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Short communication

A Novel Method for the Determination of Mercury in Herbal Preparation Using an Inhibitive Assay Based on the Protease Papain

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Abstract

Herbal products are a major source of mercury contaminant in the diet of humans. Currently, monitoring of mercury is carried out by instrument such as Flow Injection Mercury System which utilizes the cold vapour atomic fluorescence spectroscopic technique. The handling and operation of these instruments are costly, time consuming and need skilled operators. In this work, the use of the papain assay to determine mercury in digested herbal samples after neutralization is demonstrated. About 20% of the samples tested showed positive results for mercury at levels higher than the maximum permissibility limit (MPL) and the results were validated using FIMS. The papain assay is low in cost, rapid and could be carried out in a microplate format. The papain assay can be the preliminary screening assay and only positive samples are sent for instrumental validation. This marriage between enzyme-based assay and instrumental method could increase monitoring frequency and results could be obtained faster

INTRODUCTION

The herbal industry is undergoing a revival in the current years as more and more people are consuming or using herbal preparation and formulation [1]. Herbs form a central alternative therapy to modern drug-based medicine [2]. It needs no mention that many of the drugs available today in fact originated from various herbal extracts [3-6]. The herbal market is worth at least MYR 400 million in Malaysia and USD 61 Billion globally [7-8]. The quest for QEST (Quality, Efficacy, Safety and Standardization) means that stringent screening of herbal preparation and formulation is being intensely sought. An important issue regarding the safety of herbs is the presence of heavy metals, especially mercury [9]. Contamination arises from various sources such as soils, metal from the grinding machines or a coingredient contaminant [10]. Mercury is toxic to almost all forms of organism [11]. In humans mercury 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 mercury 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. Only positive samples that show toxicity to the test biosystem is sent for instrumental validation. This approach dramatically reduces costs and monitoring time. An equivalent system is unheard of in herbal monitoring as the digestion product are highly acidic, diluted many times or if neutralized contain high salt concentrations that can mask the effect of mercury in the sample. Fortunately, the permissible level of heavy metal such as mercury is 0.5 mg/Kg [13] whereas in drinking water, the permissible limit is 1 □g/L [14], about 500 times less. Previously, protease-based inhibitive assay for heavy metals based on the protease papain [14], bromelain [15] and trypsin [16] 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 mercury. In this work, the use of papain for the biomonitoring of mercury in herbal products is presented for the first time.

MATERIALS AND METHODS

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

Sixteen herbal samples were purchased between October and December in 2012 from several outlets in Selangor. Coarse samples of herbal products like pills tablets, powders or capsules need to be grounded in mortar and pestle to produce fine powder before digestion using aqua regia. Two grams of grounded 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 [13].

Preparation of Bradford dye-binding assay

The Bradford reagent [17] 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 [18]. 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 [18].

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 ºC 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 ºC by dissolving 100 mg in a 10 m**L** solution of 50 mM sodium phosphate pH 6.5. This solution was stored at -20 ºC or -80 ºC in the form of 1 m**L** aliquots. Working solutions of papain (2.0 mg/ml) and casein (0.3 mg/ml) must be prepared fresh daily [14].

Mercury Assay using Papain

In an Eppendorf tube, 5 mL of papain working stock solution was mixed with 50 mL of 100 mM phosphate buffer pH 6.5 giving a final concentration of 0.1 mg/ml papain. Then 45 mL of the clear filtrate from the aqua regia digestion method was added and incubated for 20 min at 4 ºC. After this, 50 mL of casein working solution was added and the solution mixed. Immediately, a 20 mL aliquot was pipetted out (time zero) and rapidly mixed with 200 mL 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 40 ºC for 30 minutes. After this incubation period has ended, immediately 20 mL 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) [14].

Instrument

A Perkin Elmer Flow Injection Mercury System (FIMS 400) was used to determine the concentration of mercury in herbal samples. Deionized water (Elga) was used in this work. Control of the peristaltic pumps, data acquisition, and injection time was carried out by the Perkin Elmer AAWinLab software (Norwalk, CT, USA).The instrument comprised of a six-way injection valve with a flow meter, sample loop, a cylindrical gas–liquid separator, two peristaltic pumps, and a quartz cell. Sample injected into the instrument is transported to the reaction chemifold through an acid carrier. Here sample was mixed with sodium borohydride-the reducing agent in a reduction coil. Mercury vapor was purged from the stripping coil using an argon stream and then the concentration of mercury determined in the quartz cell. All experiments were performed in triplicate.

