Effects of Temperature and pH on Myrosinase Activity and Gluconasturtiin Hydrolysis Products in Watercress

Nurazilah Farhana Binti Aripin, Noumie Surugau*

Industrial Chemistry Program, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.

*Corresponding author. E-Mail: lnoumie@ums.edu.my; Tel: +60-88-320000; Fax: +60-88-435324

ABSTRACT: Watercress (Nasturtium officinale) is a rich source of phenyl ethyl glucosinolate (PEGLS) (or gluconasturtiin). Depending on various factors, PEGLS is hydrolyzed enzymatically by the endogenous myrosinase into phenyl ethyl isothiocyanate (PEITC) and/or phenyl ethyl nitrile (PEN). Unlike PEN, PEITC is reported to possess anticancer activities. This paper described the effects of temperature (25, 45 and 65°C) and pH (3, 7 and 9) on myrosinase activity and hydrolysis products of PEGLS in watercress. The watercress samples were harvested from Kota Belud, Sabah. The hydrolysis products were extracted with dichloromethane and then analyzed with gas chromatography-mass spectrometry (GC-MS). While myrosinase activity was calculated based on the reduction of standard sinigrin concentration (as substrate) after 20 mins of endogenous enzymatic reaction. The unreacted sinigrin was then extracted with pure water and analyzed with high performance liquid chromatography-ultra violet (HPLC-UV). The results show that myrosinase activities were in the range of 1.21 – 1.23 mM min-1 with relatively higher at 45°C (1.23 mM min-1). Meanwhile, under the different pH (7 and 9), the myrosinase activities were slightly higher (1.35 – 1.36 mM min-1). As for the hydrolysis products, the highest concentrations of PEITC were recorded at 25°C (601.1 ppm) and pH 9 (561.1 ppm). PEITC concentration was much higher than PEN in all these conditions. The results suggest that mild temperature and neutral to slightly alkaline are favourable for myrosinase activity and formation of PEITC. These findings are especially relevant since food preparations often involved heating and addition of additives that may alter the final pH.

KEYWORDS: Watercress; Gluconasturtiin; PEITC; Myrosinase activity

Received 6 April 2016 **Revised** 4 May 2016 **Accepted** 27 May 2016 **Inpress** 2 June 2016 **Online** 20 December 2016

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

INTRODUCTION

Glucosinolates (GLS) are sulphur-containing secondary metabolites found largely in cruciferous vegetables. Certain GLS are precursor to health-promoting isothiocyanates (ITCs). Watercress (*Nasturtium officinale*) contains phenyl ethyl GLS (PEGLS) or its common name, gluconasturtiin (Williams *et al*., 2009). The hydrolysis product of PEGLS, i.e. phenyl ethyl ITC (PEITC), is proven to restrain the growth of cancer cells (Gill *et al*., 2007). Gupta *et al*. (2014) have published a comprehensive review on the anti-cancer effects of PEITC. However, the formation of PEITC is easily affected by various factors such as temperature, pH and presence of additives (Eylen *et al*., 2008). PEGLS is hydrolyzed into PEITC by the naturally-occurring enzyme myrosinase in plant. This aspect needs to be investigated further because food preparation commonly involved cutting, heating and addition of other additives which may affect the PEITC formation. Currently, there are still scarce reports on the dynamic of hydrolysis of PEGLS in watercress under various external factors. Thus, this paper described the effects of temperature and pH on myrosinase activity and PEGLS hydrolysis products in watercress.

METHODOLOGY

Sample and Sampling

Fresh and healthy watercress samples were harvested from the riverside of Panataran River at Kg Melangkap, Kota Belud. The samples were immediately preserved in liquid nitrogen to prevent GLS hydrolysis during sampling and transporting to the lab (Hong *et al.*, 2011). Upon arrival at UMS lab, the samples were stored at -20 °C freezer if not analyzed immediately.

