1991; Parsell and Lindquist, 1994; Downs and Heckathorn, 1998; Guy and Li, 1998; Heckathorn *et al.*, 1998). Members of the 70 kDa heat shock protein (HSP70) family are ubiquitous in plants, animals and microorganisms, and their structure and function are highly conserved among diverse organisms, from algae to mammals (Wu *et al.*, 1994). HSP70s are involved in preventing protein aggregation and in refolding of denatured proteins produced in response to cellular stress caused by an insult. In the present research, methods of screening AITC toxicity were developed and the following questions were addressed:

1. How different isoforms of HSP70 in *C. elegans* are phylogenetically related?
2. Does AITC induce expression of HSP70?
3. Is toxicity contributed by AITC alone or is there synergistic effects of other components of mustard seed meal?

4.3.1 Sequence alignments and phylogenetic relationships among HSP70s in *C. elegans*

The HSP70 multigene family in *C. elegans* has been characterized (Heschl and Baillie, 1998). Bioinformatic studies were done on the different forms of HSP70 viz., HSP70A, HSP70C, HSP70D and HSP70F, to understand level of sequence identity and phylogenetic relationships. These isoforms of HSP70 showed high homology (Figure 4-15), but they have been shown to perform different functions (Table 4-2). The phylogenetic tree made with Clustal-W showed that among the various isoforms of HSP70 in *C. elegans*, HSP70C and HSP70D were the most closely related (Figure 4-16). Phylogenetic analysis showed that HSP70A and HSP70F of *C. elegans* are distantly related. Because HSP70A is a cytoplasmic protein known to be expressed in response to stress or toxicity, further studies were focused on HSP70A.

Figure 4-15. Sequence alignment of HSP70A, HSP70C, HSP70D and HSP70F showing high identity in peptide sequences.

Table 4-2. Accession numbers of various known HSP70s in *C. elegans* and their Functions (Heschi and Baillie, 1998).

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Gene	Accession No. (NCBI)	Function
HSP70A	M18540	Expressed in response to variety of stresses
HSP70C	T15513	Expressed in the Endoplasmic reticulum (ER), not increased in response to heat stress
HSP70D	P20163	Prevents secretion from the ER
HSP70F	P11141	Expressed in the mitochondria

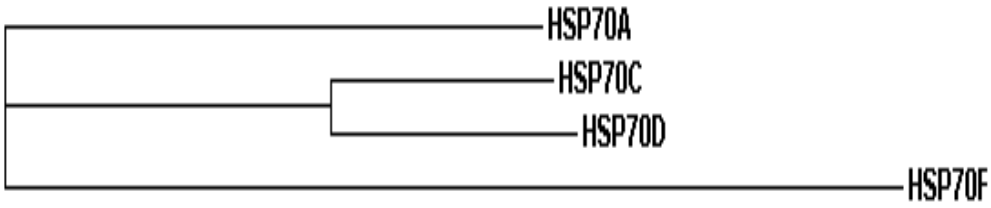

Figure 4-16. Phylogenetic relationships among various HSP70s in *C. elegans*.

4.3.2 Expression of HSP70 protein as an indicator of stress

Western blotting is a technique to understand the accumulation of a protein in response to treatments, because the protein of interest can be detected in whole extracts by using a protein specific antibody. Western blotting can also be used to confirm the size of the protein of interest.

4.3.2.1 Expression of HSP70A transcripts in response to AITC

It is important to understand the transcription of genes within the HSP70 domain. The bioinformatic research discussed above provided a reason for selecting one of the various isoforms of HSP70 in *C. elegans.* Therefore, expression of HSP70A transcripts in response to AITC treatment was investigated. Preliminary trials were performed using reverse transcriptase polymerase chain reaction (RT-PCR) technique.

4.3.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

The heat-shock-induced accumulation of *HSP70A* transcripts is shown in Figure 4-17. In order to determine the level of *HSP70A* transcripts, RT-PCR was conducted on total RNA isolated from *C. elegans* exposed to different concentrations of AITC for 1 h. Although *HSP70A* transcripts were constitutively present at low levels, there was a considerable increase in expression at all AITC concentrations over 1 µM (Figure 4-17). Based on densitometeric scoring of bands obtained by RT-PCR, the maximum level of *HSP70*A transcription was observed in *C. elegans* exposed to AITC at a concentration of 5 µM. A slight decline in the expression of *HSP70A* at the highest concentration (10 µM) of AITC might be due to its lethality. No change in the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene, was observed, which indicated that the same amount of RNA was taken in each sample. These results confirm that AITC exposure increased the expression of the *HSP70A* gene in *C. elegans.*

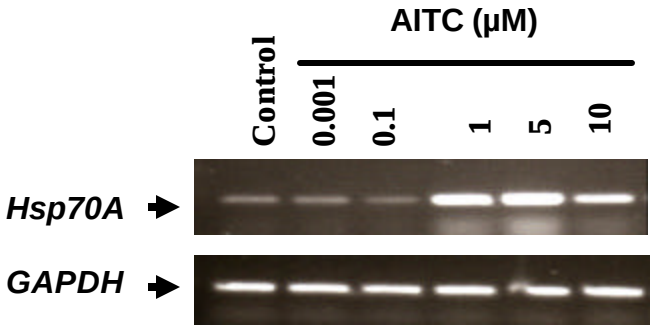

Figure 4-17. RT-PCR showing the expression of *HSP70A* (Accession No. M18540, NCBI) in response to stress caused by different concentrations of AITC.
GAPDH (Accession No. X04818, NCBI), a house keeping gene was used as control.

