PROFILLING OF GLUCOSINOLATE AND MYROSINASE ACTIVITY IN *CARICA PAPAYA* (PAPAYA) VS EKSOTIKA UNDER DIFFERENT CONDITIONS

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FACULTY OF SCIENCE AND NATURAL RESOURCES
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FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH
2015

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledge.

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Annie Johanna Ahmad
6 May 2015

ABSTRACT

Glucosinolates are sulfur-containing secondary metabolites found largely in Brassicaceae family. Glucosinolates undergo hydrolysis readily upon cell rupture (such as cutting and heating) by the naturally-occurring enzyme myrosinase to form mainly isothiocyanates and/or nitriles. Isothiocyanates are known to possess anticarcinogenic properties while nitriles are largely inactive. Benzyl isothiocyanate (BITC), a hydrolysis product of benzyl glucosinolate (BGSL), is one of the most potent anticancer agents. Papaya (*Carica papaya*) is known to contain has high amount of BITC. In Malaysia, beside ripe fruit, young leaves and young or unripe fruit of papaya are widely consumed as vegetables. Also, flower and seeds of papaya are used as traditional therapeutic remedies for various ailments. Although many studies have been done on BITC content and other medicinal properties of papaya, but very few reports on the distribution and concentration of BGSL in papaya. Thus, the first objective of this study is to profile BGSL in different parts of papaya namely seeds, leaf and unripe fruit pulp. The papaya (*C.papaya)* plant studied here was of Eksotika variety grown in the district of Kota Belud, Sabah. Because BITC is only formed when its precursor, BGSL, being enzymatically hydrolyzed by the endogenous myrosinase, therefore the second objective of the current study is to study enzymolysis and myrosinase activity under different conditions. The motivation to carry out myrosinase activity is the facts that agricultural and food processing may have varied effects on the myrosinase-GSL breakdown mechanism in which ultimately influenced the formation of the health-promoting compound, BITC. The conditions studied in enzymolysis (with sinigrin as a substrate) was the effect of hydrolysis time (10, 20, 30, 40 and 50 min). While for the myrosinase activity the condition studied were temperature (30 °C, 40 °C, 60 °C and 80 °C), pH (3.0, 4.0, 5.0, 6.0, 7.0 , 8.0 and 9.0) and concentration of ascorbic acid (2.0, 4.0, 6.0, 8.0 and 10.0 mM), ferrous and ferric ions (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM for both irons). The BGSL was extracted using deionized water and then analyzed using HPLC. For the enzymolysis and myrosinase activity, crude myrosinase extracted from seed, leaf and unripe fruit pulp of papaya was mixed with a known concentration of standard sinigrin (as substrate). After a predetermined reaction time, the unreacted (remaining) sinigrin was then extracted and analyzed using HPLC in the same manners as for BGSL. The results showed that seed has the highest content (in mg/100g dry weight) of BGSL (i.e. 490 ± 14.14) compared to leaf (14.7 ± 3.56) and unripe fruit pulp (12.5 ± 0.71). The optimum conditions for the activitiy of crude myrosinase extract from the different papaya parts are as follows: hydrolysis time, seed at 20 min (99.16%), leaf at 60 min (100 %) and unripe fruit pulp at 30 min (83.30%) ; temperature, 30 – 40 ^{o}C (all parts); pH, 8 (seed), 7 (leaf), 9 (unripe fruit pulp); concentration of ascorbic acid, 2.0 mM (all parts); no effect for all the iron concentrations tested. The findings show that myrosinase activity could occur in optimally in mild temperature within a short time and in neutral condition. As for the additives, higher concentration of ascorbic acid inhibit the activity while presence of iron does not affect myrosinase activity. Overall, this work shows that papaya seed is a rich source of BGSL, the precursor for the anticancer BITC. To ensure optimum uptake of BITC, food preparation methods must be performed in optimum conditions for the endogenous myrosinase to take place.

