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EFFECTS OF ASCORBIC ACID AND FERUM IONS CONCENTRATION ON THE HYDROLYSIS OF GLUCOSINOLATE AND MYROSINASE ACTIVITY IN THE WATERCRESS (*Nasturtium Officinale sp.*)

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Graphical abstract

WATERCRESS

Abstract

Watercress *(Nasturtium officinale sp.)* from the Brassicae family contains phenethyl glucosinolates (gluconasturtiin) as the main glucosinolate (GLS). The enzymatic hydrolysis products by naturally-occuring myrosinase produced phenethyl isothicyanate (PEITC) which reported to possess anti-carcinogenic activity. Depending on several factors, its counterpart, phenethyl nitrile (PEN) can also be formed as hydrolysis product. This study investigated the effects of adding ascorbic acid and Fe^{2+} ions at different concentration on the hydrolysis of gluconasturtiin. Hydrolysis products were extracted using dichloromethane and analyzed semi-quantitatively by using GCMS. The results showed that PEITC increased at the low concentration of ascorbic acid (up to 0.06M). Similarly, addition of up to 0.06M Fe^{2+} ions increased PEITC; higher than 0.06M inhibits the formation of PEITC. Interestingly, similar trend for the production of PEN was detected. This study also investigated myrosinase activity both by exogenous and endogenous methods at different concentrations of ascorbic acid and Fe^{2+} ions using standard sinigrin as subsrat. Overall, the myrosinase activity was more active at the low concentrations of ascorbic acid. Also, the exogenous method is more efficient than endogenous. This study proved that the presence of reducing agents such as ascorbic acid and Fe^{2+} ions during the preparation of watercress as food would affect the production of the health-promoting PEITC.

Keywords: Watercress, glucosinolates, phenyl ethyl isothiocyanate, myrosinase enzymes

Abstrak

Tumbuhan selada air (*Nasturtium officinale sp.*) dari keluarga Brassicaceae mengandungi glukosinolat utama jenis feniletil glukosinolat (glukonasturtin). Hasil hidrolisis oleh enzim mirosinas menghasilkan feniletil isotiosianat (PEITC) iaitu suatu sebatian anti kanser. Bergantung kepada beberapa faktor, molekul nitril (tiada ciri anti kanser) turut hadir sebagai produk sampingan. Kajian ini bertujuan untuk mengkaji kesan kepekatan asid askorbik dan ion Fe^{2+} ke atas hasil hidrolisis glukonasturtin yang terdapat dalam selada air. Lima kepekatan asid askorbik dan ion Fe^{2+} yang berbeza telah dikaji iaitu 0.02M, 0.04M, 0.06M, 0.08M dan 0.10M. Hasil hidrolisis diekstrak dengan menggunakan pelarut diklorometana dan dianalisis secara semi-kuantitatif dengan menggunakan GCMS. Hasil kajian menunjukkan jumlah PEITC meningkat pada kepekatan asid askorbik yang rendah (sehingga 0.06M). Manakala pengaruh kepekatan ion Fe^{2+} yang rendah membantutkan pembentukan PEITC tetapi ia lebih menggalakkan pembentukan nitril. Kajian ini turut mengkaji aktiviti mirosinas selada air secara eksogen dan endogen di bawah kepekatan asid askorbik dan ion Fe^{2+} yang berbeza dengan menggunakan HPLC. Secara relatif, aktiviti mirosinas lebih aktif pada kepekatan asid askorbik yang rendah manakala bagi ion Fe^{2+}, aktiviti mirosinas meningkat seiring dengan peningkatan kepekatannya. Walau bagaimanapun, kaedah secara eksogen lebih cekap berbanding dengan endogen. Hasil kajian ini menunjukkan bahawa kehadiran agen-agen penurun seperti asid askorbik dan ion Fe^{2+} semasa penyediaan selada air sebagai bahan makanan akan

mempengaruhi penghasilan PEITC.