4.3.2.3 Quantitative real time RT-PCR

Real time RT-PCR is one of the most sensitive techniques available to quantify levels of gene transcripts in response to a treatment. The *GAPDH* gene was used as the reference housekeeping gene and relative expression levels were calculated after correction for expression of *GAPDH.*

Specificity of the primer sets was evident from higher annealing temperature i.e. 58°C and 60°C for forward and reverse primers, respectively (Table 4-3). These primers were 21 bases long. Higher temperature and longer length of primers are two well known factors that determine specificity. Alignment of nucleotide sequences and position of these primers (Figure 4-18) also showed specificity of primers for *HSP70A*. Specificity of primers was confirmed by sequencing amplified product which was identical to *HSP70A* transcript (Accession No. M18540).

Fluorescence (dRn) values of the SYBR green and normalizing ROX obtained for *GAPDH* and *HSP70A* from a single sample when plotted against number of amplification cycles showed showed a sigmoid curve (Figure 4-19).

No significant change in the levels of *HSP70A* transcripts over the control was observed at concentrations of AITC of 0.1 µM or lower (Figure 4-20). However, *C. elegans* treated with higher concentrations of AITC (1 to 10 µM) responded with a 4- to 5-fold increase in expression of *HSP70A* transcripts over the control. These results clearly indicate that AITC causes stress in *C. elegans* and *HSP70A* could be used as an indicator of this property of AITC.

Table 4-3: Properties of primers used for real time RT-PCR.

Gene	Primer sequences (5’ to 3’)	Primer length	% GC	Tm (°C)	Product size
HSP70A	**Forward:** ATGAGTAAGC ATAACGCTGTT	21	38.09	58	200 bp
	Reverse: ACAGTGTTATGTGGGTTCATG	21	42.85	60	

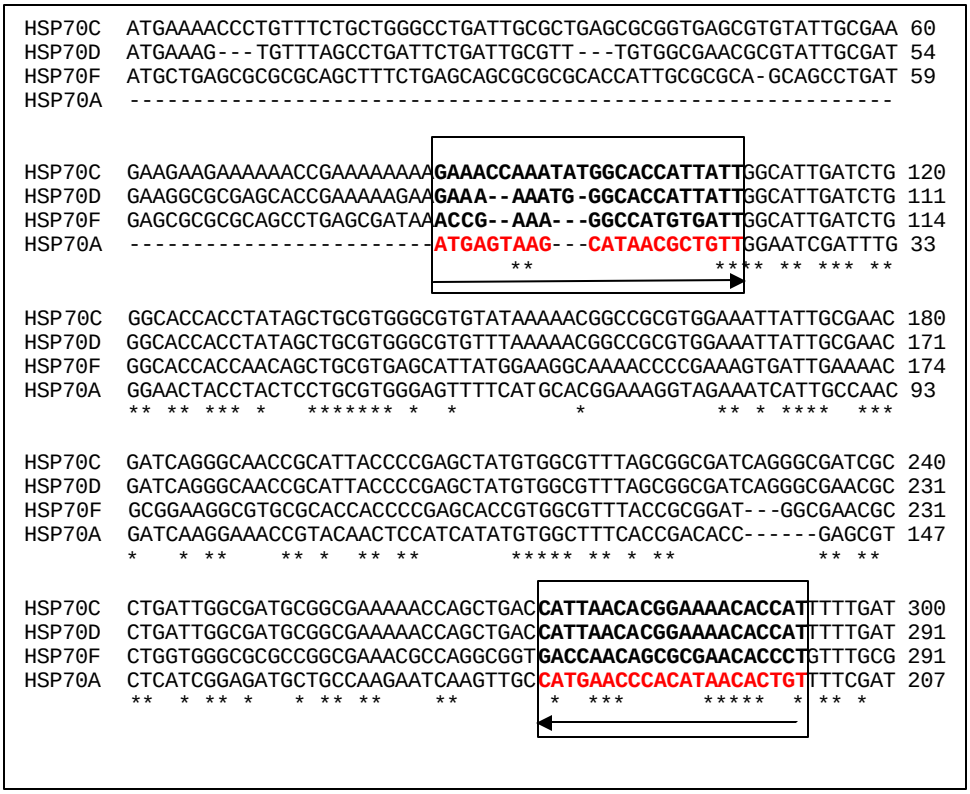

Figure 4-18. Nucleotide sequence alignment of various isoforms of *HSP70A* and position of primers for the amplification of *HSP70A* showing primer specificity.

