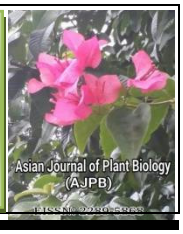
HIBISCUS

ASIAN JOURNAL OF PLANT BIOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/AJPB



Screening of Heavy Metals in Selected Vegetables using the Papain Inhibitive Assay

Masdor, N.A.[1]*, Izuan, E.H.[2], Baskaran, G.[2] And R. Sulaiman[3]

[1]Biotechnology Research Centre, Mardi, P. O. Box 12301, 50774 Kuala Lumpur, Malaysia.
[2]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.
[3]School of Food Science & Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

*Corresponding Author: azlina@mardi.gov.my/azlina.mardi@gmail.com
Noor Azlina Masdor
Biotechnology Research Centre,
Malaysian Agricultural Research and Development Institute
Mardi, P. O. Box 12301,
50774 Kuala Lumpur, Malaysia

HISTORY

Received: 5th December 2014
Received In Revised Form: 24th December 2014
Accepted:27th December 2014

KEYWORDS

heavy metals
vegetables
enzyme assay
FIMS

ABSTRACT

Vegetables are major source of heavy metals contaminant in the diet of humans. Currently, monitoring of heavy metals in vegetables is carried out by instruments such as Inductively-Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) or Flow Injection Mercury System (FIMS). Instrumental method alone is costly, need skilled personnel and time consuming. In this work, the papain assay was used to screen for the presence of heavy metals in twelve digested vegetables samples after neutralization. None of the non-spiked samples tested shows levels of heavy metals above the maximum residue limit (MRL) allowed for heavy metals in vegetables using both instrumental and papain assay. The papain assay was able to detect by mercury-spiked vegetable samples indicating that the assay could tolerate the high salt of the digested sample matrix. Papain assay is simple, rapid and low in cost. It requires small sample volumes and could be carried out in a microplate format. The assay can act as a preliminary screening assay. Positive samples are sent for heavy metals level using instruments. Using this approach, higher frequency and number of monitoring can be carried out.

INTRODUCTION

Malaysia, being a tropical country, is blessed with conditions that produce abundant plants that have medicinal [1–3] or agricultural values [4,5]. Studies have shown widespread contamination of heavy metals in vegetables [6], food [7,8] and herbal products [9,10]. In addition, agriproduce from ex-mining land has been shown to contain significant level of heavy metals [9] that have caused a decline in consumption of fruits from such area.

Contamination of heavy metals arises from various sources such as soils, metal from the grinding machines or a coingredient contaminant [9]. Heavy metals are toxic to almost all forms of organism [11]. In humans heavy metals accumulated in organs such as the gastrointestinal tract, kidney, the nervous system, and the reproductive system [12]. As production and consumption increase, incidence of chronic heavy metals poisoning is an impending reality and its monitoring is thus immediately needed. Large scale monitoring using conventional instrumental alone is very costly and time consuming. In the environmental field, researchers have begun using biological systems and organisms to form a preliminary screening tool. Enzyme-based or microbial-based biomonitoring is an alternative modern approach compared to purely instrumental methods in detecting xenobiotics in samples.

Only positive samples that show toxicity to the testing biosystem is sent for instrumental validation. This approach dramatically reduces costs and monitoring time. An equivalent system is unheard of in vegetables monitoring of heavy metals as the digestion product are highly acidic, diluted many times or if neutralized contain high salt concentration that can mask the effect of heavy metals in the sample.

Previously, protease-based inhibitive assay for heavy metals based on the protease papain [13], bromelain [14] and trypsin [15]

have been developed. These proteases exhibit high salt, broad pH and temperature tolerance. Of all the protease, papain is the cheapest and amongst the most sensitive to heavy metals. In this work, the use of papain for the biomonitoring of heavy metals in vegetables products is presented for the first time.

