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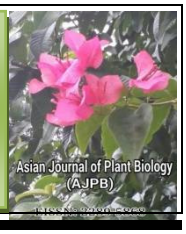

The Application of Plant Proteases from Garlic (*Allium sativum*) for Biomonitoring of Heavy Metals in the Environment

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HISTORY

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

KEYWORDS

garlic (*Allium sativum*)
proteases
iomonitor
heavy metals

ABSTRACT

In this work, a novel source of protease for the bioassay of heavy metals has been developed using proteases extracted from garlic (*Allium sativum*). The principle of casein-Coomassie-dye binding assay has been applied for this purpose. The optimum concentration of enzyme and substrate were determined at 0.7 mg/mL and 0.175 mg/mL, respectively. The crude proteases of *Allium sativum* exhibited optimal activity at pH 6.0, temperature at 40oC and incubation time of 60 minutes. The IC50 values for mercury, copper, cadmium, nickel and chromium were 0.0590 mg/L, 0.6398 mg/L, 1.291 mg/L, 0.9865 mg/L and 1.871 mg/L, respectively. The calculated limits of quantitation (LOQ) value for mercury, copper, cadmium, nickel and chromium were 0.0188 mg/L, 0.025 mg/L, 0.50 mg/L, 0.10 mg/L and 0.5 mg/L, respectively. The calculated LOD values for mercury, copper, cadmium, nickel and chromium were 0.0002 mg/L, 0.006 mg/L, 0.05 mg/L, 0.02mg/L and 0.1 mg/L, respectively. The crude proteases extracted from *Allium sativum* showed good potential for the development of a rapid, sensitive, and economic inhibitive assay for the biomonitoring of mercury, copper, cadmium, nickel and chromium in

INTRODUCTION

Water is one of the most important sources to human after air. The necessity of water to sustain life is a major concern as most waterways nowadays are being polluted with untreated wastes from industrial and agriculture activities [1]. In Malaysia, as evidenced from recent reports [2,3,4], water pollution is approaching to dangerous level due to heavily industrialized activities. At present, industrial wastes are prevalent in the composition of the waste waters from giant industrial cities resulting in the escalation of heavy metals that could eventually be released to the aquatic environment [5]. According to Malaysian Department of Environment, these toxic heavy metals contribute to severe water pollution to water bodies in Malaysia [6].

The importance in monitoring and preventing the ongoing metals pollution is paramount to safeguard public health. Heavy metal pollutions have been identified to cause harmful effects to humans such as haematotoxic, neurotoxic and nephratoxic and all of these diseases are almost untreatable due to irreversible effect towards biological systems [7]. As persistent polluters, heavy metals also accumulate in body causing permanent damages to tissues [8]. Therefore the accumulation of toxic heavy metals in waterways have attracted scientists to develop numerous methods

with consideration of cheap, fast and stability as a tool to detect the presence of heavy metals in environment [7,9,10,11].

To date various enzyme-based assays for heavy metal detections have been developed including molybdenum-reducing enzyme [12], urease [13] and proteases such as bromelain [14], papain[9] and trypsin[15]. Urease assay is the most sensitive to detect mercury pollution but its dual-sensitivity towards environmental ammonia present a problem for its inclusion as an accepted method for heavy metals biomonitoring by health agencies worldwide. Furthermore, the assay is conducted at temperature strictly 15°C preventing sites monitoring in tropical countries [16]. Bioassays using plant proteases such as papain, bromelain and another protease trypsin, have superior pH and temperature stabilities making them tolerant to sample pH and assay temperature fluctuations. However, the IC_{50} of certain heavy metals in these assays are not as sensitive enough as required by the Department of Environmental (DOE), Malaysia. Hence, there is an urgent need for screening for more sensitive proteases.

Allium sativum, commonly known as garlic is a species in the onion genus, *Allium*. It is one of the most studied herbal remedies as it is traditionally used to treat infection, diabetes, heart disease, and other disorders [17]. The application of this herbal is not limited only for health and industrial practices but it can be applied for environmental purposes. The presence of high activity of proteases in garlic bulb [18,19] has added valuable value to garlic as a tool for detection of heavy metals in environment. In this work, we reported garlic as a candidate for biomonitoring of heavy metals. The proteases from garlic were extracted at crude level and the crude was optimized for the optimum activity of proteases. The sensitivity of proteases from garlic toward heavy metals was evaluated in this study.

MATERIALS AND METHODOLOGY

Chemicals

All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Fisher (Malaysia) and Merck (Darmstadt, Germany).

