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An Inhibitive Determination Method for Heavy Metals Using Bromelain, A Cysteine Protease

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Abstract A heavy-metal assay has been developed using bromelain, a protease. The enzyme is assayed using casein as a substrate with Coomassie dye to track completion of hydrolysis of casein. In the absence of inhibitors, casein is hydrolysed to completion, and the solution is brown. In the presence of metal ions such as Hg^{2+} and Cu^{2+}, the hydrolysis of casein is inhibited, and the solution remains blue. Exclusion of sulfhydryl protective agent and ethylenediaminetetraacetic in the original assay improved sensitivity to heavy metals several fold. The assay is sensitive to Hg^{2+} and Cu^{2+}, exhibiting a dose–response curve with an IC_{50} of 0.15 mg l^{-1} for Hg^{2+} and a one-phase binding curve with an IC_{50} of 0.23 mg l^{-1} for Cu^{2+}. The IC_{50} value for Hg^{2+} is found to be lower to several other assays such as immobilized urease and papain assay, whilst the IC_{50} value for Cu^{2+} is lower than immobilized urease, 15-min Microtox™, and rainbow trout.

Keywords Bromelain · Cysteine protease · Inhibitive determination method

Biosensors and bioindicators provide rapid and simple measurements for the analysis of heavy metal compounds. Bioassay using bacteria has been developed and commercialized such as the Lux-Fluoro™ [1], Polytox™ [2] and the Microtox™ assays [3]. Despite this, enzyme-based assays provide a simpler and less expensive method, as no specialized

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equipment is required [4]. On the other hand, microbial-based bioassay is not specific and will detect both heavy metals and pesticides. Enzyme assays are also tolerant to toxicant-permeability problems often experienced in microbial-based bioassay. Enzymatic-based assays are more specific to a certain group of xenobiotics, e.g. acetylcholinesterase is sensitive to carbamate and organophosphate pesticides [5], whilst urease [6] is selective for heavy metals. Numerous enzymes have been used for inhibitive determination of heavy metal traces, e.g. peroxidase, xanthine oxidase, invertase, glucose oxidase, isocitric dehydrogenase and the proteases trypsin [7], but the most frequently applied is urease, as it is relatively cheap and easily available, although it suffers from high interference [8].

Bromelain (EC 3.4.22.4) is a mixture of bromelain A and B, the proteolytic enzymes of pineapple fruit, *Ananas comosus*. Bromelain is a cysteine protease containing a single thiol group in the active sites. This thiol group has been reported to be inhibited by trace amounts of heavy metals normally present in all buffers [9]. The concentration of heavy metals giving 50% inhibition or IC_{50} for bromelain, however, was never studied in the absence of the ethylenediaminetetraacetic (EDTA) and 2-mercaptoethanol or dithiothreitol (DTT). Previously, we have developed a similar inhibitive assay of heavy metals using papain, another cysteine protease. The papain assay is sensitive to several heavy metals. The IC_{50} of Hg^{2+}, Ag^{2+}, Pb^{2} and Zn^{2+} is 0.39, 0.40, 2.16 and 2.11 mg l^{-1}, respectively, whilst for Cu^{2+} and Cd^{2+}, the limit of quantitation (LOQ) is 0.004 and 0.1 mg l^{-1}, respectively [10]. In this work, the potential of bromelain as another assay for heavy metals is presented for the first time.

Materials and Methods

Preparation of Buffer Solutions

All buffers were prepared by mixing the appropriate amount of salts and acids forms of the reagent. Minor adjustment of buffer was made using 5 N NaOH and 5 N HCl.

Bradford Coomassie-Dye Binding Assay

Approximately 100 mg of Coomassie Brilliant Blue G-250 from Sigma was weighed and dissolved in a mixture of 50 ml 95% (*v*/*v*) ethanol and 100 ml 85% (*v*/*v*) phosphoric acid. The solution was made up to 1,000 ml and stirred overnight. The solution was filtered through Whatman Filter Paper no. 1 and stored in dark bottle. Alternatively, a commercial Bradford dye-binding reagent from Bio-Rad may be used. The commercial preparation was used according to manufacturer's instructions.