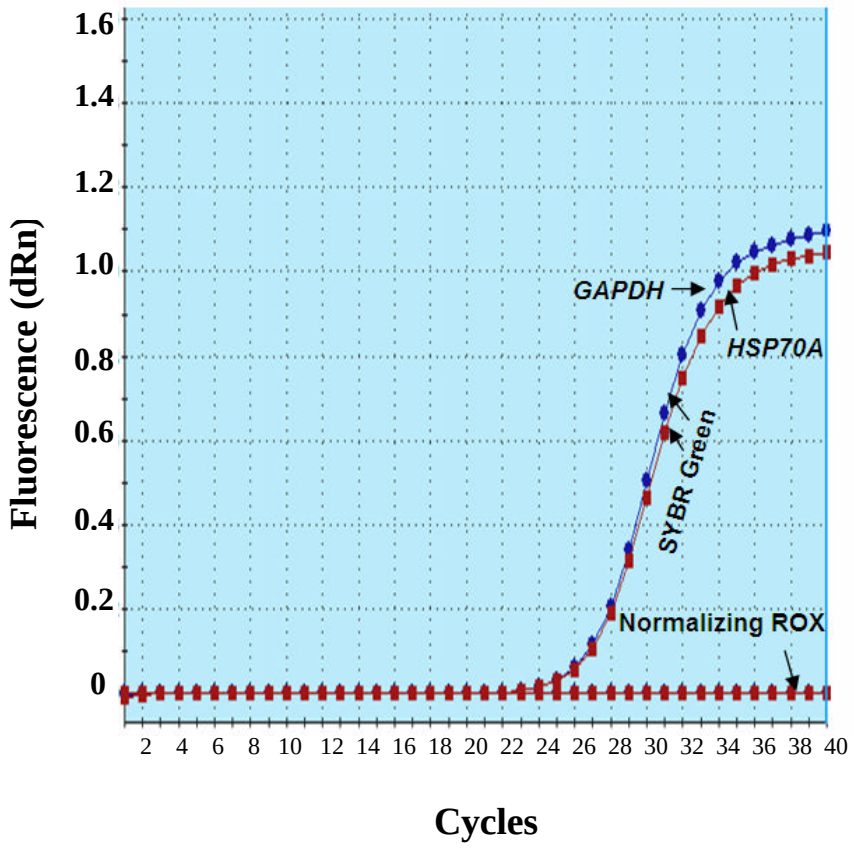

Figure 4-19. Fluorescence (dRn) values of the SYBR green and normalizing ROX obtained for *GAPDH* and *HSP70A* from a single sample showed a sigmoid curve.

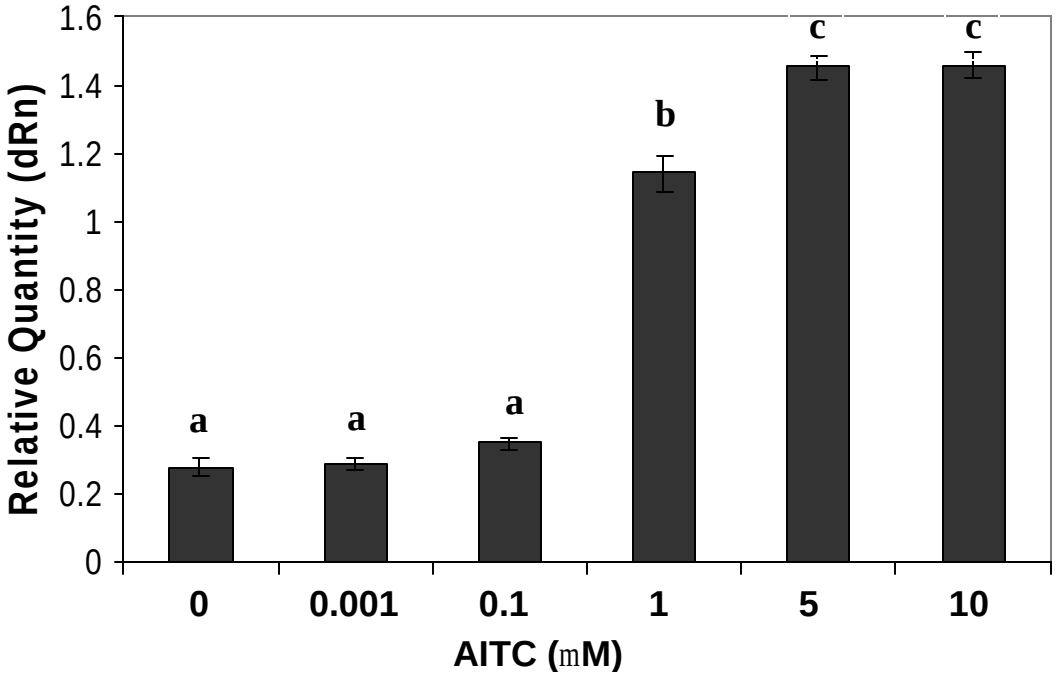

Figure 4-20. Real time RT-PCR for the expression of *HSP70A* in response to stress caused by different concentrations of AITC.

C. elegans was treated with different concentrations of AITC (0-10 μ M) for 2 h at room temperature. Real time RT-PCR was performed to amplify *HSP70A*. Bars on the columns represent standard deviations of three values. Data was analysed by one-way ANOVA. Values marked with different letters were significantly different from each other ($P<0.01$).

4.3.2.4 Western blotting

Although the transcription of the *HSP70A* gene in response to AITC was confirmed by real time RT-PCR, all the transcripts are not necessarily translated to mature, completely functional protein. Therefore, it was necessary to confirm the expression of HSP70 proteins using Western blotting.

C. elegans was exposed to different concentrations of AITC (0-20 µM) for 2 h at room temperature and the expression of HSP70 protein was investigated by Western blotting followed by immunoblotting with anti-HSP70 antibody. The specificity of the anti-HSP70 monoclonal antibody to the HSP70 protein in *C. elegans* was tested by sequence alignment. Based on the information provided by AbCam, antibody was raised against a synthetic peptide corresponding to residues near the C-terminus of human HSP70 (GenBank accession No. NM_005346). Precise sequences against which antibody was raised is their commercial confidfentility. Then, human HSP70 amino acid sequences were aligned with HSP70A (GenBank accession No. M18540) in *C. elegans* by using web-based ClustalW version 1.83 software (EMBL-EBI). Results showed 80% identity in these sequences (Figure 4-21).

Western blotting followed by immunodetection with anti-HSP70 antibody revealed the expression of a 70 kDa band that represents HSP70 protein. These results clearly showed accumulation of HSP70 protein in *C. elegans* exposed to concentrations of AITC above 1 µM for 2h at room temperature. The highest accumulation of HSP70 protein was observed in *C. elegans* exposed to 5 µM AITC (Figure 4.22). These results showed a concentration- and time-dependent increase in expression of the HSP70 protein(s), which confirmed the toxic effect of AITC on *C. elegans*. Therefore, it was concluded that AITC causes stress/toxicity in *C. elegans* and this system can be used to test toxicity of AITC in ground seed.

