ANALYSIS OF ACTIVITY OF EPITHIOSPECIFIER PROTEIN (ESP) IN DIFFERENT PARTS (FLOWERS AND LEAVES) OF *Carica papaya*

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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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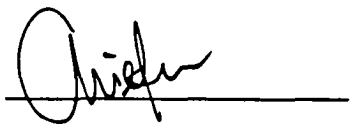

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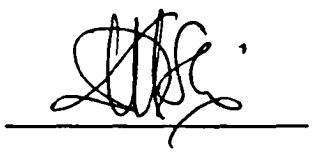

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ABSTRACT

Glucosinolate are sulphur-containing glucosides found in Brassicaceous plants that can be hydrolyzed enzymatically by plants myrosinase or non-enzymatically to form primarily isothiocyanates and/or simple nitriles. From a human health perspective, isothiocyanates are quite important because they are the major inducers of carcinogen detoxifying enzymes. Specifier proteins such as ESP may occur in some but not all glucosinolate-containing plants and promote the formation of biologically active non-isothiocyanate products upon myrosinase-catalyzed glucosinolate breakdown. In this study, the hydrolysis products of glucosinolates in leaf and flower in *Carica papaya* have been identified by using Gas Chromatography-Mass Spectrometry (GC-MS). Benzyl isothiocyanate was found as one of the hydrolysis product. Surprisingly, benzyl thiocyanate was found in *Carica papaya* may suggest the activity of TFP. New finding of benzene propanenitrile suggested the presence of gluconasturin in leaf and flower of *Carica papaya*. As the presence of benzene propanenitrile at room temperature in flower and leaf are suspected due to the presence of ESP, further studies on ESP could confirm this postulation. The ratio of epithionitrile to simple nitrile is used to analyze the ESP activity. In this study, there was no epithionitrile found as hydrolysis product. The possible explanation for this may be due to the low concentration of ferrous ion, high pH and absent of alkenyl glucosinolate in the papaya samples. More research should focus on the conditions for the ESP activity in *Carica papaya* as this will bring a new understanding in health benefit.

ABSTRAK

TAJUK: ANALISIS AKTIVITI PROTEIN EPITIOSPECIFIER (ESP) DALAM PELBAGAI BAHAGIAN POKOK BETIK (BUNGA DAN DAUN)

Glukosinolat merupakan glikosida yang mengandungi sulfur dalam tumbuh-tumbuhan Brassicaceous yang boleh melalui hidrolisis sebatian oleh enzim mirosinas atau membentuk isotiosianat dan/atau nitril. Dari perspektif kesihatan manusia, isotiosianat memainkan peranan penting sebagai "inducer" utama enzim nyah-toksik karsinogen. "Protein specifier" seperti ESP mungkin wujud di dalam tumbuh-tumbuhan yang mengandungi glukosinolat dan menggalakkan pembentukan produk biologi aktif bukan isotiosianat apabila glukosinolat mengurai bermangkinkan mirosinas. Dalam kajian ini, produk hidrolisis glukosinolat dalam daun dan bunga betik Carica papaya telah dikenal pasti dengan menggunakan Kromatografi Gas-Spektrometri Jisim (GC-MS). Benzil isotiosianat dikesan sebagai salah satu produk hidrolisis. Benzil tiosianat telah dikesan dalam Carica papaya dan mencadangkan aktiviti TFP. Penemuan baru benzena propanenitril dalam daun dan bunga Carica papaya mencadangkan kewujudan gluconasturin. Hasilan penemuan benzena propanenitril dalam bunga dan daun mencadangkan kehadiran ESP, kajian lanjut mengenai ESP dapat mengesahkan cadangan ini. Nisbah epitionitril kepada nitril mudah digunakan untuk menganalisis aktiviti ESP. Dalam kajian ini, tiada epitionitril yang dikesan sebagai produk hidrolisis. Hal ini berlaku kemungkinan disebabkan oleh kepekatan ion ferus yang rendah, pH yang tinggi dan tiada alkenil glukosinolat terdapat di dalam sampel betik. Lebih banyak penyelidikan perlu dijalankan dengan memberi fokus kepada keadaan optima untuk aktiviti ESP dalam Carica papaya kerana ini akan membawa kepada pemahaman yang baru dalam manfaat kesihatan.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
α	Alpha
ß	Beta
g	Gram
hrs	Hour
L	Litre
mM	Milli Molar Concentration
mg	Milligram
min	minutes
mL	millilitre
µL	microlitre
ESP	Epithiospecifier Protein
GC/MS	Gas Chromatography Mass Spectrometry