Preparation of Casein and Bromelain Solution

Approximately 2 g of casein (Sigma) was weighed and dissolved into 100 ml of deionised water adjusted to pH 8.0 with 5 N NaOH and 5 N HCl. The resulting precipitous solution was incubated overnight with stirring at 60°C. The casein stock solution (10 mg ml^{-1}) was initially filtered through several layers of cheesecloth. The filtrate was then centrifuged at 10,000×*g* for 15 min, and the protein concentration of casein in the clear supernatant was measured using the Bradford dye binding using crystalline bovine serine albumin (BSA, Sigma) as the standard. Bromelain (Sigma), E.C. 3.4.22.32, Lot no. 118C-9002, grade 2, 2,300 U/gm was prepared at 4°C in 50 mM sodium phosphate pH 6.5 as a 22.1-mg ml^{-1}

stock solution. Bromelain (1.1 mg ml^{-1}) and casein (0.3 mg ml^{-1}) working solutions were prepared fresh daily by diluting in 100 mM phosphate buffer pH 6.5.

Preparation of Heavy-Metal Solutions

Heavy metals and metals such as chromium (vi) ($K_2Cr_2O_7$, BDH), selenium (vi) (Na_2SeO_4, BDH), nickel (ii) ($NiCl_2$, Ajax Chemicals), zinc (ii) ($ZnSO_4$ anhydrous, J.T. Baker), iron (ii) [$(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, BDH], tungsten (vi) ($Na_2WO_4{\cdot}2H_2O$, BDH), tin (ii) ($SnCl_2{\cdot}2H_2O$, BDH) manganese (ii) ($MnSO_4{\cdot}H_2O$, BDH), borate (iii) ($H_3BO_3$, anhydrous, BDH), cobalt (ii) ($CoCl_2{\cdot}6H_2O$, J.T. Baker) and aluminium (iii) [$Al_2(SO_4)_3$, anhydrous, BHD] were prepared from commercial salts or from atomic absorption spectrometry standard solutions from Merck such as mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii). Common non-toxic metals such as potassium (K), calcium (Ca) and magnesium (Mg) were added in the form of KCl, $CaCl_2$ and $MgSO_4$, respectively, at the final concentration of 50 mg l^{-1}.

Preparation of Pesticides and Other Xenobiotic Solutions

Pesticides, with chemical purity >99% from Ehrenstorfer (Augsburg, Germany) and Pestanal®, (Riedel de Häen, Germany) such as carbaryl, flucythrinate, metolachlor, glyphosate, diuron, diazinon, endosulfan sulphate, atrazine, coumaphos, imidacloprid, dicamba and paraquat were prepared by dissolving the pesticides in the appropriate solvent or used directly from the liquid solutions. Stocks solutions at 30 mg l^{-1} were prepared in deionized water and added into the reaction mixture to a final concentration of 5 mg l^{-1}.

The xenobiotics tested include detergents, solvents and various industrial effluent-related chemicals. The xenobiotics are acetonitrile (Merck), acetone (Merck), ethylene glycol (Sigma), *n*-Hexane (Merck), ethyl acetate (Merck), ethanol (BDH), dimethyl sulfoxide (DMSO, Sigma), isopropanol (BDH), methanol (BDH), pyridine (Sigma), toluene (Merck), triethanolamine (Sigma), acrylic acid (Sigma), polyethylene glycol 600 (PEG 600, Sigma), polyethylene glycol 400 (PEG 400, Sigma), diethylamine (Sigma), sodium dodecyl sulphate (SDS, Sigma), Triton-X-100 (Sigma), Nonidet-P40 (Sigma) and ethanolamine (Sigma). These xenobiotics were prepared as a 2% (*w*/*v*) solution in deionized water and added into the reaction mixture to a final concentration of 0.25% (*v*/*v*), with the exception of toluene where it was prepared as a 0.01% (*v*/*v*) solution to account for its limited solubility in water. Toluene was added into the reaction mixture to a final concentration of 0.005% (*v*/*v*).