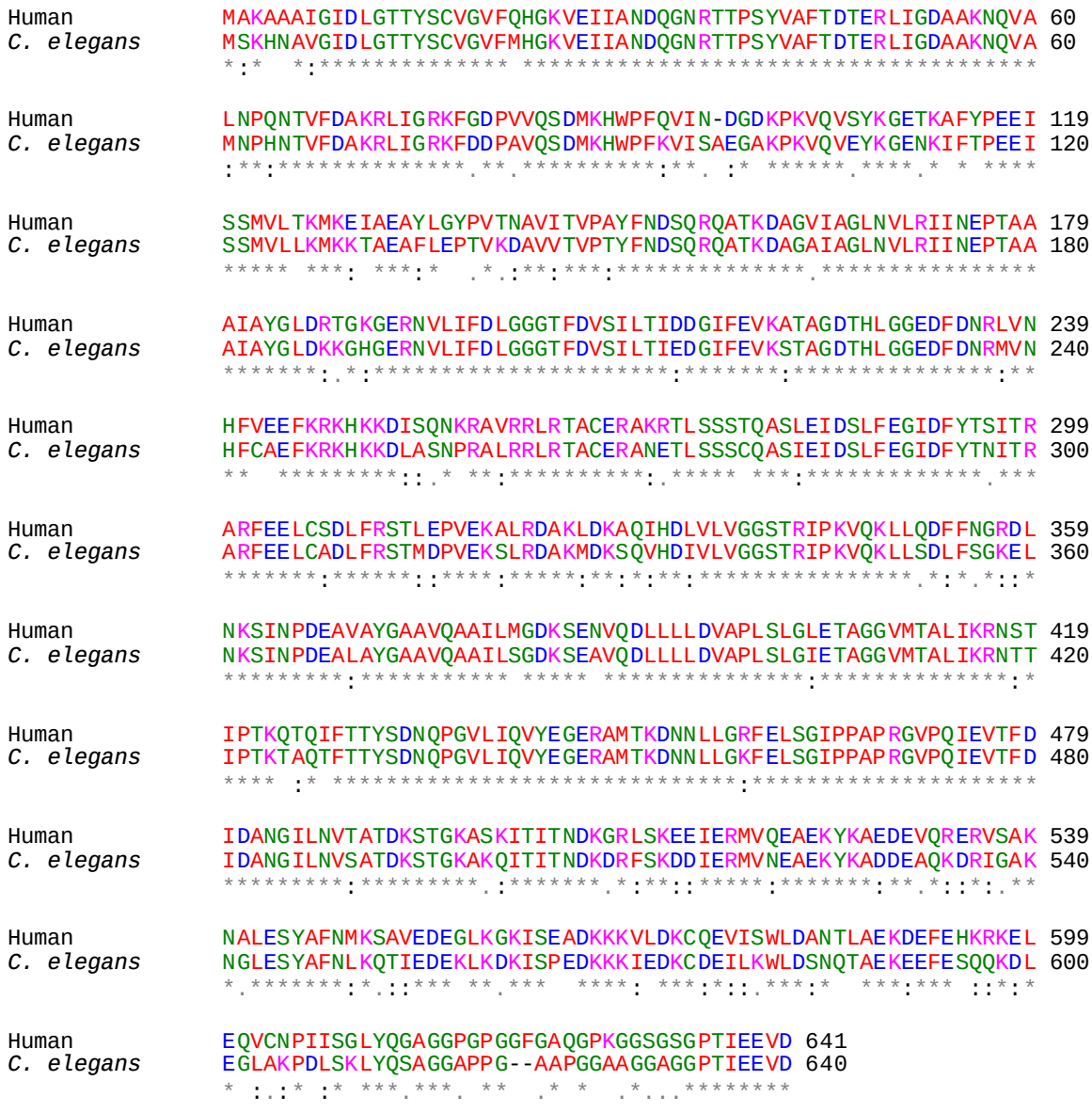

Figure 4-21. Amino acid sequence alignment of human (Accession No. NM_005346) and *C. elegans* (Accession No. M18540) HSP70 protein, showed 80% identity. Sequences were aligned using ClustalW version 1.83 (EMBL-EBI) software.

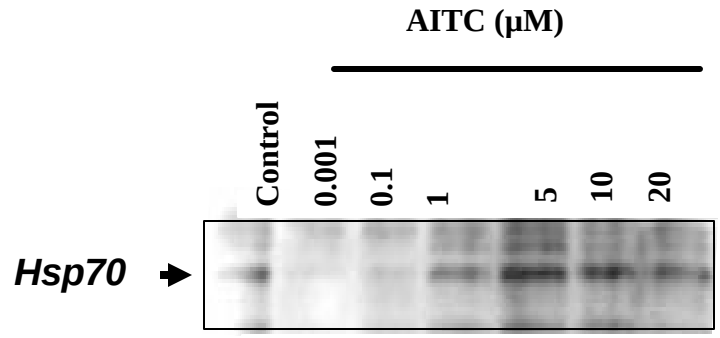

Figure 4-22. Western blot analysis showing induced expression of HSP70 protein in response to stress caused by AITC.

C. elegans was treated with different concentrations of AITC (0-10 µM) for 2 h at room temperature. Proteins in cell lysates were separated on SDS-PAGE gel and HSP70 protein was detected using anti-HSP70 antibody.

4.4 Mustard toxicity is contributed by AITC

From the above experiments on the toxicity aspects of AITC, it was concluded that all concentrations of AITC over 1 µM caused significant stress in *C. elegans.* However, it is important to know whether compounds in ground mustard seed, other than AITC, also contributed to mustard toxicity. The advantage of protein expression over the expression of mRNA transcripts was discussed earlier. At the same time, it is important to have some quantitative method of analysis of protein. Therefore, the expression of HSP70s was measured by an enzyme-linked immunosorbant assay (ELISA).