CHAPTER 1

INTRODUCTION

1.1 Specifier protein

Specifier protein is a protein that combines with an enzyme or enzyme-substrate complex which modifies that catalytic specificity of the system. An example is α-lactalbumin, which modifies the substrate (acceptor) specificity of N-acetyllactosamine synthase from N-acetylglucosamine to glucose. Specifier proteins occur in some but not all glucosinolate-containing plants and promote the formation of biologically active non-isothiocyanate products upon myrosinase-catalyzed glucosinolate breakdown (Kuchering *et al.*, 2012). In the previous research, previous study had found three types of specifier protein in the Brassicaceae family. The three types of specifier protein are NSP, nitrile-specifier protein, ESP, epithiospecifier protein, TFP, thiocyanate-forming protein. As a structural feature, specifier protein is predicted contain a series of sheets (Adams *et al.*, 2000). Specifier protein causes impact on the outcome of glucosinolate hydrolysis without having hydrolytic activity on glucosinolates themselves

(Wittstock and Burow, 2007). In the presence of specifier proteins, glucosinolates hydrolysis result in nitriles, epithionitriles and organic thiocyanates whose biological function are currently unknown (Wittstock and Burow, 2007). The biological role of specifier proteins has therefore remained an open question.

1.2 The Epithiospecifier Protein (ESP)

Glucosinolates co-occur with myrosinase isoenzymes (Thioglucosidase; EC 3.2.1.147), which catalyze the hydrolysis of the β-Dthioglucopyranoside bond (Bellostas *et al.*, 2003; Bjergegaard *et al.*, 1994; Bjergegaard *et al.*, 2003; Petersen *et al.*, 2003). ESP was describe as a relatively labile protein with a molecular weight of 30-40kDa that could stabilize by the addition of Fe^{2+}. The most common products are isothiocyanates, which arise from a 'Lossen' like arrangement to give isothiocyanates. The first observation of a nitrile containing sulphur was made by (VanEtten *et al.*, 1996). The structures of these sulphur containing nitriles were epithionitriles (Daxenbichler *et al.*, 1968). It was originally thought that nitrile formation was non-enzymic and depended only on ferrous ions and protons. However, it is now accepted that epithiospecifier protein is responsible for nitrile formation although the actual mechanism is still an open question. Epithiospecifier protein uses the unstable thiohydroximate generated by myrosinase to produce nitriles and epithionitriles. ESP was first partially characterised by Tookey (1973) and more recently was purified and characterised by Foo *et al.*, (2000) and Bernardi *et al.* (2000). ESP is a relatively stable protein which has been shown to be important in plant-insect interactions (Lambrix *et al.*, 2001). The ESP encoding gene has been cloned from *Arabidopsis thaliana* (Lambrix *et al.*, 2001). The reaction requires ferrous

ions for activity (Torres Zabala *et al.*, 2005), which presumably allows the formation of an intermediate between the thiohydroximate and epithiospecifier protein. Insertion of sulphur has been shown (Brocker and Benn, 1983) to occur via an intramolecular reaction by using ^{35}S labeled sulphur. Recently, it was shown that it was possible to modify the glucosinolate hydrolysis profile by overexpressing ESP in *Arabidopsis thaliana* thereby switching from production of isothiocyanates to nitriles (Torres Zabala *et al.*, 2005). ESP is an important factor in determining isothiocyanate levels in broccoli as it relatively heat sensitive in relation to myrosinase (Matusheski *et al.*, 2003, 2004).

