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SHORT COMMUNICATION

Assay for Mercury in Herbal Preparation using an Inhibitive Enzyme Assay based on Bromelain

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ABSTRACT

Mercury contamination of herbal products is a major source of concern globally. The high cost of instrumental determination of mercury means that a lot of cases of contamination would not be detected. Instrumental method alone is time consuming, costly and need skilled personnel. A recent trend in heavy metal monitoring is to employ biological-base system such as using microbes and their enzyme. The use of these kinds of assays in herbal monitoring has not been reported. In this work, a bromelain-based inhibitive assay was successfully used to determine mercury in digested herbal samples. Three of the twenty samples tested showed positive results for mercury with one of the sample exhibiting mercury at levels higher than the maximum permissibility limit (MPL) as found using the instrument Flow Injection Mercury System or FIMS. The bromelain assay requires little reagents and sample volumes, is rapid and low in cost. The bromelain assay can be part of the system involving the use of biological system together with instrumental method. In this system, the enzyme forms a first screening phase. In the second phase, positive samples are validated suing FIMS. Hence monitoring frequency can be increased and results could be obtained faster.

INTRODUCTION

Herbs and herbal preparation and formulation consumption and use will be increasing in the years to come as more and more people are going back to traditional healing practices [1,2]. In fact drugs used in modern medicines came from herbs [3,4]. Globally, the herbal market is worth about USD 60 billion and is expected to rise. In Malaysia alone, herbs and herbal formulation consumption and usage has increase several fold over the years [5,6]. Herbs and herbal preparation are known source of heavy metals including mercury throughout the world [7]. Elevated level of mercury could come from various ways such as from the soil [8], machineries used in their processing, through contamination of co-ingredients, or deliberately added [9]. Mercury has adverse effects not only to humans but to almost all organisms [10]. They affect all aspects of human health through modes of action that occurs in the kidney, gastrointestinal tract, central nervous system, and male reproductive system [11]. The high incidence of mercury contamination is a worrying trend and instrumental-based method alone could not cope with the increasing number of herbal preparation and formulation products each year. An alternative is urgently needed. Enzyme-based assay are potentially good candidate for biomonitoring of heavy metals in herbs. The recent development of proteases as robust enzyme systems for inhibitive assay for heavy metals in general [12-14] have opened up the possibility for biomonitoring in herbal preparation and formulations. In this work, we presented for the first time the use of an enzyme inhibitive assay using bromelain for the determination of mercury in herbal samples.

MATERIALS AND METHODOLOGY

Instrument

The determination of mercury in the samples was carried out using a Perkin Elmer Flow Injection Mercury System (FIMS 400). The instrument consisted of a six-way injection valve with a sample loop, flow meter, two peristaltic pumps, a cylindrical gas–liquid separator, and a quartz cell. The injected sample was transported through an acid carrier to the reaction chemifold where mixture of sample and the reducing agent (sodium borohydride) occurs in a reduction coil. Purged mercury vapor using an argon stream in the stripping coil then enters the gas–liquid separator before quantization was carried out in the quartz cell. A Perkin Elmer AAWinLab software (Norwalk, CT, USA)

was used to control data acquisition, peristaltic pumps and injection time. Deionized water (Elga) was used throughout the study. All experiments were performed in triplicate.

Treatment of glasswares

All glasswares were soaked with aqua regia ($HCl:HNO_3$ in a ratio of 3:2) for 2 h and then washed copiously with deionized water. All reagents were of analytical grade. Nitric acid 65% (Merck), hydrochloride acid 37% (Merck) and sodium borohydride (Sigma) were used in this study. Sodium borohydride was dissolved in 1% NaOH (w/v) to a final concentration of 0.75% (w/v). Mercury working solutions was prepared fresh from a stock solution 1000 mg/L (BDH) [9].