Kata kunci: Selada air, glukosinolat, fenil etil isotiosianat, enzim mirosinas

1.0 INTRODUCTION

Foods that contain bioactive phytochemicals play an important role in human health like prevention of cancer. Vegetables such as cabbage, broccoli, spinach, tomatoes and watercress act as a powerful weapon to prevent and reduce the possibility of cancer growth [1]. Watercress (*Nasturtium officinale sp.)* is a popular traditional salad which comes from the family Brassicaceae or Cruciferae. The previous scientific studies has shown that watercress is the richest natural source of a compound called phenethyl isothiocyanate (PEITC) that have powerful anti cancer properties, anti-inflammatory and antioxidants properties [2].

Glucosinolates (GSLs) are organic anion containing β-D-thioglucose and sulphonated oxime moieties which can be found on the seeds, roots, stems and leaves of plants [3]. The first glucosinolates were discovered in 1831 when Robiquet and Boutron isolated the sinalbin (4-hydroxylbenzyl glucosinolates) from the seeds of white mustard (*Sinapsis alba*). Since then, more than 120 different GSLs (β-thioglucose-*N*-hydroxysulfates) have been isolated from the species of Brassicaceae [4]. Glucosinolates, the precursors of isothiocyanates are present in sixteen families of dicotyledonous angiosperms especially in the Brassicaceae [5] and can be divided into three groups: aliphatic GSLs, aromatic GSLs and indole GSLs based on R group from amino acid precursor. The side chain (R) can determine whether the group consists of aliphatic GSLs (glucoraphanin, sinigrin), aromatic GSLs (glucotropheolin) or indole GSLs (glucobrassicin). Cultural conditional including day length, temperature and agronomic factor can influence the glucosinolates variation. Holst *et al.* [6] states during the hydrolysis reaction, glucosinolates are hydrolyzed by the myrosinase enzyme to produce isothiocyanates, thiocyanates, nitriles, epithionitrile and oxazolidine-2-tions by chewing vegetables or tissues damage during handling the plants [7]. These hydrolysis products depend on several factors such as side chain, pH value, temperature, concentration of ascorbic acid, the presence of epithiospesifier protein and ferrous ions [8].

In the present study, we determine the effect of ascorbic acid and ferrous ions concentration on the hydrolysis of glucosinolates and myrosinase activity in the watercress (*Nasturtium Officinale sp.*). According to Shen *et al.* [9], different concentration of ascorbic acid and Fe^{2+} ions will affect the myrosinase activities with the absorption of sinigrin in HPLC analysis.

2.0 MATERIALS AND METHODS

2.1 Sample Preparation

The fresh, healthy watercress samples were collected from Kampung Melangkap, Kota Belud, Sabah. The sample were frozen in liquid nitrogen and freeze-dried.

2.2 Hydrolysis of Glucosinolates

The lyophilized watercress sample was ground into fine powder using a mortar and pestle, and stored at -80^{O}C. A 1.00 g of watercress powder were solubilized in 5 mL of 0.02M ascorbic acid and left at room temperature for 30 mins. Then 6mL dichloromethane was added into the sample and shaken using a mechanical shaker at 2500 rpm for 30 mins. The sample was then transferred into centrifuge bottles and were centrifuged at 8500 rpm for 10 mins. The samples were then filtered into beaker and the organic layer was dried by addition of sodium sulfat anhydrous. These steps were repeated three times. The filtered samples were extracted with dichloromethane. The organic layer were concentrated using rotary evaporator, filtered through 0.45 µm microfilter and then analyzed using GCMS. The same steps were repeated for ascorbic acid at different concentrations; 0.04M, 0.06M and 0.08M. Similar procedures were carried out for the effects of ferrous ions using the same concentrations

2.3 Standard Enzymolysis

Two types of myrosinase enzyme: exogenous and endogenous myrosinase at different concentrations (0.02M, 0.04M, 0.06M and 0.08M) of ascorbic acid and ferrous ions were studied.

In both myrosinase activity, a fraction of 1.00g of watercress powder were dissolved in 30mL 0.2M Tris-HCl buffer solution, filtered and centrifuged. The mixture was combined with 80% ammonium sulfate. The solution was placed in dialysis bag and soaked for 1h in a beaker containing 0.2M Tris-HCl buffer and kept overnight at 4^{o}C. The reaction mixture consisted of 1mL of 0.02M ascorbic acid, 1mL extract of myrosinase and 100 µL 24mM sinigrin was dialyzed for 2h in exogenous myrosinase but 8h for endogenous myrosinase. Then the sample was

boiled at 100°C for 5min before analyzed using HPLC. This steps were repeated at different concentrations; 0.04M, 0.06M, 0.08M and 0.10M of ascorbic acid and ferrous ions.