ABSTRAK

GLUKOSINOLAT DAN AKTIVITI MIROSINAS DENGAN KEADAAN YANG BERBEZA DI DALAM BETIK (Carica papaya)

Glukosinolat adalah metabolit sekunder yang mengandungi sulfur di mana dapat ditemui di sebahagian besar dalam keluarga Brassicaceae. Gluosinolat akan menjalani hidrolisis apabila sel mengalami perpecahan (seperti pemotongan dan pemanasan) kemudian di tindak balaskan oleh enzim mirosinas secara semulajadi untuk membentuk produk utama, isotiosianate dan/atau nitril. Isotiosianat mengandungi ciri-ciri anti karsinogenik manakala sebahagian besar nitril adalah tidak aktif. Benzil Isotiosianat (BITC), adalah produk hidrolisis daripada benzyl glukosinolat (BGSL) juga merupakan salah satu agen anti kanser yang paling mujarab. Betik (Carica papaya) mempunyai kandungan BITC yang tinggi. Di Malaysia, selain daripada buah telah masak (matang), daun muda dan buah muda atau yang belum matang di makan sebagai sayuran secara meluas. Manakala, bunga dan biji betik di gunakan sebagai ubat terapeutik tradisional untuk mengubati pelbagai penyakit. Walaupun banyak kajian telah di jalankan terhadap kandungan BITC dan ciri-ciri perubatannya, namun laporan mengenai taburan dan kepekatan BGSL di dalam betik adalah sangat sedikit. Oleh itu, objektif pertama bagi kajian ini adalah untuk memprofilkan kandungan BGSL di berlainan bahagian iaitu biji, daun dan buah muda. Jenis kultivar betik (Carica papaya) yang di gunakan di dalam kajian ini adalah daripada kultivar Eksotika yang di tanam di daerah Kota Belud, Sabah. Oleh kerana BITC hanya akan terbentuk daripada pemulanya iaitu BGSL secara hidrolisis yang di lakukan oleh enzim mirosinas, maka objektif yang kedua bagi kajian ini adalah mengkaji enzimolisis (sinigrin sebagai subdtrat) dan aktiviti mirosinas endogen di dalam keadaan yang berbeza. Matlamat kajian aktiviti mirosinas adalah berdasarkan fakta bahawa aktiviti agrikultural dan pemprosesan makanan mungkin memberi kesan yang berbeza-beza terhadap mekanisme tindak balas mirosinas-GSL yang akhirnya mempengaruhi pembentukan sebatian kesihatan iaitu BITC. Keadaan yang di kaji di dalam enzimolisis sinigrin adalah dengan kesan masa hidrolisis (10, 20, 30, 40 dan 50 min), manakala bagi aktiviti mirosinas keaadaan yang di kaji ialah suhu (30 ° C, 40 ° C, 60 ° C dan 80 ° C), pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 dan 9.0) kepekatan asid askorbik (2.0, 4.0, 6.0, 8.0, 10.0) ion ferus dan ferik (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM untuk kedua-dua ferum). BGSL telah diekstrak menggunakan air ternyahion dan kemudian dianalisis dengan menggunakan HPLC. Untuk aktiviti enzimolisis dan mirosinas, mirosinas mentah diekstrak daripada biji, daun dan pulpa buah muda, kemudiannya dicampur dengan sinigrin (sebagai substrat) yang diketahui kepekatannya. Selepas tindak balas dengan masa yang telah di tetapkan, sinigrin *yang tidak ditindakbalaskan (baki) kemudiannya diekstrak dan dianalisis dengan mengunakan HPLC dengan aturan yang sama seperti BGSL. Hasil kajian menunjukkan bahawa biji mempunyai kandungan tertinggi (dalam mg / berat kering 100g) BGSL (iaitu 490 ± 14.14) berbanding daun (14.7 ± 3.56) dan pulpa buah muda (12.5 ± 0.71). Keaadan aktiviti optimum bagi mirosinase mentah daripada bahagian betik yang berbeza adalah seperti berikut: masa hidrolisis untuk biji pada 20 minit (99.16%), daun pada 60 minit (100%) dan pulpa buah muda pada 30 minit (83.30%); suhu, 30 - 40°C (semua bahagaian); pH, 8 (biji(, 7 (daun), 9 (pulpa buah muda); kepekatan asid askorbik, 2.0 mM (semua bahagian); penambahan untuk semua kepekatan ferum yang di uji tidak memberi kesan kepada aktiviti mirosinas. Hasil kajian menunjukkan bahawa aktiviti mirosinas boleh berlaku pada suhu optimum yang sederhana, masa yang singkat dan keaadan yang neutral. Bagi bahan penambah, kepekatan asid askorbik yang tinggi menghalang aktiviti, manakala kehadiran ferum tidak menjejaskan aktiviti mirosinas. Secara keseluruhan, kajian ini menunjukkan bahawa biji betik adalah sumber yang kaya dengan BGSL, pemula anti kanser iaitu BITC. Bagi memastikan pengambilan BITC yang optimum, kaedah penyediaan makanan mesti dilakukan dalam keaadan optimum bagi memastikan aktiviti mirosinas endogen berlaku.*

