STUDY OF THE EFFECTS OF L-ASCORBIC ACID AND FERROUS IONS ON MYROSINASE ENZYME ACTIVITY BY USING SPECTROPHOTOMETRIC METHOD

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THIS DISSERTATION IS SUBMITTED AS A PARTIAL REQUIREMENT TO OBTAIN DEGREE OF BACHELOR OF SCIENCE WITH HONOURS

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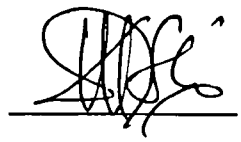

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KAJIAN TENTANG KESAN KEHADIRAN ASID L-ASKORBIK DAN ION FERUM TERHADAP AKTIVITI ENZIM MIROSINAS DENGAN MENGGUNAKAN KAEDAH SPEKTROFOTOMETRI

ABSTRAK

Glukosinolat (GS) adalah sebatian sekunder mengandungi sulfur yang terdapat di dalam famili Brassicaceae dengan banyak. GS mengalami hidrolisis secara terus oleh enzim mirosinas yang wujud secara semulajadi apabila sel pecahan berlaku untuk membentuk produk utama isotiosianat dan/atau nitril ringkas. Isotiosianat diketahui mempunyai ciri-ciri antikarsinogenik. Cerakin enzim pada degradasi glukosinolat memerlukan keadaan yang optimum untuk mengadakan keadah yang sensetif dan boleh dipercayai. Di dalam kajian ini, dua ujikaji utama telah dijalankan. Pertama, satau kaedah spektrometrik yang dioptimakan untuk cerakin enzim-substrat dengan menggunakan sebatian piawai mirosinas (enzim) dan sinigrin (substrat) telah dibangunkan secara sistematik. Kedua, dengan menggunakan kaedah yang telah dibangunkan ini, kesan ion-ion L-askorbik dan ferus ke atas aktiviti mirosinas telah dikaji. Keputusan kajian menunjukkan bahawa komposisi optima sinigrin dan mirosinas ialah masing-masing 0.20 mM and 0.065 mM. Keadaan-keadaan optima yang lain ialah pH 6.5 (dalam penimbal fosfat) dan pada suhu bilik dalam julat 22-25℃. Jarak gelombang pengesanan UV yang optima ialah pada 234 nm. Telah ditunjukkan di sini bahawa, kepekatan asid askorbik yang rendah meningkatkan aktiviti mirosinas tetapi kemudian merencatkannya apabila kepekatan meningkat. Aktiviti mirosinas yang tertinggi oleh asid askorbik adalah pada kepekatan 0.5 mM. Ion-ion ferus telah memberi impak ke atas aktiviti mirosinas dengan amat berkesan walaupun pada amaun yang rendah. Pada 10 mM ion ferus, aktiviti mirosinas telah direncatkan hampir 100%. Kaedah yang baru dibangun dengan menggunakan spektrofotometer UV-Vis ini adalah mudah, pantas dan jimat untuk penentuan aktiviti mirosinas. Kaedah baru yang dibangunkan dengan penggunakan UV-Vis spektrofotometer menunjukkan sebagai kaedah yang mudah, cepat dan kos rendah dalam penentuan aktiviti mirosinas.

ABSTRACT

Glucosinolates (GS) are sulphur-containing secondary metabolites found largely in *Brassicaceae* family. GS undergo hydrolysis readily upon cell rupture by the naturally-occurring enzyme myrosinase to form mainly isothiocyanates and/or simple nitriles. Isothiocyanates are known to possess anti-carcinogenic properties. An enzymatic assay of glucosinolate degradation requires an optimized condition for sensitive and reliable methods. In this study, two main experiments have been carried out. Firstly, an optimized spectrophotometric method for enzyme-substrate assay using standard myrosinase (enzyme) and sinigrin (substrate) have been systematically developed. Secondly, by using the developed method, the effects of L-ascorbic acid and ferrous ions on the myrosinase activity were studied. The results showed that the optimum composition of sinigrin and myrosinase were 0.20 mM and 0.065 mM, respectively. The other optimum conditions were at pH 6.5 (in phosphate buffer) and at room temperature in the range of 22-25 °C. The optimum UV detection wavelength was at 234 nm. It was shown that, low concentration of ascorbic acid enhanced the myrosinase activity but eventually inhibited it as the concentration increased. The highest myrosinase activity by L-ascorbic was at the concentration of 0.5 mM. Ferrous ions impacted on myrosinase activity significantly even with a trace amount. At 10 mM ferrous ions, the myrosinase activity was inhibited almost 100%. The newly-developed method using UV-Vis spectrophotometer appears easy, rapid and cost-effective for determination of myrosinase activity.