MATERIALS AND METHODOLOGY

Treatment of Glassware and Chemicals

All reagents were of analytical grade. All glasswares were initially soaked for 2 hours with aqua regia ($HCl:HNO_3$ in a ratio of 3:2) and then washed extensively with deionized water.

Wet digestion of samples

Twelve vegetables samples were purchased between 2007 and 2010 from several outlets in Selangor. The vegetables include cauliflower, chinese cabbage, kale, lettuce, green pepper, eggplant, cucumber, carrot, potato, red onions, celery, tomato and okra. Ten grams of samples were oven dried for three days at 65 oC. The dried samples were grounded in mortar and pestle to produce fine powder before digestion using aqua regia. Two gram of round sample was placed in a 100 ml round bottom flask (Quickfit). Aqua regia (25.0 ml) was then added and the mixture refluxed for between 6 and 8 h on a water-bath. The digested sample was allowed to cool at room temperature on completion and carefully washed with deionized water before topped up to 50.0 ml with deionized water. The blank were aqua regia alone to replace sample [9]. One of the vegetable-okra was spiked with 1 mg/L mercury after digestion as a positive sample control.

Preparation of Bradford dye-binding assay

The Bradford reagent [16] of choice in this work is the commercial preparation from Bio-Rad that gave a linear protein range of up to 0.7 absorbance unit at 595 nm. Alternatively, the Coomassie dye-binding protein assay can be also be prepared using the method of Scopes . In this method, 100 mg of Coomassie Brilliant Blue G-250 (Sigma Chemical Co., St. Louis, USA) was dissolved in a mixture comprising of one hundred millilitre of 85% phosphoric acid and fifty millilitre of 95% ethanol. The solution was made up to 1 L before stirred vigorously overnight. The solution was filtered through Whatman Filter Paper No. 1 and stored in dark bottles.

Preparation of Papain and Casein Working Solution

Casein is a protein that is mostly not soluble in water. For the papain assay to work reproducibly, a relatively clear solution is needed. To prepare this solution about 2 g of casein (Fluka) was dissolved in one hundred millilitre of deionised water. The solution needs to be adjusted to pH 8.0 using 5 N NaOH to improve dissolution. An overnight incubation with stirring at 60^{o}C allows maximal dissolution. The solution was then filtered using several layers of cheesecloth. The filtrate was then centrifuged at 15,000 x g for 20 minute to obtain a stock solution of 10.0 mg/ml measured using the Bradford protein assay above using BSA [Sigma] as the standard protein. Papain (SIGMA, E.C. 3.4.22.2, lot no: 32K2619, crude dried papaya latex. 0.5 Units/mg) stock solution (10.0 mg/ml) was prepared at 4 ^{o}C by dissolving 100 mg in a 10 ml solution of 50 mM sodium phosphate pH 6.5. This solution was stored at -20 ^{o}C or -80 ^{o}C in the form of 1 ml aliquots. Working solutions of papain (2.0 mg/ml) and casein (0.3 mg/ml) must be prepared fresh daily (13).

Heavy metals Assay using Papain

In an Eppendorf tube, 5 microlitre of papain working stock solution was mixed with 50 microlitre of 100 mM phosphate buffer pH 6.5 giving a final concentration of 0.1 mg/ml papain. Then 45 microlitre of the clear filtrate from the aqua regia digestion method was added and incubated for 20 min at 4 oC. After this, 50 microlitre of casein working solution was added and the solution mixed. Immediately, a 20 microlitre aliquot was pipetted out [time zero] and rapidly mixed with 200 □l of the Bradford dye-binding reagent. The color was allowed to develop for 5 minutes at room temperature. The absorbance at 595 nm for this time zero was taken. The remainder of the solution was incubated at 40oC for 30 minutes. After this incubation period has ended, immediately 20 microlitre was pipetted out and assay for remaining protein using the Bradford assay at 595 nm as above. Absorbance was measured on a Stat Fax® 3200 Microplate Reader (Awareness Technology Inc., USA) [18].