2.4 HPLC Analysis

Myrosinase activity was determined by evaluating the rate of hydrolysis of sinigrin using HPLC. HPLC analysis was performed using the Agilent 1100 Series with C18 column (250 mm × 4.6 mm, 5µm). The HPLC parameter was as follows: injection volume, 10 µL; column temperature, 25^{O}C; flow rate, 1.0 mL/min; Binary solvent mixture acetonitrile: water with a ratio of 80:20 (v/v) as the mobile phase and UV detection at 228nm.

3.0 RESULTS AND DISCUSSIONS

3.1 Identification of Hydrolysis Products

Glucosinolate degraded when myrosinase catalyzed hydrolysis due to the tissue disruption in plants such as cutting and heating [10]. Myrosinase enzyme reacts with β-thioglucoside bond of phenethyl glucosinolate molecules leads to an unstable aglycone which rapidly undergo intramolecular (Lossen) rearrangement to yield the main hydrolysis product, phenethyl isothiocyanate (PEITC) (Figure 1) [11]. On GC chromatogram, a noticeable peak of standard PEITC is seen at 13.71 min (Figure 2).

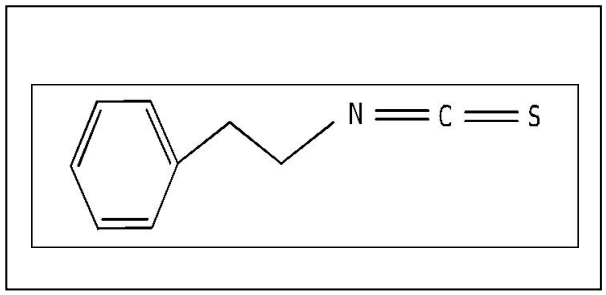

Figure 1 Structure of phenyl ethyl isothiocyanate (PEITC)

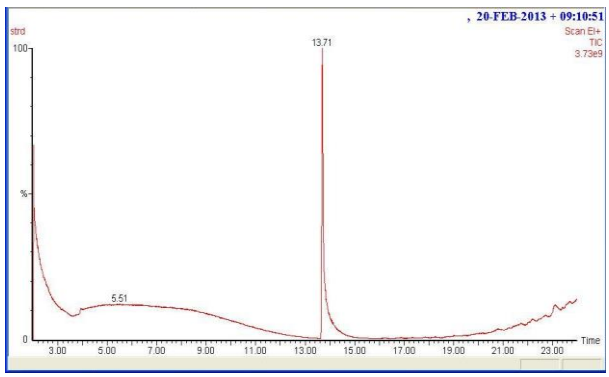

Figure 2 GC chromatogram of standard PEITC at retention time 13.71 min

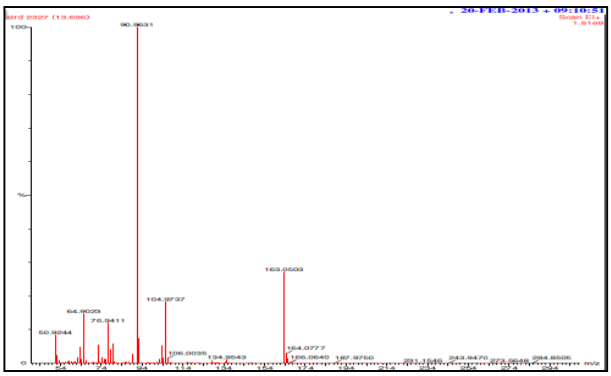

Figure 3 MS chromatogram of standard PEITC at retention time 13.71 min

The molecular ions of standard PEITC at 13.71 minutes are m/z 77, 91, 105, and 163 (Figure 3). The molecular ion (M^+) peak of PEITC with odd mass value, 163 g/mol due to the presence of nitrogen atom in the structure [12].

The influence of different concentration of ascorbic acid and ferrous ions on the hydrolysis product, PEITC and PEN are shown in Figures 4 and 5.