ABSTRACT

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LIST OF SYMBOLS AND ABBREVIATIONS

^0C	Degree Celcius
%	Percentage
AU	Absorbance Unit
ESP	Epithiospecifier Protein
g	Gram
g/mol	Gram Per Mole
GLS	Glucosinolate
HPLC	High Performance Liquid Chromatography
ITC	Isothiocyanate
M	Molarity
mg	Milligram
mg/mL	Milligram Per Milliliter
mL	Milliliter
ml/min	Millilitre Per Minute
mM	Millimolar
MPa	Megapascal
nm	Nanometer
R-	Ally-
U	Unit
λ_{max}	Lambda Max
μM/min	Micromolar Per Minute

CHAPTER 1

INTRODUCTION

1.1 Background of Study

With Rapid development of technology, people found that plants have plenty of nutrition as stimulant, curative or medicinal. People believe that food is the most effective medicine for all kinds of disease since it is natural compare to the artificial man-made medicine. The Father of Medicine, Hippocrates of Cos said that 'let food be the medicine' (Hasler, 1999). Many studies found that vegetables and fruits are containing rich sources dietary micronutrients and fibers (Zimmermann *et al.*, 2001) and recently it has been recognized as important natural medicine (Ubani, 2011). In particular, researchers find out that in a range of biological activities affecting herbivorous insects, plants and fungi contains glucosinolate-myrosinase system (Atle & John, 2006).

Myrosinase is a protein with enzymatic properties and specific activation power towards substrate without altered at the end of the reaction (Alberts *et al.*, 2002). It is a biological catalyst that enhances the rate of the hydrolysis of the glucosinolate (Bellostas *et al.*, 2008). Myrosinase is also involved in the production of some phyto-hormones from their inactive precursor glucosinolate, potentially in sulphur and nitrogen metabolism and the growth regulation (Kliebenstein *et al.*, 2002; Mikkelsen *et al.*, 2003)

Glucosinolate (GLS) are secondary plant metabolites. Native glucosinolates are biologically inactive. The enzyme myrosinase catalysed hydrolysis of glucosinolates initially involves cleavage of the thioglucoside linkage yielding D-glucose and an unstable thiohydroximate-O-sulphonate that spontaneously rearranges to a variety of compound (Isothiocyanate (ITC), nitriles, thiocyanates, epithionitriles, oxazolidines), depending on such factors as substrate, pH or the availability of ferrous ions (Atle & John, 2006).

Isothiocyanate (ITC) has ability to trigger a number of health promoting effects (Traka & Mithen, 2009), most importantly inhibition of tumorigenesis, anti-inflammatory properties and prevention of heart diseases (Wu *et al.*, 2004). It can reduce the occurrence of cancer in different organs including oesophagus, lung and stomach in animal models (Figueiredo *et al.,* 2013). Several mechanisms have been proposed to underlie anticarcinogenic benefits of ITC. ITC involve in decreasing the apoptosis rate of mitosis and stimulation in human tumour cells (Alberts e*t al.*, 2002). Isothiocyanate has multi-nutrient in which is substantial health benefit to human (National Research Council, 2006). Its chemopreventive activity and potential use as novel chemotherapeutics lead to the researchers' interest in it. Most recently, the isothiocyanates is found to inhibit NF-kB and reduce myeloma growth in nude mice in *vivo* (Brunelli *et al.*, 2010). By contrast, nitriles and thiocyanates have no beneficial effects on human, the type of GLS hydrolysis products could be influenced by several factors. Thus, it is crucial to study the myrosinase activity to ensure optimum conversion of GLS to the cancer-preventing product, ITC.