Instrument

The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on a Perkin Elmer ICP-OES (Optima 3700DV, Perkin-Elmer, USA) and a Perkin Elmer Flow Injection Mercury System (FIMS 400). All experiments were performed in triplicate

Data and statistical analysis

The percent inhibition was computed according to following formula:

$$\% \text{ Inhibition} = \frac{\text{Test activity of control - test activity of sample x 100}}{\text{Test activity of control}}$$

Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

All of the non-spiked vegetable samples demonstrated mild inhibition to the papain activity with between 10 and 20% inhibition. Only the spiked okra sample showed 100% inhibition (**Fig**. 1). Heavy metal analysis showed that all of the vegetable samples had less than 0.5 mg/kg of all heavy metals with the exception of the mercury-spiked okra, the latter shows that it had about 0.924 mg/kg mercury. Cauliflower, chinese cabbage, green pepper and celery showed higher than 0.5 mg/kg copper with the highest copper concentration was in cauliflower and green pepper with no significant difference [p>0.05] between them. Carrot exhibited the highest concentration of lead and mercury at 0.32

and 0.043 mg/kg, respectively. Cadmium was the highest in potato at 0.133 mg/kg (**Fig.** 2).

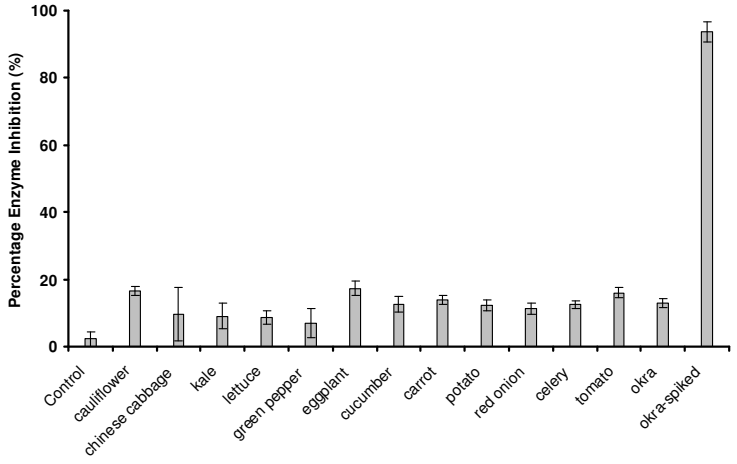


Fig. 1. Papain activity inhibition by digested vegetable samples. Results are mean ± standard deviation [n=3].

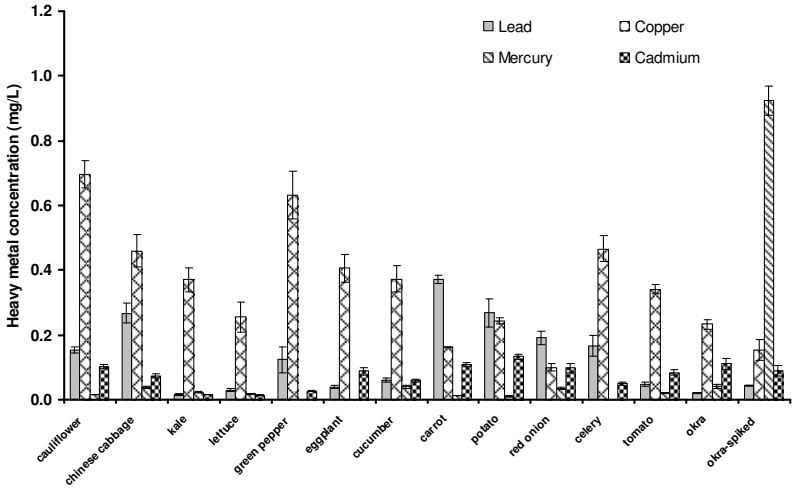


Fig. 2. Heavy metal concentration in digested vegetable samples. Results are mean ± standard deviation [n=3].