B. juncea cv. Arrid does not contain sinigrin, a substrate for myrosinase, and was used for treating *C. elegans* with or without AITC. The dose was based on AITC levels of 1% the total seed meal. *C. elegans* strain N2 was treated with AITC (0-10 µM), *B. juncea* cv. Arrid (0-114.5 µg/mL) or AITC (0-10 µM) + *B. juncea* cv. Arrid (0-114.5 µg/mL) for 2 h and the total protein extract was analyzed by ELISA. Lower concentrations of *B. juncea* cv. Arrid (0-72.25 µg/mL) had no effect on the expression of HSP70s, only the highest concentration (114.5 µg/mL) resulted in elevated expression of HSP70s (Figure 4-23). All of the concentrations of AITC alone resulted in significant increases in the expression of HSP70. AITC + ground mustard seed resulted in an increased expression of HSP70s, but the levels were still less than those observed with AITC alone.

These results indicate that 1) ground mustard seed toxicity is contributed by AITC and not by other compounds present in the meal, and 2) some components of the meal might act as AITC antagonists or the oil content in the meal inhibits the absorption of AITC, thereby reducing toxicity in *C. elegans.*

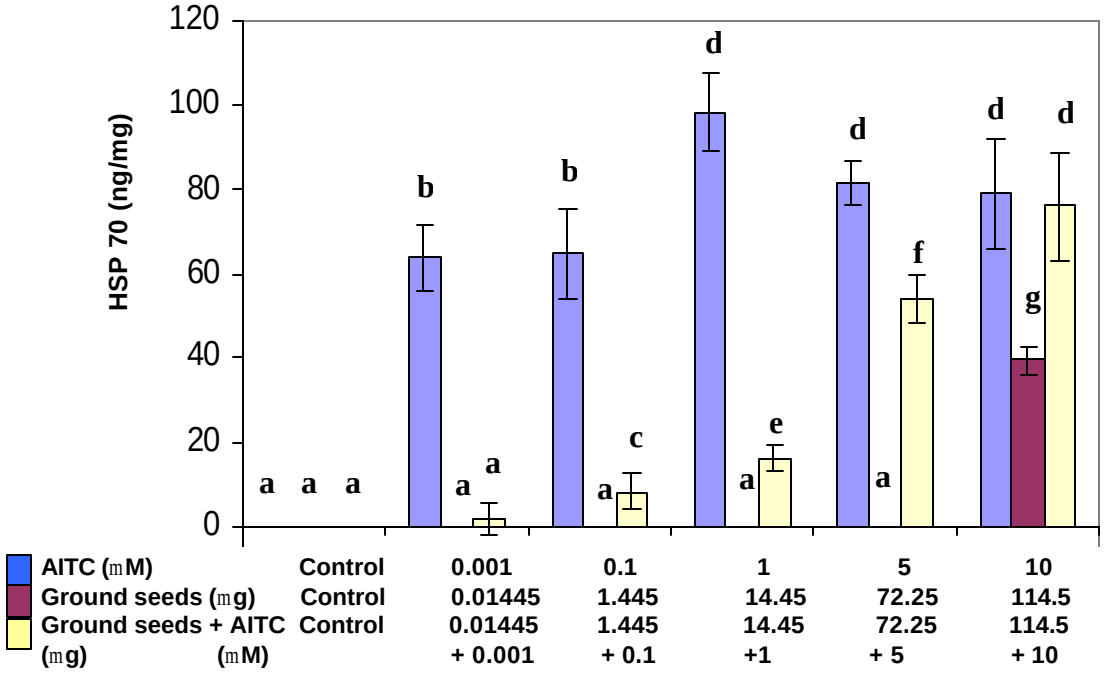

Figure 4-23. Expression of HSP70 protein in *C. elegans* in response to exposure to AITC, ground seeds and AITC+ground seeds.
C. elegans strain N2 was treated with AITC (0-10 µM), *B. juncea* cv. *Arrid* (0-114.5 µg/mL) or AITC (0-10 µM) + *B. juncea* cv. *Arrid* (0-114.5 µg/mL) for 2 h at room temperature and the total protein extract was analyzed by ELISA for the presence of HSP70. Bars on the columns represent standard deviations of three values. Data was analysed by multiple ANOVA. Values marked with different letters were significantly different from each other ($P<0.01$).

Chapter 5: Discussion

Western Canada has been one of the major producers of mustard seed since World War II, when supplies from Europe were disrupted. Canadian mustard seed production is concentrated in the Prairie Provinces, particularly in Saskatchewan. Mustard seed has the advantage of being tolerant to drought, heat and frost. Canada produces three mustard types: yellow mustard (*Sinapis alba*), brown mustard (*Brassica juncea*), and oriental mustard (*Brassica juncea*). Over the last ten years, research stations such as the Saskatoon Research Centre (SRC) of Agriculture and Agri-Food Canada have bred all three types of mustard for the condiment market, and are working on developing mustard varieties suited to non-food applications such as biodiesel and other industrial products. The SRC has released progressively improved varieties of this crop and Agriculture and Agri-Food Canada (AAFC), the Natural Sciences and Engineering Research Council of Canada (NSERC), the Agriculture Development Fund (ADF) and other major funding agencies have significantly supported improvement of mustard and mustard-based products. According to the Saskatchewan Ministry of Agriculture, the main funding for mustard research is contributed by AAFC, the Saskatchewan Mustard Development Commission (SDMC) and Saskatchewan Agriculture.

Mustard is the only cultivated oilseed crop adapted to the drier regions of the Canadian prairies and provides a suitable crop for rotation with wheat (Kirkegaard et al., 1994; Marcia *et al.*, 1999). Mustard is also resistant to many pests and fungal diseases and thus requires fewer inputs for its cultivation (Bones and Rossiter, 1996). Co-cropping *Brassica* has been tested for weed management (Haramoto and Gallandt, 2004, 2005).