Due to the beneficial activity of ITC, glucosinolate-myrosinase system has drawn researchers' attention. Controlling the glucosinolate-myrosinase system condition can lead to high yield of ITC in a short period. The activation and inhibition of myrosinase activity is depending on several extrinsic (pH and temperature) (Ludikhuyze *et al.*, 2000) and intrinsic factors (L-ascorbic acid and ferrous ions) (Bones & Rossiter, 1996). The presence of these factors will influence on myrosinase activity and stability, leading to increase or decrease the efficiency of hydrolysis of glucosinolates. Hence, the investigation should be carried out.

The myrosinase activity might be interfered by the other factors such as the protein or other components that existed in a plant (Ludikhuyze *et al.*, 2000). In the past, several methods on determination of myrosinase activity have been applied. However, the researchers are more interested on the extracted myrosinase from the *Brassica* plants. Hence, a standard of the enzyme and substrate is suggested to be used in my study.

The dietary habits by the consumer maybe affect the myrosinase activity (Yuan *et al.*, 2009). Vitamin C is one of the most important nutrients in many other horticultural crops and has many biological functions in the human body (Lee & Kader, 2000). Besides, the trace amount of iron is taken in diets to avoid of iron deficiency anaemia (Zimmermann *et al.*, 2005). The amount of vitamin and ferrous ions present in the food might contribute or deactivate the myrosinase activity. Hence, the effect of L-ascorbic acid (the predominant form of vitamin C) and ferrous ions should be tested on the myrosinase activity.

For determining the effects of intrinsic factor towards the myrosinase activity, High Performance Liquid Chromatography (HPLC) has the potential to rapidly and non-destructively analyse multiple components of a reaction mixture. Although HPLC is very high sensitivity, good separation and quantification of multiple component mixtures, sample preparation and analysis can be time consuming, costly and not suitable for a process environment (Ewanick *et al.*, 2013).

Spectrophotometric method strongly shows the excellent advantages over traditional chromatographic methods on monitoring the reactivity of peptides with chemicals (peptide reactivity) which cost lots and time consuming (Jeong *et al.*, 2013). Besides, using a rapid and inexpensive spectrophotometric assay shows a very high sensitivity, specificity, and accuracy (Schultz *et al.*, 2005). Spectrophotometric methods can be easy, fast, and high-throughput screening tools. Hence, an optimized condition was introduced for the investigation of the existence of the intrinsic factors by spectrophotometric assay by using a spectrophotometer (Jeong *et al.*, 2013).

1.2 Objective of Study

The objectives of this study include:

a. To optimize the enzymatic assay on myrosinase activity using spectrophotometer.
b. To investigate the effect intrinsic factors (L-ascorbic acid and ferrous ions) on myrosinase activity.

1.3 Scope of study

In this study, spectrophotometric method was applied on the enzymatic assay between the standard compounds of myrosinase and glucosinolate (sinigrin). The extrinsic factors (pH, myrosinase and glucosinolate mixture ratio) were regulated and optimized to ensure myrosinase work in maximum activity. After that, the myrosinase activity was determined under the inferences of intrinsic factors (L-ascorbic acid and ferrous ions). Intrinsic factors were introduced into the myrosinase-glucosinolate reaction with a range of concentration. The myrosinase activity was calculated by the declination of glucosinolate concentration over 30 minutes' reaction. The initial absorbance was read, the second absorbance was taken after 30 minutes of reaction. The changing of the glucosinolate concentration was calculated by the absorbance versus concentration in the linearity graph.

CHAPTER 2

LITERATURE REVIEW

2.1 Myrosinase

Myrosinase is also called thioglucoside glucohydrolase (E.C. 3.2.1.147). It is the trivial name for plants endogenous enzyme which functions to hydrolysis of a variety of plant anionic 1-thio-β-D-glucosides called glucosinolates. Myrosinase exist as multiple forms of several myrosinase isoenzymes (MacGibbon & Allison, 1970; Buchwaldt *et al.*, 1986). Myrosinase can be found in myrosin cell which be distributed in plants' seeds, leaves, stems and roots. Myrosinase have been proved that it is localized in the subcellular in the protein bodies or vacuole of myrosin cells (Thangstad *et al.*, 1991). Glucosinolate-myrosinase activity is shown higher especially in the young tissue in plant (Bones & Rossiter, 1996).