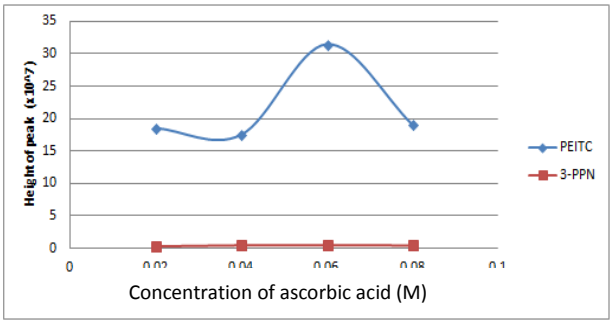

Figure 4 Production of PEITC at different concentration of ascorbic acid

Based on Figure 4, the presence of PEITC show the highest peak at 0.06M ascorbic acid concentration. This optimum peak proves that the presence of ascorbic acid increases the production of PEITC in the hydrolysis reaction. However production of PEITC decreases at 0.08M ascorbic acid due to the disturbance at myrosinase enzyme where the structure of myrosinase enzymes is deionized. Previous studies [13] proved that myrosinase activity is retarded at high concentrations of ascorbic acid. This shows that low concentrations of ascorbic acid, 0.02M will promote the activity of myrosinase to give high production of PEITC.

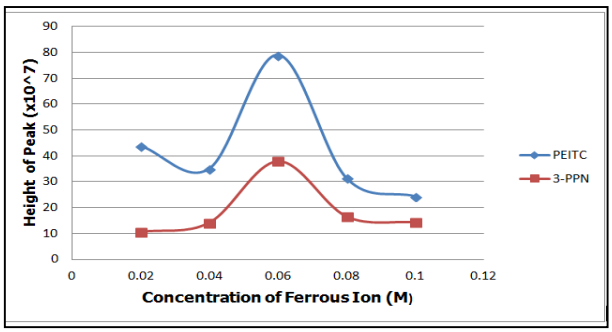

Figure 5 Production of PEITC at different concentration of Fe^{2+} ions

Interestingly, at 0.06M Fe^{2+}, the highest formation of both PEITC and PEN was recorded (Figure 5). Also, PEITC was higher at 0.02M compared to 0.04M, 0.08M and 0.10M of ferrous ions. Liang *et al*. [11] state that the formation of PEITC is low at high concentration of ferrous ion. Unlike PEITC, the production of PEN at 0.02M and 0.04M was lower but higher at 0.10M Fe^{2+}. This is because formation of nitrile is more dependent on ferrous ions [7]. According Serra *et al*. [14], ferrous ions act as inhibitor for the formation of isothiocyanate but encourage the formation of nitrile.

3.2 Myrosinase Activity

Using standard sinigrin as substrat, the myrosinase activity was calculated using equation y = 283.52x + 170.04. Figure 6 shows calibration curve of the standard sinigrin over the range of 1.0 to 5.0mM.

$$\text{Myrosinase activity} = \frac{[Initial\ sinigrin] - [Final\ sinigrin]}{time\ (min)}$$

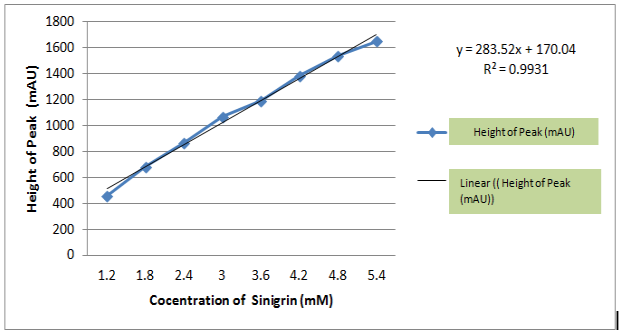

Figure 6 Height of peak for sinigrin standard at different concentration

The influence of different concentration of ascorbic acid and ferrous ions on the myrosinase activity are shown in Figures 7 and 8.