The average deviations (three replicates) of the effect of xenobiotics and pesticides were statistically analyzed using one-way analysis of variance (ANOVA; α=0.05) followed by the Dunnett post test to detect any differences among the tested groups compared to control.

Bromelain Inhibition Studies

The inhibition assay is based on the assay developed by Shukor et al. [10] which was modified from Burokerkilgore and Wang [11]. In an Eppendorf tube, 20 μl of bromelain from the stock solution was added with 10 to 100 μl of heavy metals or xenobiotics from stock solutions dissolved in phosphate buffer pH 6.8. The final concentration of bromelain in the reaction mixture is 0.11 mg ml^{-1}. When using heavy metals from Atomic Absorption Spectrophotometer standard solutions, working stock solutions of 20 mg l^{-1} were made by dissolving the standard solutions in 100 mM phosphate buffer pH 6.5. Our experience shows that simply diluting the standard solutions in deionized water is not effective in

reducing the acidic effects of nitric acid in the solutions, which can be as high as 20 mM in the working solutions. The mixture was incubated at room temperature for 15 min, and then 50 μl of casein was added to the final concentration of 0.25 mg ml^{-1}. The final volume was made up to 200 μl using deionized water, and immediately, 20 μl aliquot was withdrawn and mixed with 200 μl of Bradford dye-binding reagent in a microplate well and incubated for 5 min to get the absorbance for time zero. The remaining solution was incubated at 40°C for 30 min. After this incubation period, a 20-μl aliquot was again taken and treated in the same manner with the aliquot at time zero. The absorbance at 595 nm was measured using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology, USA). When studying the inhibition curves of heavy metals which gave sigmoidal and hyperbolic dose–response curves, the values for the IC_{50} were calculated using four-parameter logistics and one-phase binding models, respectively, available from Prism non-linear regression analysis software from www.graphpad.com. The conditions employed in this section such as pH, temperature, concentrations of substrate and enzymes are optimum conditions resulted from bromelain optimization studies (data not shown). Protein was assayed according to the dye-binding method [12]. Means and standard errors were determined according to at least three independent experimental replicates.

Field Trials

Water samples were obtained locally from known polluted sites from several industrial outlets that are concentrated in the Prai Industrial estate in Penang and several streams in places that have been gazetted as clean by the Malaysian Department of Environment (DOE) [13] such as Sungai Udang Recreational Jungle, Melaka (SURJ), Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan (UBRJ) and Gunung Arong Forest Reserve, Mersing Johor (GAFR). The Prai Industrial estate, bound by the Juru River, is amongst the earliest industrial complexes built in the 1970s and is notorious for causing high levels of heavy-metal concentration in the river [14]. The rivers form the major collection points for mixed effluents coming from electric, electronic and metal-foundry industries from the estate. It is not possible to trace the composition of the effluents to the actual pollutant-emitting industries, as the effluents from these industries are channelled through underground pipes and are further mixed at merging channels before emptying their content into these rivers. All of the water samples were collected in acid-washed high-density polyethylene bottles containing several drops of 1% (*v*/*v*) HNO_3. The samples were filtered with 0.45 μm syringe filter. An aliquot of 45 ml of the clear filtrate was mixed with 50 μl of 100 mM phosphate buffer pH 6.5 followed by the addition of 5 μl of bromelain in an Eppendorf tube and again mixed thoroughly. The mixture was incubated for 20 min at room temperature and then was assayed according to the procedure outlined before. Tap water replaced samples to form the negative control, whilst copper at 1.0 mg l^{-1} formed the positive control. The determination of heavy metals in the samples was carried out using atomic emission spectrometry on a Perkin Elmer Optima 3000 inductively coupled plasma atomic emission spectroscopy (ICP-AES). All experiments were performed in triplicates.