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LIST OF SYMBOLS

Fe^{2+}	-	Ferrous ion
Fe^{3+}	-	Ferric Ion
^{o}C	-	Celsius (temperature)
Sr^{+2}	-	Strontium ion
Zn^{+2}	-	Zinc ion
Cu^{+2}	-	Copper ion
µm	-	Micromolar
mm	-	Milimeter
mg	-	Miligram
mL	-	Mililiter
mM	-	Milimolar
M	-	Molarity
V	-	Volume
n	-	Number of mole
M_1	-	Initial concentration of stock solution used for dilution (mM)
V_1	-	Initial volume of stock solution that is need for dilution (mL)
M_2	-	Final concentration of standard solution needed for analysis (mM)
V_2	-	Final volume of stock standard needed for analysis (mL)
R^2	-	coefficient correlation
y	-	Y axis
m	-	Gradient
c	-	Slope
x	-	X axis
SD	-	Standard deviation
nm	-	Nanometer
RF	-	Response Factor
A_x	-	Area/Peak height of analyte
C_x	-	Concentration of analyte (mM)
A_{is}	-	Area/Peak height of internal standard
C_{is}	-	Concentration of internal standard (mM)

μl	-	Microliter
RSD	-	Relative standard deviation
FW	-	Fresh weight
g	-	gram
mM	-	Milimolar per minute
min^{-1}		
p	-	Significant value
Ca^{2+}	-	Calcium ion
Zn^{2+}	-	Zinc ion

LIST OF ABBREVIATIONS

GSL	-	Glucosinolate
SMCSO	-	S-methylcystine sulfoxide
ITC	-	Isothiocyanate
SFN	-	Sulforaphane
PEITC	-	Phenyl ethyl isothicyanate
BITC	-	Benzyl Isothicyanate
NSP	-	Nitrile specifier protein
ESP	-	Epithiospecifier protein
TFP	-	Thiocyanate-forming protein
BGSL	-	Benzyl Glucosinolate
Ala	-	Alanine
Leu	-	Leucine
Ile	-	Isoleucine
Val	-	Valine
Met	-	Methionine
AITC	-	Allyl Isothiocyanate
GMG-ITC	-	Glucomoringin Isothiocyanate
6-MITC	-	6-(methylsulfinyl)hexyl Isothiocyanate
GST	-	Glutathione S-transferases
VEGF	-	Vascular endothelial growth factor
HIF-1α	-	Hypoxia inducible factor
IBD	-	Inflammatory bowel disease
DF	-	Dietary fibre
NaOH	-	Sodium Hydroxide
HCL	-	Hydrochloric Acid
TFA	-	Trifluoroacetic acid
Tris	-	trishydroxymethylaminomethane
R	-	Replicate
SIN	-	Sinigrin
L-phe	-	L-phenylalanine

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Fruits, vegetables and plants are known to be a major nutrient sources for human. Compounds such as secondary metabolites, vitamins and minerals supply are important in reducing disease risk, for example as chemoprotective agents against cancers (Boeing *et al.*, 2012; Talaviya, 2011). The effectiveness of cancer prevention through consumption of fruits and vegetables, mostly cruciferous vegetables have produced many epidemiological finding and public attention (Béliveau and Gingras, 2007; Block *et al.*, 1992; Hayes *et al.*, 2008; Herr and Büchler, 2010a; Johnson *et al.*, 1994; Key, 2011; Reiss *et al.*, 2012; Verhoeven *et al.*, 1996).