There are several aspects influencing the glucosinolates hydrolysis pathway such as plant species, side chain, cell pH, temperature, ascorbic acid, iron concentration and other protein elements like epithiospecifier protein (ESP), thiocyanate-forming protein (TFP) and nitrile specifier protein (NSP) which may permit alternatives products such as nitrile, epithionitriles and thiocyanates instead of isothiocyanates (Vaughn and Berhow, 2005; Bones and Rossiter, 2006). Sufficient endogenous iron content in plant tissues can trigger epithiospecifier protein (ESP) and thiocyanate forming protein (TFP) activities hence produce more nitriles and thiocyanates instead of isothiocyanate (Matusheski *et al.*, 2006). ESP hydrolyzed glucosinolates with terminal alkenyl group into epithionitrile and at the same time, all other glucosinolates hydrolyzed into simple nitrile (Williams *et al.*, 2010). Specifier such as TFP promotes thiocyanate formation (Burow and Bergner, 2007) and NSP promotes simple nitrile formation (Kiissen and Bones, 2009). All specifier proteins work by binding to myrosinase as a cofactor or by binding to the unstable aglycone

the produce from glucosinolate-myrosinase interaction before the aglygone rearrange (Burrow *et al.*, 2006).

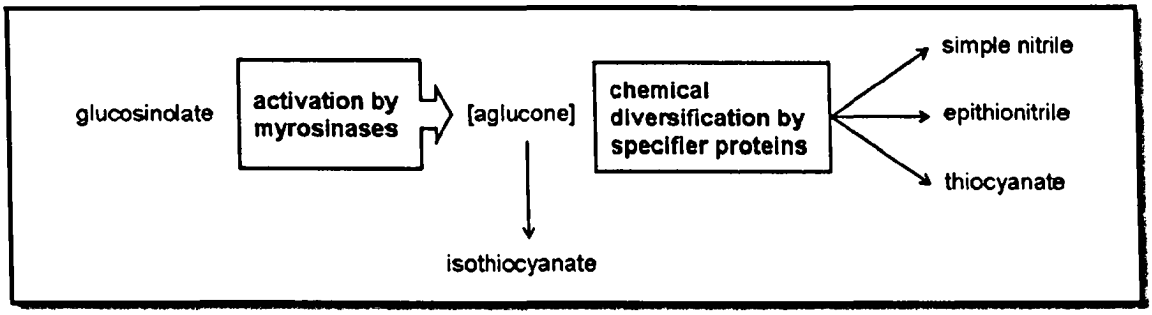

Figure 1.1 Glucosinolate to breakdown upon tissue disruption

Activation of glucosinolates is initiated by myrosinases which cleave the thioglycosidic bond in the glucosinolate sceleton. The resulting agluca spontaneously rearrange to form isothiocyanates unless specifier proteins convert them into simple nitriles, epithionitriles, or thiocyanates depending on the biochemical properties of the specifier protein and the chemical nature of the glucosinolate side chain (Wittstock and Burow, 2010).

There are various reports on glucosinolates and their hydrolysis products in various plants. Most of them were focusing on glucosinolates in crucifer vegetables such as broccoli (Tian *et al.*, 2005), cabbage (Oerlemans *et al.*, 2006) and cauliflower (Valette *et al.*, 2003). In this study, the hydrolysis co-factor ESP from *Carica papaya* was investigated. In report compromise the experiment of the analysis of the amount and activity of the ESP in leaves and flowers of *Carica papaya*. The understanding of the amount and the activity will show us about the presence of specifier protein in

Carica papaya. The ability to form non-isothiocyanate products by specifier protein activity may reduce the anticancer properties of papaya extracts.

1.3 Objective of Research

The proposed research was studied to achieve the following objectives:

1) To profile hydrolysis product in flower and leaf of *Carica papaya*
2) To analyze the activity of Epithiospecifier Protein (ESP) in different parts (flower and leaf) in *Carica papaya*

1.4 Scope of Research

The study focuses on the profiling the hydrolysis product in flower and leaf of *Carica papaya*. The hydrolysis product found can be used to determine the type of glucosinolate contained in *Carica papaya*. The activity of Epithiospecifier Protein (ESP) in different parts (flower and leaf) in *Carica papaya* is analyzed by determined the present of epithionitrile. Ratio of simple nitrile and epithionitrile is use to calculate the activity of Epithiospecifier Protein in *Carica papaya*.

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