Extraction of myrosinase from different part of plants (i.e. leaf, steam, root or seed) is giving different pattern of isoenzyme. There is two myrosinase isolated by James & Rossister (1991) that have different degree of glycosylation on different glucosinolate. However, they have found that both isoenzymes have the highest activity against aliphatic glucosinolates but have the least activity against indole glucosinolates (James & Rossister, 1991).

Myrosinase degrades glucosinolates at approximately the same rate in *vitro* (James & Rossister, 1991). However, the specificity of myrosinase is affected by associate factors like epithiospecifier protein, myrosinase-binding protein, and the presence of ascorbic acid. Besides, different myrosinase activity is contributed by plant species, organ and stage of development (Bones, 1990), as well as seasonal condition and climatic factors (Charron *et al.*, 2005).

The complexity of glucosinolate-myrosinase plays important role in cruciferous plants (Bones & Rossiter, 1996). The glucosinolate-myrosinase system may have several functions in the plant: i) plant defence function against pathogens and pest infestation (Jander, 2012); ii) sulphur and nitrogen metabolism (De *et al.*, 2002a); iii) growth regulation (Kissen *et al.*, 2009).

2.1.1 Structure and Conformation of Myrosinase

The myrosinase is made up by large number of carbohydrates (i.e. mannose residues various thiol groups, disulfide and salt bridges) and in the interface of the myrosinase subunits a zinc atom heavily glycosylated (Björkman & Janson, 1972). The protein folds into a $(\beta/\alpha)_8$-barrel structure, very similar to that of the cyanogenic β-glucosidase from white clover (Burmeister *et al.*, 1997). Myrosinase exists as a crystallographic dimer with subunits of 60-70kDa each. There is a zinc atom stabilized the dimers by linking two subunits (Burmeister *et al.*, 1997).

Myrosinase has been reported to be disulphide linked dimers of 75 kDa subunits (Bones & Slupphaug, 1989) although in the immunological studies have proven that myrosinase binding proteins and myrosinase associated proteins can actually form complexes with higher molecular weight (Rask *et al.*, 2000). Different molecular weight of myrosinase complexes in the Brassicas can be formed in the range of (500–600 kDa, 270–350 kDa and 140–200 kDa) (Bellostas *et al.*, 2008).

Myrosinase is not only a carbohydrate complexes but complexes with proteins where the oligomerization state of myrosinase is not clear. Various degrees of glycosylation account for at least part of the charge heterogeneity seen in this enzyme (Höglund *et al.*, 1991). Myrosinase is a functionally enzymes where fourteen

isoenzymes were identified in seed extraction of *Sinapsis alba*. Besides, there is enzymatic activity of two isoenzymes differed depending on the substrate glucosinolate in *Brassica napus L.* (James & Rossiter, 1991). A 3D structure of myrosinase from *Sinapsis alba* seeds is shown in Figure 2.1.

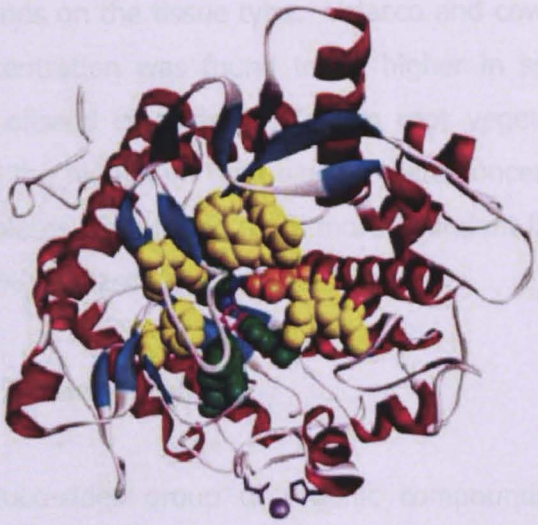

Figure 2.1 Schematic drawing of a myrosinase subunits showing the $(\beta/\alpha)_8$-barrel structure. The position of a substrate in the active site is indicated by the inhibitor 2-deoxy-2-fluroglucotropaelin molecule in magenta covalently bound to the nucleophile (centre). The view is from the substrate entrance part of enzyme. N-terminus is at the bottom left and the C-terminus at the bottom centre (Rask *et al.*, 2000).