Wet digestion of samples

Herbal samples were procured randomly from several outlets in Selangor between June and October in 2012. Coarse herbal particles like pills or powders, tablets, capsules were first grounded to fine powder or particles using a mortar and pestle prior to wet digestion A total of 16 samples were wet digested using the method above (aqua regia). About 2 g of samples were placed in a quickfit round bottom flask of 100 ml. 25ml of aqua regia was then added. The mixture was refluxed for 6–8 h on a water-bath and then allowed to cool at room temperature and then filtered. Deionized water was used to carefully wash the residue. The mixture was then topped up to 50.0 ml using deionized water. Blank samples contain aqua regia [9].

Bradford dye-binding assay

Commercial Bradford dye-binding reagent from Bio-Rad was used in this work. Alternatively, the Coomassie dye-binding protein assay can be prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Sigma Chemical Co., St. Louis, USA) in a mixture consisting of 100 ml 85% phosphoric acid and 50 ml of 95% ethanol. The solution was topped up to 1 L and stirred overnight. Then the solution was filtered through several layers of Whatman Filter Paper No. 1. The resulting solution was stored in dark bottles [15].

Preparation of bromelain and casein solution

Casein is largely insoluble in water. To prepare an acceptably clear solution, about 2 g of casein (Sigma) was dissolved in 100 ml of deionised water. The solution was adjusted to pH 8.0 using 5 N NaOH. Then the solution was incubated overnight with stirring at 60°C. To obtain a casein stock solution of 10.0 mg/ml the overnight incubated solution was filtered through several layers of cheesecloth before centrifuged at 10,000 g for 15 minutes. The concentration of casein protein was measured using the Bradford assay (Bradford, 1976) above using BSA (Sigma) as the standard protein. Bromelain stock solution was prepared at 4 °C in a solution of 50 mM sodium phosphate pH 6.5 to a 10.0 mg/ml stock solution. This solution was aliquoted into small portions and stored at -20 °C or -80 °C. Working solutions of casein (0.3 mg/ml) and bromelain (2.0 mg/ml) were prepared fresh daily [13].

Mercury assay using bromelain

About 5 µl of bromelain from the working stock solution was added to 50 µl of 100 mM phosphate buffer pH 6.5 in an Eppendorf tube. This is followed by the addition of 45 µl of the clear filtrate from the wet digestion method and mixed thoroughly. The mixture was incubated for 20 min at 4 °C. Then 50 µl of casein from the working solution was added and mixed. Immediately, a 20 µl aliquot was taken out (time zero) and mixed with 200 µl of Bradford dye-binding reagent. This mixture was incubated for 5 minutes at room temperature and the absorbance at time zero taken. The remaining solution was left to incubate for 30 minutes at 40°C. Then a 20 µl aliquot was immediately taken and assay for remaining protein as above. The absorbance at 595 nm was measured on a Stat Fax® 3200 Microplate Reader (Awareness Technology Inc., USA) [13].

RESULTS AND DISCUSSION

The Bradford casein dye-binding assay was used to indicate the inhibition of bromelain by heavy metals. The basis of this assay is the inability of the Bradford dye-binding reagent to stain polypeptide that has molecular weights of less than 2 kDa [12-14]. Under normal circumstances, bromelain would digest casein to polypeptide of these sizes. Since the amount of bromelain in the assay is miniscule (about 1µg), the principle dye in the Bradford assay- Coomassie would not be able to stain it and the solution remains a brownish solution. In the presence of mercury, the substrate casein will not be degraded and will be stained blue by the Coomassie reagent.