Chemicals and Reagents

Sinigrin, PEITC and PEN standards were obtained from Phytoplan (Germany), methanol (AR grade), sodium hydroxide (NaOH), hydrochloric acid (HCl), trifluoroacetic acid (TFA), HPLC-grade dichloromethane (DCM), florisil, sodium sulphate, Trizma-HCl, acetonitrile (ACN) and HPLC-grade pure water were purchased from Thermo Fisher Scientific (USA). Deionized water was produced in-house using a PureLab Maxima water purifier purchased from Chemical Laboratory and liquid nitrogen was purchased from MOX (Sabah).

Determination of Myrosinase Activity in Watercress

a. Preparation of myrosinase crude extract

The method used in preparation of myrosinase crude extract was based on Yabar *et al.*, (2011) with some modifications. A 100 mg of freeze-dried watercress was dissolved in 10 ml of pure water and then incubated at 30°C in water bath for 2 hours. It was then filtered through 0.45µm membrane microfilter to produce about 9 - 10 ml of crude extract.

b. Effects of temperature and pH

Two millilitre of myrosinase crude extract was added with 200 µl of 6.0 mM standard sinigrin and left to stand at different temperature (25°C, 45°C and 65°C) for 20 minutes in a water bath. Similar procedures were repeated for the effects of pH, but the mixtures were in 0.01M Tris buffer solution in their respective pH i.e. 3.0, 7.0 and 9.0. All experiments were carried out in triplicates.

c. Calculation of myrosinase activity

The final concentration (unreacted) sinigrin was calculated using a linear regression equation of sinigrin standard calibration (concentration range of 0.1 – 0.5 mM). The myrosinase activity was then calculated using the following equation:

$$\text{Myrosinase activity} = \frac{[\text{Initial conc. of sinigrin} - \text{Final conc. of sinigrin (mM)}]}{\text{Reaction time (minute)}}$$

HPLC Analysis

The unreacted sinigrin in the crude mixture and sinigrin standard solutions (for calibration curve) were analyzed using HPLC which was equipped with Agilent RP C18 column (150 × 4.6 mm, 5µm particle size). The solvent system consisted of 90% solvent A (0.1% TFA /water) and 10%

solvent B (0.1% TFA /ACN) in isocratic elution mode. Column oven temperature was set at 30°C, flow rate 1 ml/min and injection 20 µl. Detection was recorded at 229 nm by UV absorbance (Nakamura *et al*., 2008).

Determination of GLS Hydrolysis Products

a. Hydrolysis of GLS and extraction of GLS hydrolysis products

The method used as reported by Songsak & Lockwood (2004) and Al Gendy *et al*., (2010). One gram of freeze-dried sample (stored in -20° C) was dissolved in 10 ml of deionized water and left to stand at the desired temperature for 30 minutes. During this duration, myrosinase hydrolyzes PEGLS (and other GLS, if any) in the watercress sample. After that, the hydrolysis products of GLS were extracted using 20 ml HPLC-grade DCM where the mixture was mechanically shaken for 60 minutes at 250 rpm. The organic layer was separated and dried over anhydrous sulfate. Florisil was used for sample clean-up and then concentrated to about 1.0 ml using a rotary evaporator at 30°C. The concentrated extract was filtered using 0.2µm nylon filter and kept in a sealed vial and stored at -20°C if not analyzed immediately.

b. Effect of temperature and pH

The same procedures as described above were repeated for the effects of temperature and pH. For the temperature, the sample solutions were left at 25, 45 and 65°C, respectively, in a water bath where the temperature was maintained throughout the 30 minutes. While for pH, instead of dissolving in deionized water, the freeze-dried sample was dissolved in 0.01M Tris buffer solution with pH of 3.0, 7.0 and 9.0, respectively.

c. Identification and Quantitation of GLS hydrolysis products

The identification of PEITC and PEN peaks in the GC chromatograms was obtained by comparing their retention times with that of PEITC and PEN standard compounds. This was further confirmed by their mass spectra. The concentrations of GLS hydrolysis products (in this study, only PEITC and PEN were detected as shown in Figure 3) were quantified using the linear regression equation from their respective standard calibration curve. The concentration range was 60 - 1000 ppm for PEITC and 40 - 200 ppm for PEN.