Results and Discussion

Bromelain, similar to papain can be assayed by a variety of methods ranging from casein and azocasein to artificial substrates such as Nα-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), benzoyl-L-arginine ethyl ester (BAEE) and N-benzoyl-L-tyrosine ethyl ester

(BTEE) [15, 16], and the Bradford dye-binding reagent can be replaced with Biuret-Lowry, Folin Ciocalteu and Bichinconic acid. However, the Bradford dye-binding assay is the best system in terms of rapidity, simplicity, sensitivity and interference-free systems for the use as a protease assay [11]. The commercial Bradford dye-binding assay from Bio-Rad has a linear range of up to 0.70 absorbance unit at 595 nm (data not shown) and is adequate for this assay works. This relatively wide range of linearity allows visual differences between positive and negative results to be clearly seen, and this is important for qualitative works for monitoring at the large-scale level using color chart in the future.

The Bradford dye-binding reagent is unable to stain polypeptide with a molecular weight less than 2 kDa. Casein is a big protein with varying molecular sizes ranging from monomer to octamer on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [17]. Casein is stained by the Bradford dye-binding reagent giving a blue solution. Bromelain acts on casein by degrading it into small polypeptides that are not stained by the reagent, and the solution remains brown. In the presence of heavy metals that inhibit bromelain activity, casein would remain undigested, and the color would remain blue. This is the advantage of using the Bradford dye-binding–bromelain–casein system as an assay for heavy metals. A simple field trial test for the presence or absence of heavy metals would be visibly detected as blue or brown colors, respectively.

In order to ensure that optimum sensitivity to heavy metals is obtained, heavy metals' chelant such as EDTA as well as sulfhydryl group reducing agents such as DTT or 2-mercaptoethanol must be excluded from reaction mixture especially EDTA. An example of the detrimental effects of adding EDTA is in the microbial MTT assay. The principal microbe in the assay, *Rhizobium meliloti* is unfortunately sensitive to the non-toxic ions calcium and magnesium at several milligrams per litre levels [18]. Addition of EDTA alleviates the toxic effect of both metals but significantly lowers the inhibitive effect of other heavy metals. Exclusion of these agents improved markedly the sensitivity of the bromelain assay to mercury and copper (data not shown) similar to the results obtained from the papain assay [10].

Inhibition of Proteases by Heavy Metals

At 1 mg l^{-1}, two heavy metals, mercury and copper, show complete inhibition of bromelain activity (Fig. 1). Non-toxic metal ions such as potassium (K), calcium (Ca) and magnesium (Mg) at the final concentrations of 50 mg l^{-1} do not inhibit the activity of bromelain, whilst toxic heavy metals such as arsenic, lead, chromium zinc and nickel show no inhibition at 1 mg l^{-1}.

Table 1 shows the IC_{50} values and their 95% confidence intervals (CI) for the two heavy metals that inhibit bromelain activity. Mercury shows sigmoidal dose–response curves, and the regression model best suited this curve with high correlation coefficient values is four-parameter logistic with variable Hill slope value. The inhibition curve for mercury in bromelain is similar to the curve found for mercury in papain but bromelain is more sensitive to mercury with a lower IC_{50} (Table 2). Copper gave a linear inhibition curve in papain, whilst in bromelain copper gave a hyperbolic inhibition curve, and the best model is one-phase binding; a model often used to find the Michaelis constant, K_m, from enzyme's rectangular hyperbolic kinetics. In contrast to papain, bromelain is not inhibited by cadmium, lead and zinc [10]. Table 2 shows the comparison of bromelain assay to papain, immobilized urease, Microtox™, *Daphnia magna* and fish assays (rainbow trout).