Statistical analysis

The average deviations (three replicates) of the effect of toxicants were statistically analyzed using one way ANOVA ($\alpha = 0.05$) followed by the Dunnett post-test to detect any differences among the tested groups as compared to control. The IC_{50} values were determined using non-linear regression, Graph Pad Prism 5.0.

Preparation of buffers

All buffers were prepared according to the methods of Dawson (1969). Sodium phosphate buffer was prepared as a 50 mM solution at pH 6.5. The preparation of the buffer was as follows; 7.098 g of Na_2HPO_4 (50mM) and 7.8 g of $NaH_2PO_4.2H_2O$ (50 mM) from SIGMA were weighed and dissolved in 800 mL of deionized water each. Both solutions were mixed, slowly stirred and the pH was adjusted to pH 6.5 before the volume was made up to 1 L. Borate buffer was prepared as a 50 mM at pH 8 solution. The preparation of the buffer was as follows; 3.0915 g of boric acid from AJAX Chemicals was weighed and dissolved in 800 mL of deionized water. The solution was stirred and the pH value was slowly adjusted to pH 8 with NaOH (1 M). The volume was made up to 1 L [20].

Preparation of Bradford reagent

The following experiments were carried out to prepare the Bradford assay for quantifying casein from samples. 0.1 g of Coomasie Brilliant Blue G-250 from SIGMA was weighed and dissolved in mixture of 50 mL ethanol 95% and 100 mL phosphoric acid of 85%. Then it was added up to 1 L after the dye dissolved completely and stirred for at least 5 hours. The solution was filtered through Whatman Filter Paper No. 1 and stored in a dark bottle. Alternatively, commercial Bradford reagent from various manufacturers such as from BIO-RAD might be used. For this pre-made BIO-RAD reagent, 10 mL was taken and dissolved in 40 mL of distilled water.

Preparation of casein solution

Two grams of casein (SIGMA) was weighed and dissolved into 100 mL of deionized water and 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 (10.0 mg/mL) was initially filtered through several layers of cheesecloth. The filtrate was then centrifuged at 10,000×g for 15min and the protein concentration of casein in the clear supernatant was measured using the Bradford dye-binding assay using crystalline BSA (SIGMA) as the standard. Casein (0.3 mg mL^{-1}) working solutions were prepared fresh daily.

Preparation of heavy metals

Heavy metals such as mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii), cobalt (ii), iron (ii), chromium (vi) and silver (ii) were sourced from Atomic Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany). These heavy metal solutions were prepared at 100 mg L^{-1} concentration as stock solutions. Working solutions were prepared from the stock solutions at concentrations of heavy metals at 10 mg/ L, 8 mg/ L, 6 mg/ L, 4 mg/ L, 2 mg/ L, and 1 mg/ L and stored in acid-washed polypropylene containers. Individual heavy metal at 1 mg/ L was added into each reaction mixture as a preliminary screening. Heavy metals that inhibited the assay at 1 mg/ L were then further screened using lower heavy metal concentrations. Lower heavy metal concentrations were prepared by diluting 1 mg l^{-1} from the stock solution into 0.8 mg/ L, 0.6 mg/ L, 0.4 mg/ L, 0.2 mg/ L, 0.1 mg/ L, 0.08 mg/ L and 0.04 mg/ L.

Preparation of proteases from *Allium sativum*

Garlic (*Allium sativum*) was obtained from local market. Edible portion of garlic was chosen as raw material for the experiment. Cloves of garlic were weighed and soaked overnight with deionized water (1:2w/v) in chiller at 4°C. Fifty grams of garlic was soaked in 100mL of deionized water. Then, it was homogenized by using an ice cold steel blender. The homogenate was checked for pH value before it was centrifuged at 13,000xg for 10 min (4°C). The pH value of supernatant was again measured and appropriate buffer was used to resume the pH of homogenate. The supernatant (juice fraction) was recovered and

filtered with syringe filter 0.25 µm. This process resulted in clear crude extract. The crude extract was then kept in ice at 0°C to suppress any enzyme activity [21].