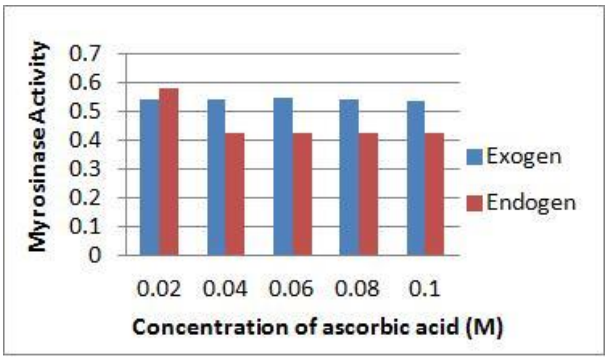

Figure 7 The height peak of Fe^{2+} ions by exogenous and endogenous enzymes at different concentrations

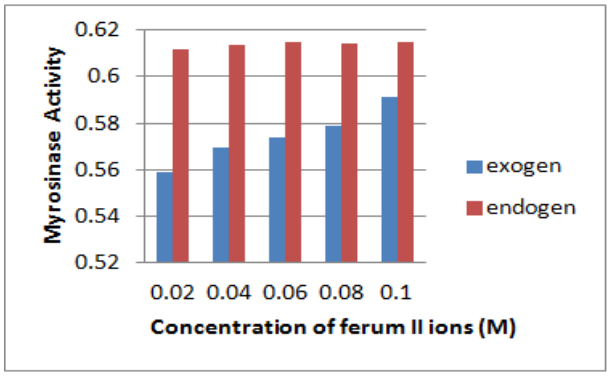

Figure 8 The height peak of Fe^{2+} ions by exogenous and endogenous enzymes at different concentrations

Figure 7 shows myrosinase activity tested using exogenous procedure maintained at around 0.54mM/min over the range of ascorbic acid concentrations. However, this drops from 0.58mM/min to 0.42mM/min when tested endogenously. Myrosinase activity will be retarded [13] at high ascorbic acid concentration due to the degradation of ascorbic acid affects the sinigrin at high level.

Figure 8 shows increasing trend of myrosinase activity as the concentration of Fe^{2+} increases. Andersson *et al.* (2009) reckoned that the presence of low concentration of ascorbic acid can increase the catalytic activity of myrosinase; however higher concentration of ascorbic acid can promotes inhibitory effect on the enzyme. Ascorbic acid can be an effective activator causing the conformational change of protein. The surface of myrosinase consists of one site for substrate and two sites for ascorbic acid. The substrate site has two moieties, one for aglycone part of GSL and one for glycone. One of the moiety consists one of the site for ascorbic acid. When ascorbic acid occupied one of the effector site, causing the alteration of the substrate site making it perfectly fit for GSL binding. However, high concentration of ascorbic acid caused an inhibitory effect for the GSL binding because ascorbic acid might bind into the site making it unavailable for GSL binding. This inhibitory effects are also known as uncompetitive inhibition [7].

3.3 Comparison between Exogenous and Endogenous Myrosinase

Chromatogram of endogenous myrosinase in Figure 10 produce more peaks compared to exogenous myrosinase in Figure 9 as Shen *et al*. [9] state that the exogenous myrosinase produce less byproducts compared to endogenous myrosinase due to less time taken during extraction of exogenous myrosinase.

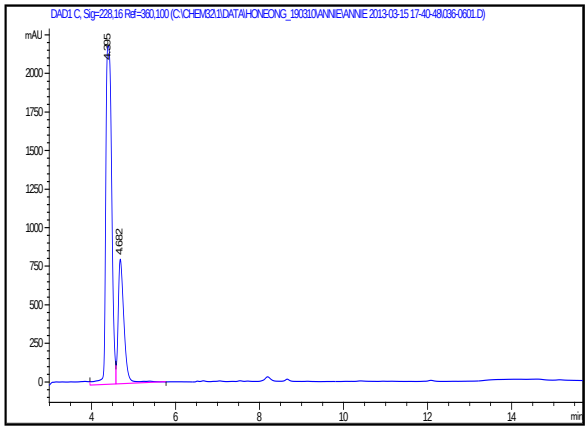

Figure 9 HPLC chromatograms of 0.02M ascorbic acid concentration using exogenous myrosinase