In daily diet, vegetables from cruciferous family including broccoli, radish, cauliflower, kale, radish, Brussel's sprouts, cabbage and watercress are mostly cultivated and consumed universally. Cruciferous vegetables can be consumed as a salad or cooked. These vegetables are rich in major nutritional components such as vitamins (ascorbic acid, tocoperols, provitamin-A and folic acid), minerals (calcium, copper, selenium, zinc and manganese) carbohydrates and proteins (Singh *et al.*, 2001). Additionally, cruciferous vegetables also rich in health beneficial secondary metabolites including glucosinolates (GSL), flavonoids, S-methylcysteine sulfoxide, anthocyanins, carotenoids, coumarins, terpenes, antioxidant enzymes and other minor components (Manchali *et al.*, 2012).

Among the phytochemicals in cruciferous vegetables, GSL is one of the major components other than S-methylcystine sulfoxide (SMCSO) (Herr and Büchler, 2010a; Manchali *et al.*, 2012). GSL are sulphur- and nitrogen-containing compounds, also known as "mustard oil GSL" found mostly in Brassica plants with potential benefits for human health (Du and Halkier, 1998; (Rosseto et al., 2008); Herr and Büchler, 2010). So far, there are more than 132 natural GSL (i.e. with different *R*-

groups) have been identified (Fahey *et al.*, 2001; Agerbirk and Olsen, 2012). GSL are mainly found in Brassicals order, which include the Brassicaceae family (Fahey *et al.*, 2001). GSL are chemically stable unless when they meet myrosinases (β-thioglucoside glucohydrolases, EC: 3.2.3.1) when the cell rupture. GSL and myrosinase are stored in different cellular compartments (Kissen & Bones, 2009; Fahey *et al.*, 2001). Food preparation activity such as cutting, chewing, thawing, cooking, fermenting or freezing cause the damage of tissue and release the myrosinase and GSL (Fahey *et al.*, 2001). The myrosinase then rapidly hydrolyzed GSL to release glucose and unstable intermediate, aglucone. Under different conditions (e.g. pH, ascorbic acid, temperature and iron), the aglucone spontaneously rearrange into different products (**Figure 1.1**) namely isothiocyanate (ITC), thiocyanate, nitrile, epithionitrile and oxazolidine-2-thiones (Blažević and Mastelić, 2009).