Mustard seed, in the form of either seed or oil, is primarily used in the condiment and food industries. It is also used as an emulsifier or a water binding agent, and for texture control, in meat products. According to Red Coat REDA Inc., Gravelbourg, SK, the oil content of yellow mustard seed is the lowest (27%) of the three types of mustard, and it is the mildest in flavour of the three types. Therefore, yellow mustard is suitable for a wide range of applications, including flour, mustard pastes, spice mixes, meat processing, and other food products. Brown and Oriental mustard seed are ingredients of hot spices. Brown mustard has the highest oil content (36%; Skrypetz, 2003). Oriental mustard seed is often used to produce spicy cooking oils. There are Oriental mustard seed varieties grown in Canada that have oil contents of up to 50%, although the average oil content is approximately 39% according to AAFC. There has been a fluctuating trend in Canadian mustard seed production in recent years from a low of 105,000 tonnes in 2001 to a high of 306,000 tonnes in 2004. Exports increased nearly 8 percent from 2004 to 2005. Canada exports mustard seed mainly to the United States of America (46%), Belgium (22%) and Japan (7%) (Jacques, 2005). Demand for mustard seed is expected to grow during the next decade as world population and the use of spices increases. Canada's ability to meet this growing international demand, as well as continued research into new crop varieties, will contribute to Canada's continued success in the mustard industry (Jacques, 2005).

According to the SMDC, since 1936 plant breeders in Canada, mainly in Saskatchewan, have worked to improve the quality of mustard. During the 1950s and 1960s, mustard production migrated east, and today, Saskatchewan accounts for nearly 90 per cent of Canadian mustard production.

The myrosinase-glucosinolate system is characteristic of members of the Brassicaceae family (Bones and Rossiter, 1996). This system is only activated following tissue damage and plays a defensive role against pathogens (Rask *et al.*, 2000). Glucosinolate hydrolysis leads to the rapid formation of hydrolysis products such as allyl isothiocyanate (AITC) (Fahey *et al.*, 2001; Mithen 2001; Fenwick *et al.*, 1983).

Mustard is characterized by its typical flavour and taste, which is contributed by glucosinolate hydrolysis products. However, glucosinolate hydrolysis products comprise a large family of compounds. A subset of these compounds has been shown to have

possible health promoting effects (Verhoeven *et al.*, 1997), they may inhibit tumorigenesis (Smith *et al.*, 1998) and may play an important role in preventing heart disease (Wu *et al.*, 2004). AITC, one of the glucosinolate hydrolysis products, may be widely used in applications where its broad spectrum of toxicity and volatility are advantageous viz., anti-fungal, anti-bacterial and bio-fumigant, which are described in Chapter 2. Due to the toxic effects of AITC, it is important to develop quick and efficient AITC detection methods and to determine myrosinase activity in mustard seed meal.

In the present investigation, an attempt was made to improve the "Herb and Spice Method", the only available "official" method to measure total isothiocyanate production in mustard meal. The "Herb and Spice Method" is crude, imprecise, time consuming (requires 2 h of extraction for the quantification of one sample) and requires incubation at 37ºC (ASTA 1997).

The method developed in the current study was rapid and more reliable and was used to indirectly measure myrosinase activity and to understand the kinetics of the enzyme. A wide range of conditions (temperatures of 7 to 97ºC and incubation times of 30 sec to 2 h) were studied to determine optimal conditions for myrosinase activity. Rapid evolution of AITC was observed at five min of incubation at all temperatures tested. Higher temperatures (>57ºC) resulted in significant inhibition of enzyme activity, and at 97ºC, no myrosinase activity was found. It was concluded that: 1) hydrolysis of glucosinolates is a rapid process and a detectable amount of AITC can be found as early as 5 min; and 2) higher temperatures inhibit myrosinase activity.

Eylen *et al.* (2006) studied the thermal and pressure stability of myrosinase at temperatures from 60 to 75ºC and pressures from ambient to 1000 MPa. Myrosinase was found to be stable at 600 MPa pressure at temperatures up to 60ºC. At low pressures, there was an antagonistic effect between temperature and pressure. They suggested that the high pressure stability of myrosinase may be a valuable alternative to thermal treatments if one wants to retain myrosinase activity. Matuscheki *et al.* (2004) have shown inactivation of myrosinase following thermal treatment. The kinetics of myrosinase inactivation during thermal treatment has also been investigated in crude

broccoli extract (Ludikhuyze *et al.*, 1999) and in red cabbage juice (Verkerk *et al.*, 2002).

The conditions for inactivation of myrosinase using microwave energy were studied with a response surface design. Inactivation of myrosinase was dependent on moisture content and exposure time to microwaves, and there was a significant increase in both the yellow colour and sulfur content of the microwave-treated seed oils (Owusu-Ansah and Marianchuk, 1991). Microwave irradiation at 2450 MHz for 2.5 min is known to inactivate myrosinase and decompose glucosinolates (Verkerk and Dekker, 2004).

Attempts were made to inhibit or reduce myrosinase activity by manipulating the pH of the extraction medium over the range of 1.5 to 13. Results indicated that myrosinase required a pH of 6.0 for optimum activity, and that a change in pH did not impact the substrate but altered the activity of the enzyme. Other compounds viz., $C_2H_4(COOH)_2$, H_3BO_3, $CaCl_2$ and C_2H_5OH were also explored for their ability to inhibit myrosinase activity. Succcinic acid, calcium chloride and ethanol significantly reduced myrosinase activity. Different acids were tested for a rapid inactivation of myrosinase without hydrolysis of the GLS. Mild acids (like succnic acid and borate) and harsh acid (hydrochloric acid) inhibited myrosinase activity in mustard grounded seeds. A base was also tesetd to inhibit myrosinase activity because the gycosidic bond is comparatively more resilient to base. Furthermore, ethanol which can denature protein without altering pH was also used that would leave the GLS intact. An extract from *Arctostaphylos uvae-ursi* has been reported to inhibit myrosinase activity (Mykytyn and Demjanchuk, 1999).