Optimization of proteases

The experiment was carried out to find the optimum concentration of enzyme that could give an optimum absorbance difference during the period of incubation. Different concentrations of enzymes in the range 0–0.5 mg ml^{-1} were tested by mixing suitable volumes of the enzyme solution with 50µl of 100 mM phosphate buffer pH 6.5. The final volume was made up to 100µl using deionized water and the mixture was incubated for 20 min at room temperature. After the incubation period, 50µl of casein from a stock solution of 0.3 mg ml^{-1} was added and mixed thoroughly. Initially, a 20µl 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 room temperature for 30 min. After this incubation period, a 20µl aliquot was 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 Inc., USA). In order to study the optimum concentration of the substrate casein, crude concentration in the reaction mixture was fixed at 0.1 mg ml^{-1} whilst for temperature and pH optimization studies, casein and crude concentrations were fixed at 0.3 mg ml^{-1} and 0.1 mg ml^{-1} respectively. The overlapping buffer system used for pH optimization studies were: citrate phosphate buffer from pH 5.0 to 6.0, sodium phosphate buffer from pH 6.0 to pH 7.5 and Tris buffer from pH 7.2 to pH 8.0. One unit of activity is defined as the amount of casein (in mg) hydrolyzed per minute by 1 mg of protease under the specified assay conditions [9].

Screening of heavy metals

The assay was conducted according to method developed by Shukor et al., [9]. In an Eppendorf tube, 50 µl of crude extract from the stock solution was added to 120 µl of 50 mM phosphate buffer pH 6.0 followed by the addition of 20 µl of heavy metals. As a control, the heavy metals were replaced with deionized water. The final volume was made up to 200 µl using deionized water and the mixture was incubated for 20 minutes at room temperature. After the incubation period, 30 µl of casein from a stock solution of 0.3 mg ml^{-1} was added and mixed thoroughly. Initially, a 20 µl aliquot was withdrawn and mixed with 200 µl of Bradford dye-binding reagent in a microplate well and incubated for 5 minutes to get the absorbance for time zero. The remaining solution was incubated at 40 °C for 60 minutes. 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 Inc., USA). The values for the IC_{50} (inhibitory concentration, 50%), was calculated using the method Shukor et al [9]. Some metals exhibit a different inhibition curves. For these curves, regression models that satisfactorily give high correlation coefficient include one-phase binding. The IC_{50} from the one-phase binding model is equal to half maximal response. The one-phase binding model itself is similar to the Michaelis-Menten rectangular hyperbolic kinetic, thus the IC_{50} is the same to the value of the Michaelis constant; Km. Regression curves are generated using the PRISM (Prism version 5.00 for Windows) non-linear regression analysis software available from GraphPad, (GraphPad Software Inc., San Diego, CA). Means and standard errors were determined according to at least three independent experimental replicates.

Field trial

Water samples were collected from Prai Industrial Estate and Juru River which are located in Prai, Pulau Pinang, about 400 km from Kuala Lumpur. Water samples were collected in acid-washed HDPE bottles containing several drops of 1% (v/v) HNO_3. The samples were filtered with 0.45 µm syringe filter. Twenty microliters of the clear filtrate was mixed with 120 µl of 50 mM phosphate buffer pH 6.5 followed by the addition of 50 µl of crude enzyme in an eppendorf tube and again mixed thoroughly. The mixture was incubated for 20 min at ambient temperature. After the incubation period with water sample, 50µl of casein from a stock solution of 0.3 mg ml^{-1} was added and mixed thoroughly and the absorbance was measured using portable spectrophotometer. The validation of heavy metals in the water samples were carried out using Inductive Couple Plasma spectrophotometer on a Perkin Elmer Optima 3000 ICP-OES. All experiments were performed in triplicate.

RESULTS AND DISCUSSIONS

Optimization of proteases activity

In this part, the optimum proteases activity was evaluated until concentration 1 mg/ml, the optimum concentration for crude concentration occurred at 0.7 mg/ml protein (**Fig.** 1). The concentration of crude above this, showed no significant different for the activity due to the saturation of the protein and the presence of other proteins in crude extract of *Allium sativum* causing hindrance of protease activity. In addition, excess concentration of enzyme might decrease sensitivity of bioassay toward heavy metals because high concentration of heavy metals required to inactive proteases activity of garlic. Therefore, the optimum concentration of the enzyme must be determined to prevent sensitivity problem. The crude can be assayed using several methods such as Lowry, Bichinconic acid and Bradford method with casein or azocasein as the substrate. In term of simplicity, rapidity and stability, Bradford dye binding assay showed many advantages and suitability to this bioassay system compared to other methods. Using this assay system, the activity of the crude was measured based on the capability of the crude ezyme to hydrolyse casein to polypeptide with a molecular weight less than 2 kDa, at this size, the Bradford dye binding reagent is unable to stain it and the color remain brown. If the activity is very low and no activity appears, the casein cannot be degraded by the proteases and dark blue color will appears indicating that the dye bind to the casein.