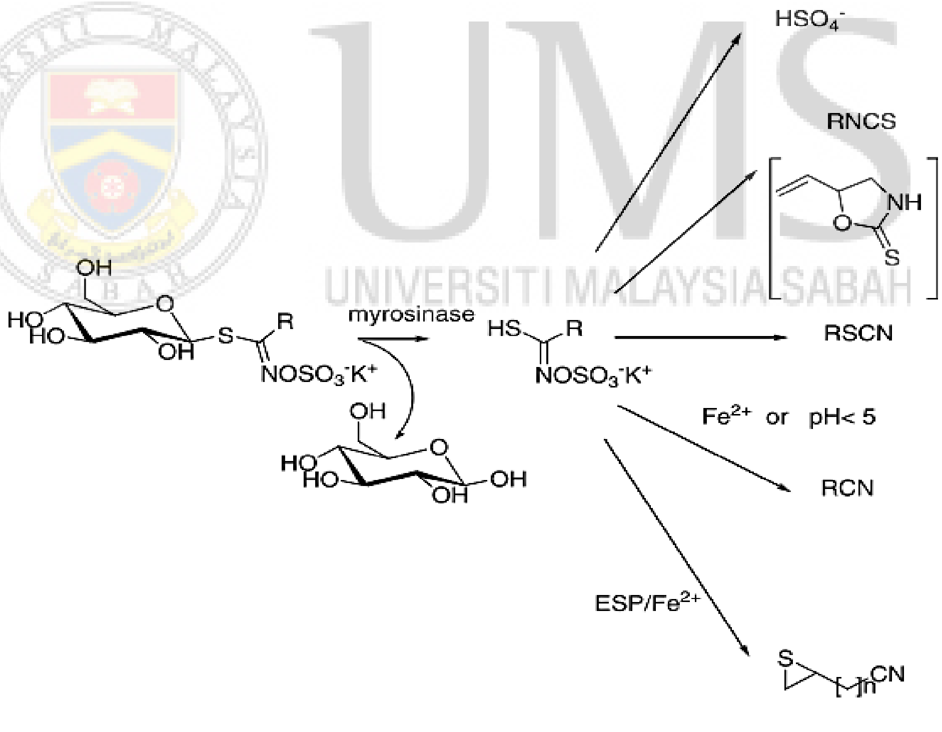

Figure 1.1: **General Diagram Showing The Main Products Of Enzymatic GSL Degradation Products. Source : Bones And Rossiter, 2006.**

GSL themselves are not directly bioactive, but their breakdown product formed through the hydrolysis by myrosinase, especially ITCs which also known as mustard oils, which has ability to prevent and to treat human diseases (Brown and Hampton, 2011). It is widely reported that ITCs have inhibited liver, lung, colon, breast, ovary, prostate, bladder and pancreas cancer (Zhang et al., 2005; Vig *et al.*, 2009; Herr and Buchler, 2010; Chang *et al.*, 2013; Chen *et al.*, 2014; Lamy *et al.*, 2013; Misra *et al.*, 2014; Rajendran *et al.*, 2013; Savio *et al.*, 2014; Wiczk *et al.*, 2012; Yang *et al.*, 2014).

The most convincing anticancer are sulforaphane (SFN), benzyl isothiocyanate (BITC) and phenylethyl isothiocyanate (PEITC) Wu *et al.*, 2005; Myzak and Dashwood, 2006; Fimognari and Hrelia, 2007; Nakamura *et al.*, 2008; Gupta *et al.*, 2013; Gupta *et al.*, 2014a; Gupta *et al.*, 2014b; Huang *et al.*, 2014; Liu *et al.*, 2013; Misra *et al.*, 2014; Nikhil *et al.*, 2014; Rao, 2013; Savio *et al.*, 2014; Sehrawat *et al.*, 2013). *In vitro* and *in vivo* studies have proven that ITC effects the steps in cancer development by inhibiting enzyme phase I which involves in the bio-activation of chemical carcinogens. ITC also induces enzyme phase II in order to protect cells or tissues against carcinogenic intermediates (Fimognari and Hrelia, 2007; Moreno *et al.*, 2006). ITC compounds show a similar biological activity such as SFN and PEITC, mostly can be found in cruciferous plant while BITC rarely exist in Brassicaceae plant (Fahey *et al.*, 2001). Other types of GSL hydrolysis products, namely nitriles and thiocyanates have no beneficial effects on human (Traka and Mithen, 2008; Jeffery and Araya, 2008). In fact, Latté and co-workers (2011) have found that they are poisonous to the animal but cause no harm to human.

Plant species, *R*-group structure, pH, temperature, processing conditions, ascorbate, cell iron concentration and protein specifier (namely nitrile specifier protein (NSP), epithiospesifier protein (ESP) and thiocyanate-forming protein (TFP)) influenced the activity of myrosinase and the production of GSL hydrolysis products (Vaughn and Berhow, 2005; Bones and Rossiter, 2006; Williams *et al.*, 2009; Guo *et al.*, 2013a; Kong *et al.*, 2012; Kuchernig *et al.*, 2012; Kuchernig *et al.*, 2011; Nong *et al.*, 2010; Oliviero et al., 2012; Piekarska *et al.*, 2013; Prakash *et al.*, 2013; Shen *et al.*, 2010; Williams *et al.*, 2010; Dosz *et al.*, 2014). Recent study shows that, the optimal pH and temperature of myrosinase activity varies among plant species, ranging from pH 4 to 9 and 20 °C to 70 °C (Travers-Martin *et al.*, 2008). Shen *et al.* (2010) found that the optimal hydrolyzed conditions of glucoraphanin to SFN by the endogenous myrosinase of broccoli were at 25 °C, pH 4.0.