In the present research, it was hypothesized that AITC might act as a plant growth promoter/regulator because AITC and ethylene share a common chemical structure (R-CH=CH_2, where R is -CH_2SCN and -H in AITC and ethylene, respectively). Therefore, AITC might act as an ethylene analogue. Ethylene is a well known plant growth regulator known to promote seed germination and overcome seed dormancy in a dose- and species-dependent manner (Esashi *et al.*, 1988; Abeles *et al.*, 1992; Kepczynski *et al.*, 1997; Nascimento 2003). Based on the similarities between AITC and ethylene, AITC may also regulate seed germination. Ethephon® and AITC were tested to determine their impact on flaxseed and tomato seed germination. Interestingly, higher

concentrations (0.1 to 1 mM) of AITC enhanced seed germination. The present study showed the seed germination enhancing properties of AITC, and thus AITC could be used in agricultural practice.

There may be more than a single mode of action for glucosinolate toxicity and biological activity. For example, AITC possesses mutagenic and DNA-modifying activity with formation of reactive oxygen species (Kassie and Knasmuller, 2000). In addition, benzyl isothiocynate has mutagenic properties (Yamaguchi, 1980) and caused DNA strand breaks (Musk *et al.*, 1995). AITC inhibits growth of PC-3 human cancer cell xenografts by inducing apoptosis and reducing mitotic activity (Srivastava *et al.*, 2003). The apoptosis and cell cycle arrest properties of AITC have been documented in leukemia cells (Xu and Thornalley, 2000), prostate cancer cells (Xiao *et al.*, 2003) and colon cancer cells (Gamet-Payrastre *et al.*, 2000). AITC induces apoptosis and rapid transient activation of Caspase-3 and Caspase-8 (Yu *et al.*, 1998; Xu and Thornalley, 2000; Xu and Thornalley, 2001). AITC is also known to arrest the cell cycle in G2/M phase (Xiao *et al.*, 2003; Smith *et al.*, 2004). In the present investigation, *C. elegans* was used to study AITC-mediated stress or toxicity.

Heat shock proteins play an important role in acclimatization of living organisms to a variety of biotic and abiotic stresses (Vierling, 1991; Parsell and Lindquist, 1994; Hamilton and Coleman, 2001). HSPs are general stress proteins and are involved in maintenance of proper cell function and, thereby, help in cell survival during stress by facilitating recovery from stress (Vierling, 1991; Parsell and Lindquist, 1994; Downs and Heckathorn, 1998; Guy and Li, 1998; Heckathorn *et al.*, 1998). HSP70 family proteins are ubiquitous in plants, animals and microorganisms and their structure and function are highly conserved among diverse organisms, from algae to mammals (Wu *et al.*, 1994). These proteins are involved in preventing protein aggregation and in refolding of denatured proteins produced as a result of cellular stress caused by an insult. In *C. elegans*, four isoforms of HSP70 are known: HSP70A, HSP70C, HSP70D and HSP70F. Each of these isoforms of HSP70 performs a different function. HSP70A is expressed in response to a variety of stresses, and HSP70C is expressed in the ER and its expression is not increased in response to heat stress (Heschl and Baillie, 1998).

However, HSP70D prevents secretion of proteins from the ER and HSP70F is expressed in the mitochondria.

In the present investigation, methods of screening AITC toxicity were developed. Western blotting revealed expression of a 70 kDa protein in *C. elegans* due to exposure to different concentrations of AITC in a dose-dependent manner. Nematodes were visibly injured by the treatments with AITC, as was indicated by their moment. Nematodes were actively moving in all the concentrations below 10µM. High concentrations appeared to be lethal as their moment stopped. Detection of HSP70 protein by ELISA confirmed the above observations. Methods were developed to screen AITC toxicity by real time RT-PCR to accomplish quantitative analysis of the expression of *HSP70A* transcripts. It is important to understand whether compounds in ground mustard seed, other than AITC, also contribute to mustard toxicity. The advantage of protein expression over expression of mRNA transcripts was discussed earlier. At the same time, it is important to have a quantitative method of analysis of protein. Therefore, the expression of HSP70 proteins was measured by an ELISA.

C. elegans strain N2 was treated with *B. juncea* cv. Arrid and/or AITC and expression of HSP70 proteins was quantified by ELISA. The dose of *B. juncea* cv. Arrid was determined based on the fact that AITC is 1% of the total seed meal. Lower concentrations of *B. juncea* cv. Arrid (0-72.25 µg/mL) had no effect on the expression of HSP70 proteins, whereas the highest concentration (114.5 µg/mL) resulted in elevated expression of HSP70 proteins. All of the concentrations of AITC alone resulted in a significant increase in the expression of HSP70 protein. AITC + ground mustard seed resulted in an increased expression of HSP70 proteins but the levels were still less than those observed with AITC alone.

These results indicate that 1) ground mustard seed toxicity is contributed by AITC, and 2) some components of the ground mustard seed act as AITC antagonists or the oil content in the meal inhibits the absorption of AITC, thereby reducing toxicity in *C. elegans.* These findings suggest that HSP70 could be used as a universal tool for studying mustard toxicity. The present work could help the mustard industry open a new market. Industry needs the tools for screening toxicity of mustard meal or oil-based products. A company in Saskatchewan plans to use mustard for nematode control.

Therefore, the possibility for a totally new market and new acreage for mustard is supported.

HSP70 expression can also be used as a universal biomarker in ecotoxicological studies. It can also be a helpful tool for the monitoring of freshwater ecological systems. Genetically modified zebra fish containing enhanced green fluorescent protein (eGFP) under the control of a 1.5 kb *HSP70* promoter have been developed (Blechinger *et al.*, 2002) which could be used for screening toxicity of mustard-based fish meals. The methods of screening AITC toxicity developed in this study are fast, reliable and quantitative.