RESULTS

Three out of the 16 samples tested showed positive indication for the presence of mercury based on the presence of intense blue color compared to control and quantitatively through absorbance measurement. Instrumental analysis showed that out of the three samples that showed strong papain inhibition activity, sample G showed level of mercury of above 0.5 mg/L while samples J and M was below this (Figure 1). All of the other samples showed low inhibition to papain activity and also exhibited negligible low level of mercury.

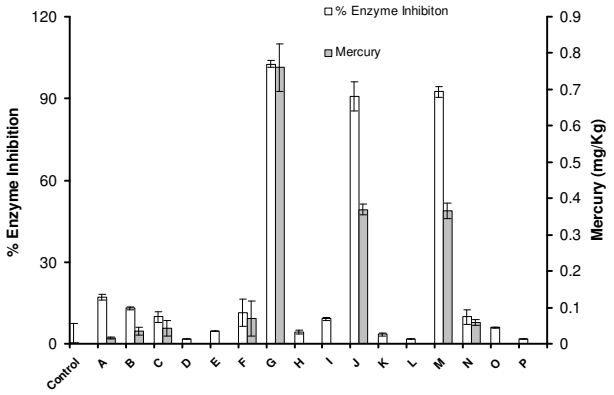

Figure 1. Papain activity inhibition by digested herbal samples and mercury concentrations in the samples. Results are mean ± standard deviation (n=3).

DISCUSSIONS

The original assay for protease using the Bradford assay requires the addition of DTT or EDTA. These agents protect the protease from inhibition by heavy metals. Once removed the protease is susceptible to heavy metals. This is the basis of the assay. The Bradford dye-binding reagent is unable to stain polypeptide or digested protein that has molecular weights of less than 2 kDa [14-16]. In addition, the Bradford assay is also unable to stain intact protein if the concentration is less than 0.05 mg [17]. The basis of the papain assay for heavy metals is that when mercury is

absent papain will digest casein and the digested protein is not stain and remains a brown solution. Once mercury is present, papain activity will be inhibited and Bradford assay will be able to stain casein in the resulting solution is blue.

The papain assay acts as the first line of defense due to its simplicity and sensitivity. The papain assay showed an IC_{50} for mercury of 0.39 mg/L and a Limit of Detection of 0.11 mg/L [15]. This is adequate to screen for mercury-contaminated sample. In this work, the dilution normally applied after aqua regia treatment of samples is minimize to 10 ml to make sure the papain assay detection capability is maximized. The papain assay successfully detected three samples with elevated level of mercury but only one sample, sample G exceeded the Maximum Permissibility Limit (MPL) for mercury allowed by the Ministry of Health, Malaysia in herbal products at 0.5 mg/Kg [13].

The cutoff point to be used to suggest the presence of mercury is a signal that registers about 10% inhibition to the assay. This is suggested since the sample has already been diluted ten times during digestion. Previous screening works of Ang and Lee [13] have shown elevated levels of mercury in several commercial Tongkat Ali preparations. The determination of mercury is very important since it is very toxic and its presence in herbal formulation and preparations is detrimental to our health. In addition, variation in mercury content from one batch to another makes routine mass monitoring even more important. The marriage between instrumental and biomonitoring methods using enzyme assay could cut cost. In addition the system could handle high volume of monitorings. The cost and time of measurements can be reduced since only positive samples would be sent for instrumental analysis.

Papain is inhibited by not only mercury but other heavy metals including silver, lead, zinc, cadmium and copper [14]. Hence, these samples could contain other toxic heavy metals as well. Herbal preparation could contain these other heavy metals but their MPL level is several folds higher than mercury and the dilution factor during sample digestion usually lowers the signal of detection. The results above indicate that mercury is a major contaminant in herbal preparation. The manufacturer of the herbal products is not revealed for legal purposes. It is only when more data are obtained such as one-year monitoring from one batch to another that the true level of mercury contamination can be determined to be an intrinsic property of the herbal source used instead of results from a single batch. The papain assay is an excellent tool for biomonitoring of mercury contaminant in herbal product due to its robust properties. It has broad pH and temperature stability and activity. In addition it is salt tolerant [14]- 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 analyses using FIMS, 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 herbal preparation 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 mercury contaminant in herbal products with several commercial samples containing mercury at levels above the MPL. The rapid and robust property of papain assay coupled with papain low cost should make it an important biomonitoring tool in the future.

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