d. GC-MS Conditions

A GC-MS (Perkin Elmer) equipped with Turbomass version 5.2 software and a 30.0 m × 0.25 mm × 0.25µm 5MS column (Perkin Elmer) with helium carrier gas at constant flow of 1ml/min was used. The temperature program was initially 50°C for 2 min and gradually increased to 270°C with the rate of 10°C /min. The injector temperature was 200°C in split ratio of 1:50, with injection volume of 1.0 µl. The transfer line temperature was 250°C and the ion source was 200°C. The MS scan was carried out in the range of 50 to 400 m/z.

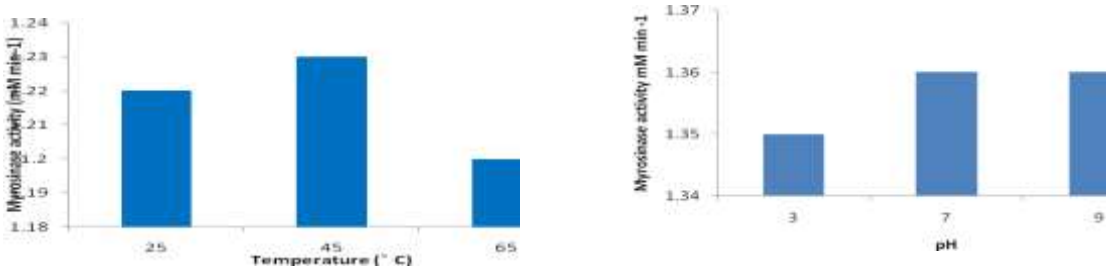
RESULTS AND DISCUSSION

Myrosinase Activity

In this study, myrosinase activity was determined by a simple method where the crude myrosinase extracted from watercress was reacted with a known concentration of a substrate i.e. commercial sinigrin. This simple method is with the assumption that non-enzymatic hydrolysis processes are negligible.

Effects of pH and Temperature on Myrosinase Activity.

Figure 1 shows the effect of temperature ranging from 25°C to 65°C on the activity of the endogenous myrosinase. As in the study of pH effects, the calculated myrosinase activities are not significantly different at these temperatures (range: 1.21 – 1.23 mM min^{-1}); with relatively higher at 45°C. It suggests that higher temperature will start denaturing the enzyme and lead to reduce its activity. The study by Eylen *et al*., (2008) stated that myrosinase activity in broccoli was stable until 60°C and as the temperature increased to 65°C the myrosinase showed reduction of their activity.



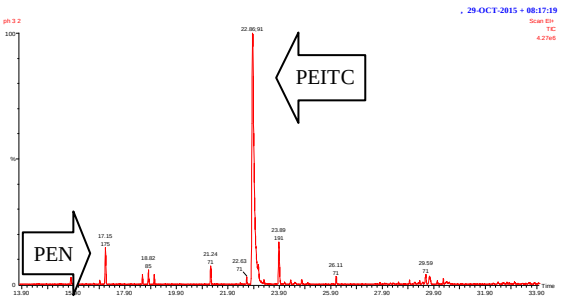
(a) Effect of temperature. (b) Effect of pH.

Figure 1. Comparison of myrosinase activity under different temperature and pH in watercress.