The effect of different concentration of casein toward proteases activity is presented in **Fig.** 2. The optimized concentration of substrate for the optimum protease activity was saturated at 0.175 mg/ml casein protein and above with no significant different. This substrate concentration was noted to be comparable with the concentration needed for the other bioassay proteases such as bromelain, papain and trypsin [9, 14, 15]. The optimized concentration at 0.175 mg/ml would ensure that degradation is complete before any auto digestion of proteases

could take place and concentration over this optimum concentration would make the casein taking a long time to be digested by proteases.

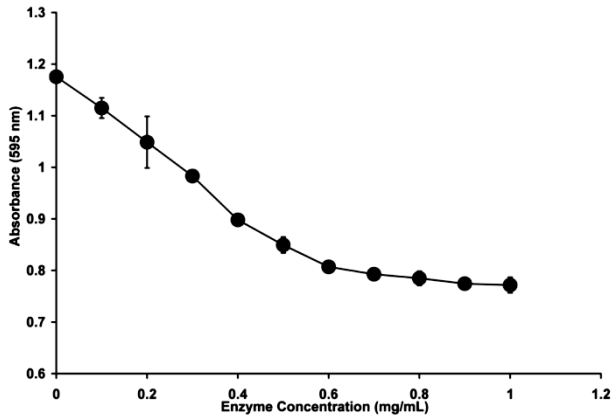

Fig. 1: The effect of enzyme concentration on the activity of proteases from garlic. Data was mean ± standard deviation of the mean (n=3).

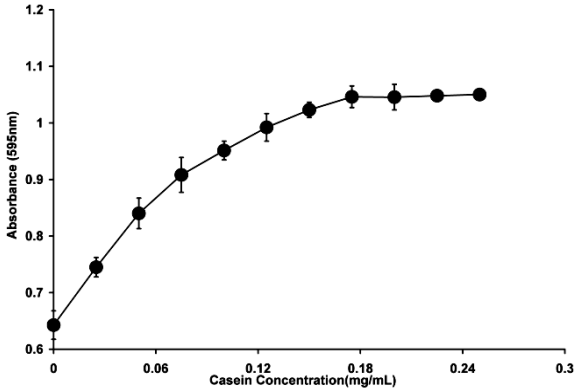

Fig. 2: The effect of substrate casein toward proteases activity. Data was mean ± standard deviation of the mean (n=3).

The most suitable pH for protease from *Allium sativum* was pH 6.0 (**Fig.** 3). Hence, pH 6.0 of sodium phosphate buffer was chosen throughout the experiment since citrate molecule of citrate phosphate buffer could act as chelating agent to prevent heavy metals from binding to the active sites of the enzyme. The catalytic activity of an enzyme is pH and temperature sensitive since intermolecular bonds that hold proteins in their secondary and tertiary structures are disrupted by changes in temperature and pH. The most favorable pH value is the point where the enzyme is most active (optimum pH). During field works, the samples will have different pH depending on where the samples are collected. Slightly changes in pH will affect the system and it will give inaccurate result during monitoring process. Therefore, the application of buffer in the bioassay system is important for preventing the pH of the samples from affecting the enzyme activity.

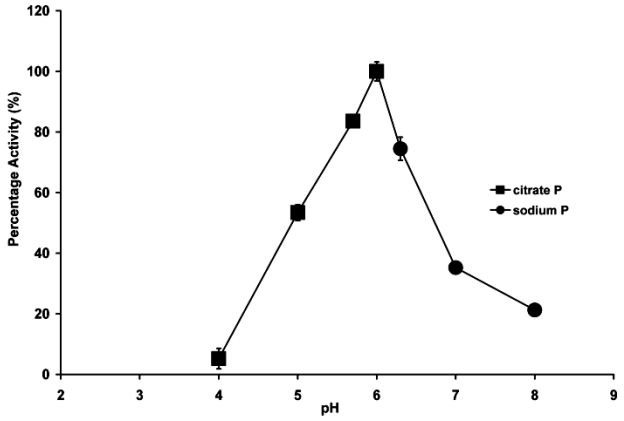

Fig. 3: Effect of pH on the activity of proteases from garlic. Data was mean ± standard deviation (n=3).