Out of the 16 samples tested, 3 of them showed positive response to the bromelain assay indicating the presence of mercury. Digested samples (4, 17 and 13) that showed strong inhibition to bromelain activity also contained high level of mercury with mercury content in sample 4 exceeding the MPL for mercury in herbal product at 0.5 mg/Kg [9]. Other samples showed both low enzyme inhibition and mercury content (Figure 1). A >10% inhibition to the assay is suggested as the cutoff point to indicate the presence of mercury since the sample has already been diluted ten times during digestion. The bromelain assay showed an IC_{50} for mercury of 0.15 mg/L [13]. Previous screening works of Ang and Lee [9] have shown elevated levels of mercury in several of the commercial Tongkat Ali preparation. As mercury is very toxic its determination in numerous herbal formulation and preparation in use not to mention variation between batches of production-makes routine mass monitoring highly important. Instrumental method alone could not handle such volumes of monitoring as it would be very expensive and time consuming. Biomonitoring using enzyme assay could cut cost and handle such high volumes 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 result indicate that the bromelain assay could be used as a preliminary screening tool for the detection of mercury in herbal products as the availability of FIMS in monitoring stations and laboratory is limited and time consuming. Using the bromelain assay only positive samples are needed to be sent for instrumental analysis, drastically cutting cost and analysis time allowing for more samples to be screened. The use of enzyme-based assay to detect heavy metal contamination in herbal preparation is almost non-existent in literature search and comparison with published works is not possible. A lot of assays for heavy metals such as microbe-based assays [16], aquatic-based organism assays [8,17,18] and enzyme-based assays [14] are interfered by high salt content in samples and would be strongly affected by salt concentration after aqua regia neutralization of digested samples. Probably this is the reason why these assay had never been attempted to monitor heavy metals in herbal

preparation. In the future, bromelain can be used in the form of biosensor either with label or label free format to monitor heavy metals contamination in herbal products. Hence this work is important in opening new areas for improvement.

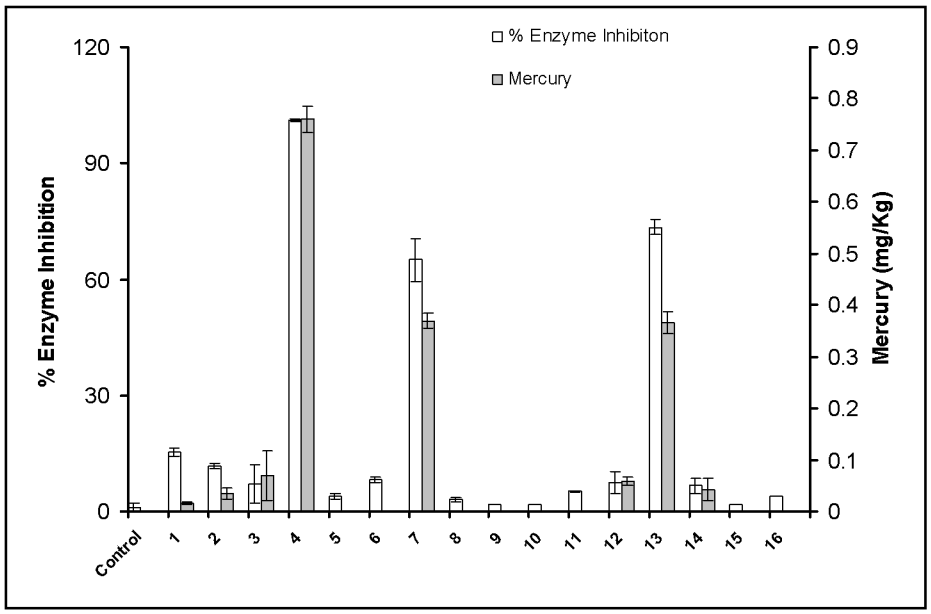

Figure 1: Bromelain activity inhibition by digested herbal samples and mercury concentrations in the samples. Results are mean ± standard deviation (n=3).

CONCLUSION

The bromelain assay was successfully used to detect elevated level of mercury in herbal samples with three of the samples tested showed the presence of mercury and out of these, one sample was detected using FIMS to contain level of mercury above the permissible limit of 0.5 mg/Kg. The rapidity and low cost of the bromelain assay makes it useful for routine biomonitoring of herbal samples.

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