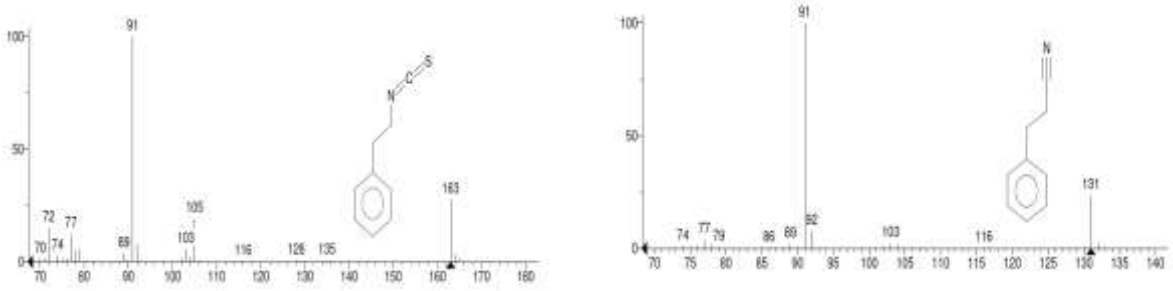
At this pH range and for a short reaction time (20 min), the myrosinase activities are not vastly different (1.35 – 1.36 mM min^{-1}). These findings are slightly different from the observations by Andersson *et al*., (2009) where myrosinase have an optimum activity at pH 4 to 7 in *Brassica napus*. At pH lower than 4, myrosinase lost its activation caused by partial deactivation, while it maintain its original activity at pH 8 and above. The previous researchers stated that at pH smaller than pH 3, the myrosinase activity cannot be detected. The differences are not unexpected because the stability of myrosinase depends on its gene which will cause different rate the activity (Bones & Rossiter, 2006).

Effects of pH and Temperature on GLS Hydrolysis Products.

Figure 2 reveals the detection of PEGLS hydrolysis products, namely PEITC and PEN, in the watercress extract. The detection of m/z = 163 and m/z = 131 correspond to the molecular ions of PEITC and PEN, respectively. Meanwhile the prominent peak at m/z = 91 in both spectra corresponds to M – 72 which is typical fragmentation of PEGLS.

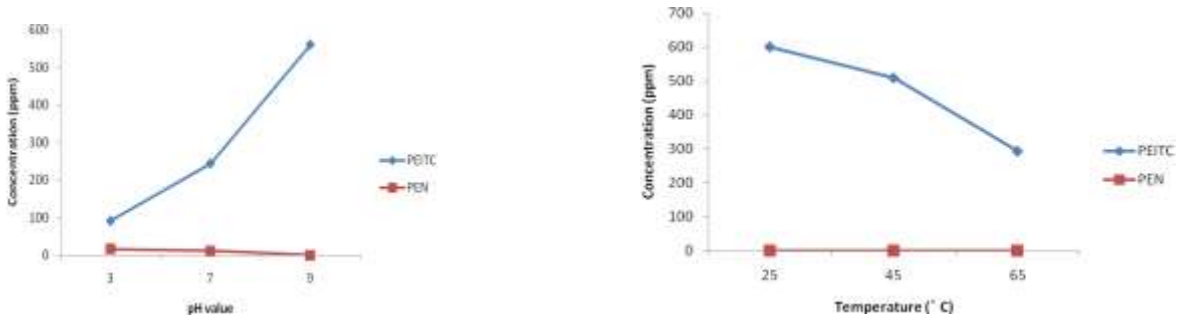


(a) GC chromatogram.



b) Mass spectra of the peaks at 17.2 min . b) Mass spectra of the peaks at 22.9 min.

Figure 2. GC-MS analysis of watercress extract.



(a) Effect of pH. (b) Effect of temperature.

Figure 3. Trend of PEITC and PEN formation under different pH and temperature in watercress.

Figure 3 shows the trend of PEITC and PEN under differing temperature and pH. The highest concentration of PEITC was recorded at room temperature (25°C) and this trend decreases as the temperature increases. Interestingly, no PEN was found in watercress although the temperature increased up to 65°C. Similar observations were reported in papaya leaves by Nagappan (2012). Higher temperature will caused enzyme denaturation and reduce the myrosinase activity and thus decrease the formation of PEITC. Increasing the pH enhances the formation of PEITC, with optimum yield up to 561.09 ppm at pH 9. Meanwhile, PEN decreased as the pH increases. These findings are in agreement with Vaughn & Berhow (2005) where acidic condition favouring nitrile formation and higher pH favouring ITC formation. It shows that the optimum myrosinase activity to convert PEGLS into PEITC is at neutral to slightly alkaline pH.