Use of mustard as a biopesticide is one of the applications based on toxicity of AITC. Toxicity of Indian mustard and AITC to masked chafer Beetle larvae has been demonstrated (Ryan *et al.*, 2002). Mustard used in biopesticide production may also yield protein meal, fertilizer and biodiesel. Bio-pesticidal (Brown and Morra, 2005), fungicidal (Brown and Morra, 2005), antibiotic (Mari *et al.*, 2002; Kiyoshi 2005; Winther and Nielsen, 2006; Tunc *et al.*, 2007), biofumigant (Brown and Morra, 2005; Tsao *et al.*, 2002a; Noble *et al.*, 2002) and nematocidal (Buskov *et al.*, 2002; Lazzeri *et al.*, 1993) properties of AITC have been studied. This suggests the potential importance of AITC and other GLS hydrolysis products in agricultural applications.

If AITC and mustard are to be used more widely in new pesticide markets, questions will arise regarding both the risks to non-target organisms and the efficacy of the compound on target organisms. The regulatory environment will often require extensive testing for compounds in the environment, yet the presence of a compound does not imply either impact or toxicity. The need for expensive analytical tests may slow the introduction of new products with potent toxicity. Conversely, simple and robust tests may aid in the development of suitable policy and regulations regarding the deployment of the compound or material. The HSP approach of using a sentinel organism could be a useful tool to allow the determination of toxicity *in situ* and to potentially enable the deployment of mustard as a biopesticide. The HSP test data has been shared with BioGreen Inc. of Hague, SK and the company is interested in using the test for both product development and in their presentations to regulatory agencies, including the US Environmental Protection Agency.

Chapter 6: Summary and Conclusions

The present study was aimed to study: 1) factors affecting myrosinase activity in mustard (*Brassica juncea* L.), 2) the effect of allyl isothiocynate (AITC) on the germination of flax and tomato seed, and 3) the toxicity of AITC and ground mustard seed using *C. elegans* as a model system.

Myrosinase hydrolyzes sinigrin to produce AITC in the presence of water. Spatial separation of the myrosinase and sinigrin in the intact plant prevents this hydrolytic reaction. However, milling and grinding processes result in the hydrolysis of sinigrin. As a result, AITC contributes to the toxicity of ground mustard seed and mustard-based products. Therefore, a quick and efficient method for measuring AITC was developed and used to measure myrosinase activity indirectly. It was revealed that sinigrin hydrolysis was a rapid process and can be inhibited by 1) higher incubation temperatures (>57ºC), 2) a pH less than or greater than 6.0, and 3) adding succinic acid, calcium chloride or ethanol to the reaction mixture. Myrosinase preferred near-neutral conditions for its activity and a change in pH did not affect the substrate but severely affected the activity of myrosinase. These results may lead to the improvement of mustard processing methods to reduce toxicity caused by sinigrin hydrolysis products.

It was hypothesized that AITC might act as a plant growth promoter/regulator based on the structural similarity of AITC to ethylene ($R\text{-}CH{=}CH_2$, where R is $-CH_2SCN$ and -H in AITC and ethylene, respectively). Therefore, AITC might act as an ethylene analogue and promote seed germination. The effects of Ethephon®, IAA and AITC on the germination of flaxseed and tomato seed were investigated. It was concluded that AITC could be useful in agricultural practice.

The third objective of this study was to investigate the toxicity of AITC and ground mustard seed using *C. elegans* as an experimental model and to identify

molecular indicators of AITC-induced stress in *C. elegans.* Members of the 70 kDa heat shock protein (HSP70) family are ubiquitous in plants, animals and microorganisms, and their structure and function are highly conserved among diverse organisms, from algae to mammals. HSP70 proteins are known to be involved in preventing protein aggregation and in refolding of denatured proteins produced as a result of cellular stress caused by an insult. Bioinformatic tools used in this study revealed high identity in the four isoforms of HSP70 in *C. elegans* viz., HSP70A, HSP70C, HSP70D and HSP70F. Phylogenetic analysis showed that HSP70C and HSP70D were the most closely related and that HSP70A and HSP70F were distinctly related. Because HSP70A is a cytosolic protein known to be expressed in response to stress or toxicity, expression of HSP70A was used as a marker of stress/toxicity in *C. elegans*.

Real-time RT-PCR results confirmed over-expression of the *HSP70A* gene in *C. elegans* strain N2 following AITC treatment. Translation of *HSP70* transcripts was confirmed by Western blotting which showed accumulation of HSP70 in *C. elegans* exposed to AITC (>1 µM) for two hours at room temperature. The highest accumulation of HSP70 was observed in *C. elegans* exposed to 5 µM AITC. Therefore, expression of HSP70A could be used as an indicator of the stress-inducing properties of AITC and to test toxicity of AITC in ground mustard seed.

Expression of HSP70 proteins was quantified by ELISA. *C. elegans* was treated with *B. juncea* cv. Arrid ground seed and/or AITC. Results showed that the highest concentration of *B. juncea* ground seed (114.5 µg/mL) caused an elevated expression of HSP70. All of the concentrations of AITC alone resulted in a significant increase in the expression of HSP70s. AITC + ground mustard seed resulted in an increased expression of HSP70s, but the levels were less than those observed with AITC alone. Therefore, it was concluded that it is AITC alone and no other component from mustard ground seed that contributes to mustard toxicity. Some components of the ground seed either act as AITC antagonists or it is may be due to presence of other compounds, which may interact rather than oil alone. Therefore, presence of other compounds in the ground seed inhibits the absorption of AITC, thereby reducing toxicity in *C. elegans.*

This work may lead to the development of better industrial mustard processing methods or new methods for testing the toxicity of mustard-based products, and has demonstrated that AITC may have practical application in enhancing seed germination. Agro-industries may benefit from these findings.

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