At low concentration of ascorbic acid, myrosinase activity was activated, but the activity was inhibited by the addition of ascorbic acid concentration (Burmeister *et al.*, 2000; Tsuruo and Hata, 1967; Nong *et al.*, 2010). While the addition of ferric and ferrous ions seems to have variation in activity depending on the source of the myrosinase enzyme (Illiams *et al.*, 2010; Liang et al., 2006; Prakash *et al.*, 2013; Uda *et al.*, 1986).

Myrosinase activity in Brassica significantly varies by season, botanical group and plant species, suggest that activity is high in fall than in spring season (Charron *et al.*, 2005). Since most of the biological effects appreciated by man are not caused by the GSL per se but by the certain breakdown products, hence the activity of enzyme myrosinase should be studied systematically in order to find an optimum condition for the high yield production of ITC to assure their effectiveness in preventing cancer.

The information about GSL content before and after the processing condition has been less studied. Recent findings show the content of GSL in Brassica was increased at high temperature, suggested due to chemical extractability of the GSL (Song and Thornalley, 2007; Verkerk and Dekker, 2004). Depending on the method that used for the cooking process, most of GSL contents reduced in boiling which may due to the leaching into the cooking water or soups (Song and Thornalley, 2007; Volden *et al.*, 2008 and 2009). In dietary intake, GSL increment through leaching cannot be considered as the dietary lose. Consumer should include the soup in order to get the health beneficial effect of the GSL compound. Furthermore, colonic micro flora that live in the gastrointestinal tract have the ability like myrosinase enzyme where it can hydrolyzed GSL into breakdown product (Li and Kushad, 2005). An understanding of the physical and biochemical changes occurring before the ingestion of the GSL-containing vegetable may help to interpret the metabolic fate of

GSL in the experimental studies and inform the subsequent formulation of dietary strategies to optimize the uptake of ITC *in vivo*.

1.2 Importance of the Study

In this study, determination of the amount of the dominant (if not sole) GSL in papaya, benzyl GSL (BGSL), was carried out. The current work is a continuation (or extension) of the work previously carried out by a member of our GSL research group i.e. Miss Gayathri Nagappan (2012). Her work was on the hydrolysis products of BGSL, which were mainly BITC and benzyl nitrile. The current study is focusing on the profiling the precursor of BITC (which is the BGSL) in papaya. It is important to analyze the amount and distribution of this BGSL in its plant-source so that uptake of the anticancer compound (BITC) can be possibly optimized. As seen in the literatures reviewed in this chapter, most of the previous studies were focusing on studies of GSL and GSL hydrolysis products in cruciferous types of brassica while research in papaya is still very limited. BGSL was first observed in papaya by (Tang, 1973). The only similar studies were done by Li *et al.*, (2012); Nakamura *et al.*, (2007) and Ascimento *et al.* (2008) on papaya from Solo and Golden cultivars. The previous works were limited to papaya seeds and ripe fruit pulp. The current study is on seed, young leaf and unripe fruit pulp of papaya from Eksotika cultivar. This is the most popular cultivated papaya cultivar in Malaysia. Eksotika papaya is resulted from a cross between Subang 6 and Hawaiian Sunrise Solo, which was released by MARDI in 1987 (Chan, 1987). This is the first report of GSL profiling in this papaya cultivar.

The GSL-myrosinase system is the crucial part for the formation of BITC. The GSL is enzymatically hydrolized into BITC by the naturally-occurring enzyme myrosinase. In another words, the amount of BITC (and/or its counterpart, benzyl nitrile) formed is very much dependent on the activity of myrosinase. Hence, it is important to understand the mechanism of myrosinase activity and, if possible, the optimum conditions where it works the best. Recently, the activity of myrosinase gene cloned from papaya was observed (Ascimento *et al.*, 2008; Nong *et al.*, 2010; Wang *et al.*, 2009). The myrosinase genes found were namely CpTGG1, CpTGG2 (from Solo cultivar) and CpTGG3 (from Golden cultivar). Each myrosinase gene was