DISCUSSIONS

Papain was originally assayed as a protease with the addition of EDTA and DTT to protect itself from heavy metals. By removing these protective agents, the protease assay for heavy metals was first developed by Shukor et al. [18]. The basis of this assay is that when heavy metals are absent papain will digest casein, its substrate, and the digested protein will not be stained by the Coomassie dye and the solution remains brownish. In the presence of heavy metals, papain is inhibited and the Coomassie dye will be able to stain casein, resulting in a blue solution.

Using the papain assay it was revealed that none of the vegetable samples inhibited the enzyme activity of more than 20% except spiked okra sample. Instrumental analysis showed that the levels of heavy metals in the samples did not exceeded the Maximum Residue Limit [MRL] for lead, mercury, cadmium, copper at 2.0, 0.05, 1.0 and 10 mg/kg, respectively [19].

The papain assay acts as the first line of defense due to its simplicity and sensitivity. The papain assay showed concentration that caused 50% inhibition or IC50s for cadmium, copper, mercury and lead at 1.0, 0.10, 0.39, 2.16 mg/L, respectively [18]. Since digestion and dilution of samples resulted in a ten fold dilution of heavy metals concentration in samples for inhibitive enzyme assay purposes a cut-off point of 20% inhibition to the papain assay would indicate that the samples contain heavy metals at elevated concentration. This is adequate to screen for heavy metals-contaminated sample. This is reflected in the overall inhibition of less than 20% towards papain activity by the vegetable samples that upon analysis by instruments showed safe level of heavy metals below the MRL values.

As heavy metals are very toxic at levels above the MRL, their routine determination in vegetable samples are warranted as vegetables are consumed everyday and at relatively large amount [20,21]. Vegetables grown at polluted sites have been shown to contain elevated levels of heavy metals [21] and this indicate the importance of routine monitoring.

The papain assay is an excellent tool for biomonitoring of heavy metals contaminant in vegetable product due to its robust properties. It has broad pH and temperature stability and activity. In addition it is salt tolerant [13]- a feature important in acid-digested samples. The use of papain assay would allow thousands of samples to be screened daily and only positive samples are sent for instrumental analyses, a move that could cost thousands of ringgit per day. To date, the use of bioassay or enzyme-based assay for monitoring heavy metals in vegetables is almost nonexistent and published works are almost nil. Thus comparison with existing literature can not be made.

In conclusion, the papain assay has been successfully used to detect heavy metals contaminant in vegetable products. All of the vegetable samples tested using the proposed papain assay showed levels of heavy metals below the MRL and the results are validated instrumentally. Biomonitoring using enzyme assay could cut cost and handle such high volume of monitoring as the required skill to carry out the work is low. Only positive samples would be sent for instrumental analysis, cutting cost and time of measurement. The rapid and robust property of papain assay coupled with papain low cost should make it an important biomonitoring tool in the future.

ACKNOWLEDGEMENT

This work was supported by the research grant from the Ministry of Science, Technology and Innovation [MOSTI] under the Science Fund grant no: 02-03-08-SF0061.

REFERENCES

[1] Hendra R, Ahmad S, Oskoueian E, Sukari A, Shukor MY. Antioxidant, Anti-inflammatory and Cytotoxicity of Phaleria macrocarpa [Boerl.] Scheff Fruit. BMC Complement Altern Med. 2011;11.

[2] Wazir D, Ahmad S, Muse R, Mahmood M, Shukor MY. Antioxidant activities of different parts of Gnetum gnemon L. J Plant Biochem Biotechnol. 2011;20[2]:234–40.

[3] Hussin NM, Muse R, Ahmad S, Ramli J, Mahmood M, Sulaiman MR, et al. Antifungal activity of extracts and phenolic compounds from Barringtonia racemosa L. [Lecythidaceae]. Afr J Biotechnol. 2009;8[12]:2835–42.