CONCLUSION

Expectedly, both set of results showed higher myrosinase activity lead to higher formation of PEITC. PEITC is higher at milder temperature and neutral to slightly alkaline. These parameters

are easily influenced by factors like temperature and pH. It is also implying that although watercress is rich with the precursor, PEGLS, but it does not necessarily provide a high uptake of the anticancer PEITC. In the future, more research on the effects of other parameters on myrosinase activity to yield the higher concentration of the health-benefiting PEITC.

ACKNOWLEDGEMENTS

We would like to thank Universiti Malaysia Sabah for their financial support on glucosinolate research through UMS Research Grants Scheme (SGPUMS SBK0001-ST-2012).

REFERENCES

[1] Al-Gendy, A. A., El-gindi, O. D., Hafez, A. S. & Ateya, A. M. (2010). Glucosinolates, volatile constituents and biological activities of *Erysimum corinthium* Boiss. (Brassicaceae). *Food Chemistry*, **118**(3), 519–524.

[2] Andersson, D., Chakrabarty, R., Bejai, S., Zhang, J., Rask, L. & Meijer, J. (2009). Myrosinases from root and leaves of Arabidopsis thaliana have different catalytic properties. *Phytochemistry*, **70**(11-12), 1345–1354.

[3] Bones, A. M. & Rossiter, J. T. (2006). The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry*, **67**(11), 1053–1067.

[4] Van Eylen, D., Oey, I., Hendrickx, M. & Van Loey, A. (2008). Effects of pressure / temperature treatments on stability and activity of endogenous broccoli (*Brassica oleracea* L . cv . Italica) myrosinase and on cell permeability. *Journal of Food Engineering*, **89**, 178–186.

[5] Gupta, P., Wright, S. E., Kim, S. H. & Srivastava, S. K. (2014). Phenethyl isothiocyanate: A comprehensive review of anti-cancer mechanisms. *Biochimica et Biophysica Acta - Reviews on Cancer*, **1846**(2), 405–424.

[6] Gill, C. I. R., Haldar, S., Boyd, L. A., Bennett, R., Whiteford, J., Butler, M., Pearson, J. R., Bradbury, I. & Rowland, I. R. (2007). Watercress supplementation in diet reduces lymphocyte DNA damage and alters blood antioxidant status in healthy adults. *Journal of Clinical Nutrition*, **85,** 504–510.

[7] Hong, E., Kim, S. & Kim, G. (2011). Identification and quantitative determination of glucosinolates in seeds and edible parts of Korean Chinese cabbage. *Food Chemistry*, **128**(4), 1115–1120.

[8] Nagappan, G. (2012). *Effects of additives and food processing conditions on benzyl glucosinolate hydrolysis products in Carica papaya*. MSc Thesis, Universiti Malaysia Sabah, Malaysia.

[9] Nakamura, Y., Nakamura, K., Asai, Y., Wada, T., Tanaka, K., Matsuo, T. & Ohtsuki, K. (2008). Comparison of the glucosinolate-myrosinase systems among Daikon (Raphanus sativus, Japanese white radish) varieties. *Journal of Agricultural and Food Chemistry*, **56**(8), 2702–2707.

[10] Songsak, T. & Lockwood, G. B. (2004). Production of two volatile glucosinolate hydrolysis compounds in *Nasturtium montanum* and *Cleome chelidonii* plant cell cultures. *Fitoterapia*, **75**(3-4), 296–301.

[11] Vaughn, S. F. & Berhow, M. A. (2005). Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Industrial Crops and Products*, **21**(2), 193–202.

[12] Williams, D. J., Critchley, C., Pun, S., Chaliha, M. & Hare, T. J. O. (2009). Phytochemistry Differing mechanisms of simple nitrile formation on glucosinolate degradation in *Lepidium sativum* and *Nasturtium officinale* seeds. *Phytochemistry*, **70**(11-12), 1401–1409.