The IC_{50} value for copper is within the IC_{50}, LC_{50} (lethal concentration to 50% of subjects exposed) and EC_{50} (concentration responsible for a 50% reduction of response) range of immobilized urease, 96-h rainbow trout, and 15-min Microtox™. Due to the linear

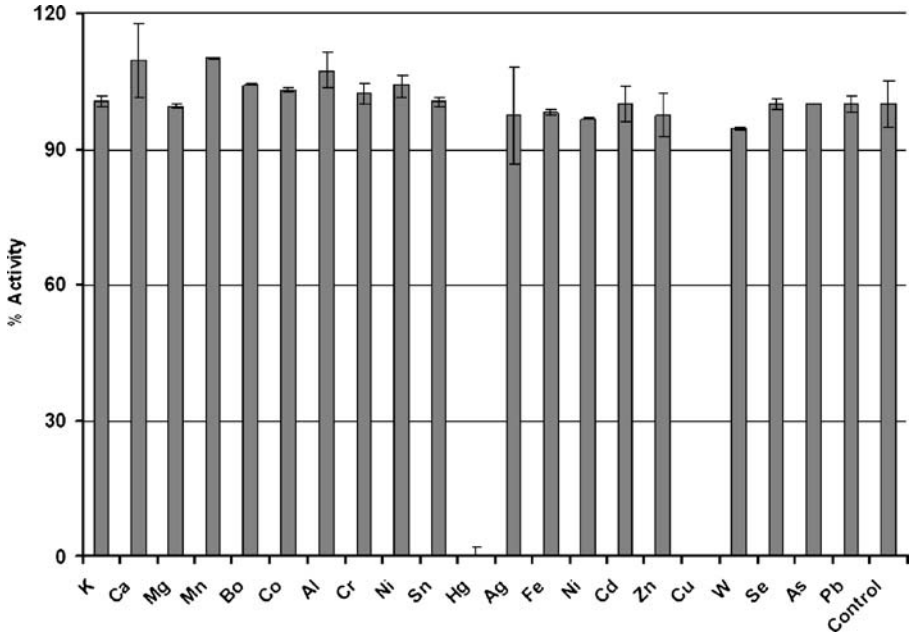

Fig. 1 Screening for heavy-metal inhibition of bromelain activity. Data are mean±SE of the mean (n=3)

relationship exhibit by copper and cadmium to the papain assay, the IC_{50} can not be determined, as the limit of response is limited to the linearity of the Bradford dye-binding assay. Thus, a LOQ is used instead. The IC_{50} value for mercury is within the IC_{50}, LC_{50} and EC_{50} range of all of the assays with the exception of 15-min Microtox™. The IC_{50} value of immobilized urease is used instead of free urease, as the ubiquitous presence of high level of background ammonia in samples usually prevent environmental analysis; hence there is the need to immobilize the urease [6]. Repeated measurement of bromelain inhibition by all of the heavy metals suggests the assay is reproducible with coefficient of variation of the replicated data ranging from 6 to 13%.

Interference Study

The influence of foreign species on the proposed method was investigated. Pesticides such as carbaryl, flucythrinate, metolachlor, glyphosate, diuron, diazinon, endosulfan sulphate, atrazine, coumaphos, imidacloprid, dicamba and paraquat showed no effect to the activity of bromelain relative to control (one-way ANOVA, $F_{12,26}$=0.3527, P>0.05). Pesticides were tested at the final concentrations of 5 mg ml^{-1}, and the signals obtained by this method were compared with the signals obtained without the interference. The concentration of pesticide chosen in this work is generally much higher than normally found in natural water and also limited to the solubility of pesticide in water.

Table 1 IC_{50} values for heavy metals that inhibit the activity of bromelain.