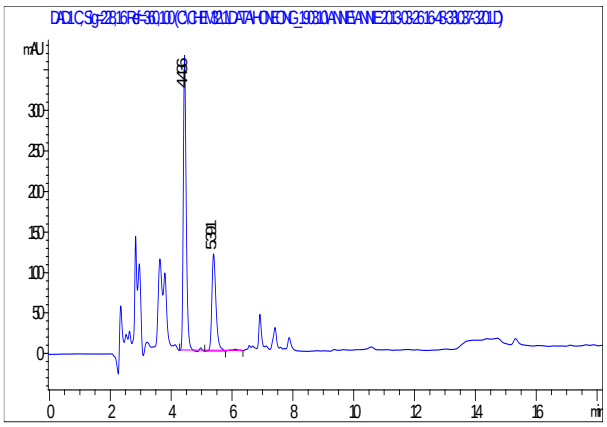

Figure 10 HPLC chromatograms of 0.02M concentrations of ascorbic acid using endogenous myrosinase

The chromatogram of exogenous myrosinase in Figure 11 is highly expressed at 4.687min than endogenous myrosinase at 4.780min (Figure 12). Due to time limitation, the exogenous myrosinase produce less amount of byproduct compared to myrosinase endogenous. However, the final concentration of sinigrin for Fe^{2+} ions showed a negative value. This is probably due to period of storage of the sinigrin are too long before HPLC analysis. Comparison between exogenous and endogen myrosinase is shown in Table 1.

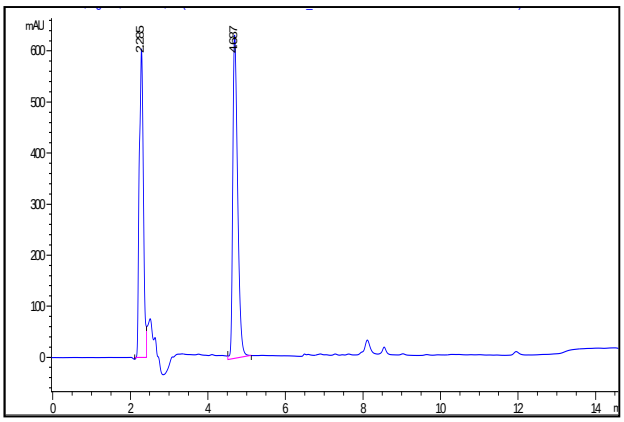

Figure 11 HPLC chromatograms at 0.02M concentration of Fe^{2+} ions using exogenous myrosinase

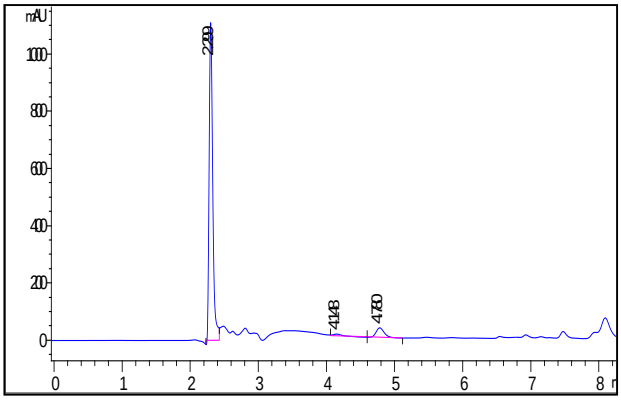

Figure 12 HPLC chromatograms at 0.02M concentration of Fe^{2+} ions using endogenous myrosinase

Table 1 Comparison of exogenous and endogen myrosinase

Exogenous Myrosinase	Endogen Myrosinase
Time to extract PEGSLs is short	Time to extract PEGSLs is longer
Quantity of byproducts is less	Quantity of byproducts is high
High enzymolysis rate of PEGSLs	Low enzymolysis rate of PEGSLs
No need to centrifuge for extraction	No need to centrifuge for extraction
High cost for enzymolysis PEGSLs	Low cost for enzymolysis PEGSLs

4.0 CONCLUSION

This study has showed that the presence of reducing ions such as ascorbate and ferrous can influence the production of the anticancer, PEITC, in natural source like watercress. As little as a few mM of these ions could alter the amount and type of PEGLS hydrolysis products. It is also shown here

that myrosinase activity is crucial to determine the amount of the health-promoting PEITC. Effects of other factors such as temperature and pH are also important and have been studied in our group. The results of this study could be used as reference in order to get optimum uptake of the health-promoting effect of PEITC from watercress as a plant source.

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