2.2 Glucosinolates

Glucosinolates is the compound derived from glucose and amino acids which normally occur as secondary metabolites of most the Brassicales plants (Rask *et al.*, 2000). Glucosinolates can be found in all parts of the plant with different concentration and depends on the tissue type. Velasco and coworkers (2008) found that glucosinolate concentration was found to be higher in seeds than in leaves, differ from 3.8-fold in oilseed crops to 7.1-fold in root vegetable crops. Aliphatic glucosinolates occupied the highest in total glucosinolate concentration in seeds and leaves. Indole glucosinolates were found to be more abundant in leaves (5% to 17%) than in seeds (5% to 8%) (Velasco *et al.*, 2008).

2.2.1 Structure and Nomenclature

Glucosinolates is a gluco-sides group of organic compounds; thioethers contain sulphur and nitrogen atoms in the molecule. They generally consist of a sugar entity, β-D-thioglucose, with an ester bond to an organic aglycone (i.e. alkyl group yielding isothiocyanate, nitrile, thiocyanate or a similar compound upon hydrolysis) (Fahey *et al.*, 2001). There is a central carbon atom binds to the thioglucose group by sulfur atom and making a sulphated aldoxime as well as binds to a sulphate group via a nitrogen atom (Fahey *et al.*, 2001). Besides, the central carbon is bounded to a side group which derived from amino acids and is highly variable. It can be aliphatic (e.g. alkyl, alkenyl, hydroxyalkenyl, w-methylthioalkyl), aromatic (e.g. benzyl, substituted benzyl) or heterocyclic (e.g. indolyl). The type of bound side group depends on the type of glucosinotes which responsible in the variety of biological activities of these plant compounds (James, 1996). The *R*-group (side chain) and the sulphate group have anti-stereochemical configuration.

The sulphate group present makes glucosinolates strongly acidic properties results to its water-soluble anions properties counterbalanced by a potassium cation since it is the most abundant cations in the plants tissue (Fahey *et al.*, 2001). The sulphate group and the thioglucose part impart non-volatile and hydrophilic properties to all glucosinolates, the *R*-group is variable in properties from lipophilic to

mark hydrophilic (Fahey *et al.*, 2001). The general structure of glucosinolates is shown in Figure 2.2.

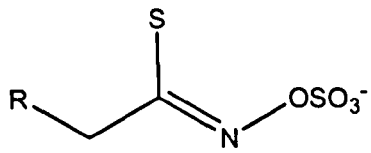

Figure 2.2 The general structure of glucosinolate (*R* is an organic aglycone and is an alkyl group) (Freeman *et al.*, 2008).

The structural variations among more than 140 known glucosinolates are caused by the variations in the R-groups and owing to acyl substituents on the thioglucose part (Bellostas *et al.*, 2007). The structural diversity arises from side chain elongation of amino acid precursors prior to the formation of the glucosinolate core structure and from a wide range of secondary modifications including oxidation, desaturation, hydroxylation, methoxylation, sulphation and glucosylation (Wittstock *et al.*, 2002). With the discovery of more glucosinolates, a semi-systematic system for their naming arose, based on the structure of the side chain. Table 2.1 shows trivial name for some glucosinolates 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 glucosinolates.

Glucosinolate breakdown depends on structural requirements regarding the glucosinolate side chain as well as on the functionality of specifier proteins (Wittstock *et al.*, 2010). Hence, the actual glucosinolate structure, including stereochemistry, and to the mechanism of the myrosinase catalysed glucosinolate hydrolysis are important as the basis for the wanted bioactive glucosinolate products (Lin *et al.*, 2013).

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