Heavy metals	Regression model	R^2	IC_{50} mg l^{-1} (95% CI)
Hg	Four-parameter logistic	0.973	0.13 to 0.16
Cu	One-phase binding	0.999	0.1631 to 0.3048

Table 2 Comparison of bromelain assay to immobilised urease, Microtox™, *Daphnia magna* fish assays (rainbow trout) and papain.

Metals	IC_{50} immobilized urease[a]	EC_{50} 15-min Microtox™ [b]	LC_{50} 48-h *Daphnia magna*[a]	LC_{50} 96h rainbow trout[c]	IC_{50} papain (mg l^{-1})[c]	IC_{50} bromelain (mg l^{-1})
Cu	0.41±0.14	0.076–3.8	0.020–0.093	0.25	0.004 (LOQ)	0.16 to 0.30
Hg	0.33±0.021	0.029–0.05	0.0052–0.21	0.033–0.21	0.24–0.62	0.13 to 0.16

[a] Jung et al. [6]

[b] Hsieh et al. [3]

[c] Shukor et al. [10]

About 16 out of 20 xenobiotics tested; acetonitrile, acetone, ethylene glycol, *n*-hexane, ethyl acetate, ethanol, DMSO, isopropanol, methanol, pyridine, toluene, triethanolamine, PEG 600, PEG 400, diethylamine and acrylamide all at the final concentration of 0.25% (*v*/*v*) show no significant difference than control (one-way ANOVA, $F_{16,34}=0.058$, $P>0.05$) whilst SDS, Triton X-100, Nonidet-P40 and ethanolamine gave positive interference with percent activity at 155, 186, 162 and 140%, respectively, compared to control (100%). The results are similar to papain assay [10] and are probably because both are plant cysteine proteases.

Field Trials

The results (Fig. 2) show that all three locations sampled from the Prai Industrial estates gave positive toxicity results, whilst all of the three non-polluted sites gave negative results

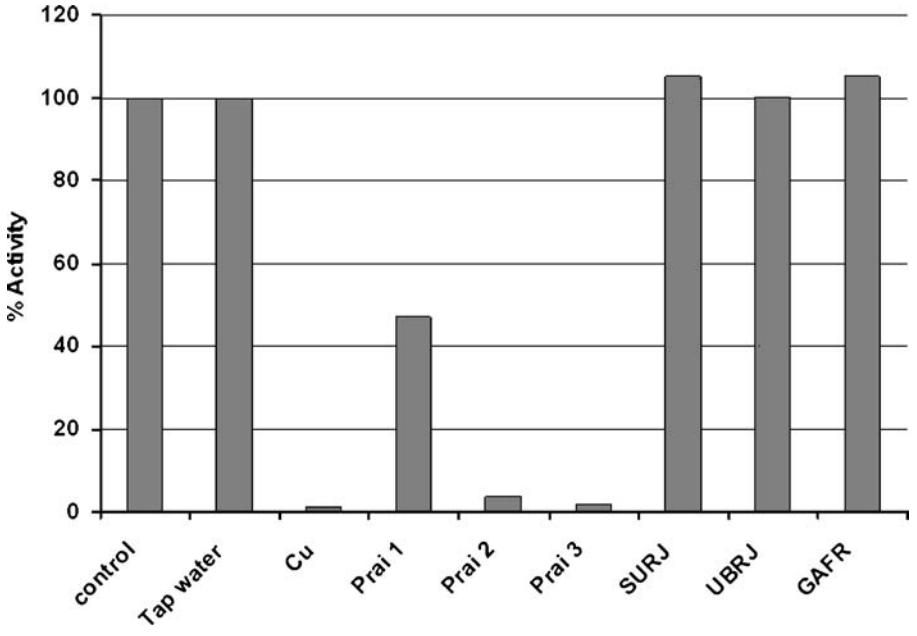

Fig. 2 Inhibition of bromelain activity by locally collected water samples. Prai 1, 2 and 3 are water samples collected from the Prai Industrial Estate, whilst SURJ, UBRJ and GAFR are samples collected from Sungai Udang Recreational Jungle, Ulu Bendul Recreational Jungle, and Gunung Arong Forest Reserve

Table 3 Concentrations of Hg and Cu in samples as determined using Perkin Elmer Optima 3000 ICP-AES. Data are mean±SE of the mean for three replicates.