[4] Kiong ALP, Then CN, Hussein S. Callus induction from leaf explants of Ficus deltoidea Jack. Int J Agric Res. 2007;2[5]:468–75.

[5] Hussein S, Ibrahim R, Kiong ALP, Fadzillah NM, Daud SK. Multiple shoots formation of an important tropical medicinal plant, Eurycoma longifolia Jack. Plant Biotechnol. 2005;22[4]:349–51.

[6] Karak T, Bhattacharyya P, Paul RK, Das DK. Metal accumulation, biochemical response and yield of Indian mustard grown in soil amended with rural roadside pond sediment. Ecotoxicol Environ Saf. 2013;92:161–73.

[7] Nurnadia AA, Azrina A, Amin I, Yunus ASM, Effendi HMI. Mineral contents of selected marine fish and shellfish from the west coast of Peninsular Malaysia. Int Food Res J. 2013;20[1]:431–7.

[8] Alina M, Azrina A, Mohd Yunus AS, Mohd Zakiuddin S, Mohd Izuan Effendi H, Muhammad Rizal R. Heavy metals [mercury, arsenic, cadmium, plumbum] in selected marine fish and shellfish along the straits of malacca. Int Food Res J. 2012;19[1]:135–40.

[9] Ang HH, Lee KL. Contamination of mercury in tongkat Ali hitam herbal preparations. Food Chem Toxicol. 2006;44[8]:1245–50.

[10] Ling APK, Tan KP, Hussein S. Comparative effects of plant growth regulators on leaf and stem explants of Labisia pumila var. alata. J Zhejiang Univ Sci B. 2013;14[7]:621–31.

[11] Shariati F, Pourkazemi M, Shariati S, Mahboobnia B, Pourdehghani M, Azizzadeh L. Tissue-dependent metallothionein and cytosolic metal distribution in a native Caspian Sea fish, Acipenser persicus. Fresenius Environ Bull. 2013;22[5]:1408–14.

[12] Antonovich VP. Speciation of mercury in environmental samples. J Anal Chem. 1996;51[2]:106–13.

[13] Shukor Y, Baharom NA, Rahman FA, Abdullah MP, Shamaan NA, Syed MA. Development of a heavy metals enzymatic-based assay using papain. Anal Chim Acta. 2006;566[2]:283–9.

[14] Shukor MY., Masdor N., Baharom NA., Jamal JA., Abdullah MPA., Shamaan NA., et al. An inhibitive determination method for heavy metals using bromelain, a cysteine protease. Appl Biochem Biotechnol. 2008;144[3]:283–91.

[15] Shukor MY, Baharom NA, Masdor NA, Abdullah MPA, Shamaan NA, Jamal JA, et al. The development of an inhibitive determination method for zinc using a serine protease. J Environ Biol. 2009;30[1]:17–22.

[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 1976;72[1-2]:248–54.

[17] Scopes RK. Protein Purification: Principles and Practice. 3 edition. New York: Springer; 1993. 404 p.

[18] Shukor Y, Baharom NA, Rahman FA, Abdullah MP, Shamaan NA, Syed MA. Development of a heavy metals enzymatic-based assay using papain. Anal Chim Acta. 2006;566[2]:283–9.

[19] Anonymous. Food Act 1983 and Regulations 1985, Regulation 38 - Fourteenth Schedule Malaysian Food Regulations. Kuala Lumpur, Malaysia: International Law Book Services; 2006.

[20] Ismail BS, Farihah K, Khairiah J. Bioaccumulation of heavy metals in vegetables from selected agricultural areas. Bull Environ Contam Toxicol. 2005;74[2]:320–7.

[21] Maimon A, Khairiah J, Mahir AR, Aminah A, Ismail BS. Comparative accumulation of heavy metals in selected vegetables, their availability and correlation in lithogenic and nonlithogenic fractions of soils from some agricultural areas in Malaysia. Adv Environ Biol. 2009;3[3]:314–21.