Locations	GPS location	Concentration of heavy metal (mg l^{-1})	
		Hg	Cu
Prai Industrial Estate, Penang (Prai 1)	05°20.96′N, 100°24.17′E	18.46±1.34	0.13±0.05
Prai Industrial Estate, Penang (Prai 2)	05°21.06′N, 100°24.21′E	16.67±0.39	n.d.
Prai Industrial Estate, Penang (Prai 3)	05°21.09′N, 100°24.39′E	17.89±0.69	n.d.
Sungai Udang Recreational Jungle, Melaka (SURJ)	02°18.102′N, 102°07.837′E	n.d.	n.d.
Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan (UBRJ)	02°43.767′N, 102°04.668′E	n.d.	n.d.
Gunung Arong Forest Reserve, Mersing Johor (GAFR)	02°33.197′N, 102°45.340′E	n.d.	n.d.
Tap water	Universiti Putra Malaysia	n.d.	n.d.

n.d. Not detected

for the presence of toxic heavy metals studied. The almost complete inhibition (99%) by 1.0 mg l^{-1} copper forms a positive control, and full activity (100%) of tap water forms a negative control for the experiment. It was found that mercury forms the major contaminant in the estate (Table 3) with levels far exceeding the Malaysian DOE maximum permissible limit for class II (Fishery) at 0.005 mg l^{-1} [13]. Copper in sample Prai 1 at 0.13 mg l^{-1} also exceeded the DOE standard which states 0.02 mg l^{-1} as the maximum permissible limit for the same class. Although sample Prai 1 contains similar concentrations of mercury as samples Prai 2 and 3, the inhibition of bromelain activity was only 43.8% compared to 97.6 and 98.9% for samples Prai 2 and 3 (Fig. 2), respectively, suggesting bioavailability of mercury is probably lower in sample Prai 1 compared to Prai 2 and 3. Previous field trial in the same locations using papain was successful in detecting toxicity of the water due to copper [10]. However, mercury was not measured at the time, and it is probably the main cause of papain inhibition. The unpolluted status of tap water, water samples taken from recreational jungle sites and a forest reserve determined by the proposed method is in agreement with ICP-AES data (Table 3). The waters of Prai Industrial Estate empty their content into the Juru Estuary, holding one of the largest *Anadara granosa* cockle cultures in Malaysia [14], making the results obtained in this work highly significant to be a basis for more exhaustive field trials in the Juru Industrial Estate in the future for signs of environmental pollution.

Conclusion

A novel assay for several heavy metals using an inhibitive-enzyme system was developed using a dye-binding-bromelain-casein assay system as the principal assay and omitting sulfhydryl-protecting and metal-chelating agents. The assay could detect copper and mercury at sub-parts-per-millions. Trials using samples obtained from polluted and non-polluted sites show promising results. The bromelain assay is not interfered with

xenobiotics and, combined with its broad pH and temperature range for activity, suggests that the assay is suitable for large-scale environmental biomonitoring. As stem bromelain obtained from Sigma consists of Bromelain A and B and can be separated by chromatography, we are currently attempting to purify these individual components and subjecting each component to heavy metals. We hope to find some differences or similarities in terms of inhibitory effect of heavy metals. We will also use different substrates for bromelain such as BAPNA, BAEE and BTEE using the same conditions obtained in this work. We are also currently evaluating extensive field-trial results using papain and bromelain and comparing enzyme inhibition data for bioavailable and nonavailable heavy metals and total heavy metals using atomic emission spectrometry.

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