The proteases exhibited high protease activity in a broad range of temperature which was between 35-45^{o}C (**Fig.** 4). The instability of enzymes at temperature more than 45^{o}C is possibly caused by several factors including tertiary and quaternary protein denaturation through thermal vibration leading to loss of cofactors and accelerated oxidation of sulfhydryl groups at higher temperatures. A broad temperature range for protease activity is an advantage for field site monitoring in tropical country and the optimized temperature was comparable with the other proteases like bromelain, papain and trypsin [9, 14, 15]. Temperature stability is one of the main problem for on-site biomonitoring because some of the commercial toxicity bioassay kit working at temperature below than tropical temperature, for examples Microtox™ based on Vibrio fischery works at 15^{o}C making on site monitoring in tropical country unsuitable and expensive due to the application of refrigerated system for temperature maintaining [10]. The stability of protease at higher temperature would make this bioassay system cheap and simple without the application of expensive refrigerated system.

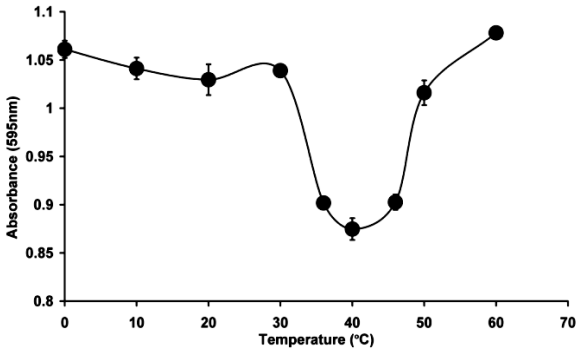

Fig. 4: Effect of temperature on the activity of proteases from garlic. Data was mean± standard deviation (n=3).

The incubation time for this enzyme occurred at 50 to 70 minutes at 40^{o}C which was longer compared to the other proteases such as bromelain, papain and trypsin, It needed just 30 minutes to complete digestion process (**Fig.** 5) but it still could be consider as a fast bioassay because the result can be obtained less than one and half hour including incubation of the enzyme with heavy

metals. The problem might due to the purity of the crude is low, the presence of other proteins in crude extract of *Allium sativum* that have caused proteases need a long period of time to react and hydrolyze casein. Therefore, purification of the crude is required in the future to remove unrelated protein in order to improve the incubation time.

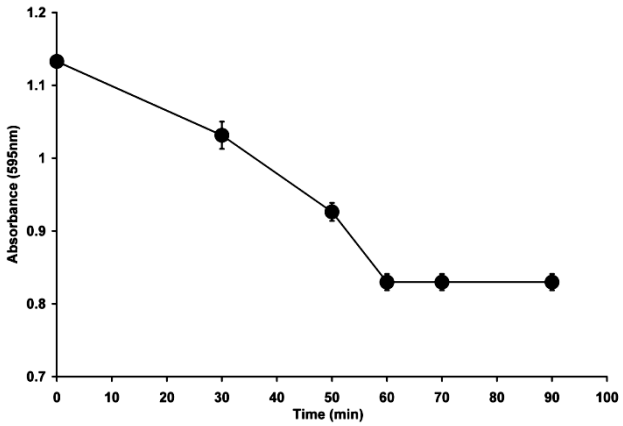

Fig. 5: Effect of incubation time on activity of protease from garlic. Data was mean ± standard deviation (n=3).

Inhibition of proteases by heavy metals

Among ten heavy metals tested, only five heavy metals inhibited proteolytic activity of garlic crude with inhibition more than 30% at 1 mg L^{-1} (**Fig.** 6). The inhibitions shown by the heavy metals on protease activity for mercury, copper, cadmium, nickel and chromium were 64%, 73%, 37%, 38% and 37% respectively. Therefore proteases from garlic have potential to be used in biomonitoring of those heavy metals in an environment. During inhibition of proteases by heavy metals, non-degradable substrate casein with size more than 2kDa can be stained by Bradford reagent to form blue complex structure [9]. The dye appears to bind most readily on basic (arginine) and aromatic amino acids residues [23]. Light blue colour notes that enzyme is not effectively inhibited by heavy metals, thus it can partially hydrolyze the substrate. Since the substrate was degraded into very small structure of end products with less than 2kDa, they cannot be stained by the Bradford reagent give brown color indicating the proteases of garlic do not inhibited by heavy metals. It has been reported that the toxic effect of metal ions on enzymes was usually due to their binding to thiol groups present in or near the center of active sites, producing an irreversible inhibition. Hence, it can be assumed that the presence of metal ion inhibits the activity of the enzyme by binding to the cysteine group of the enzyme at the active site causing alteration of enzyme's structure and inactivate the enzyme [22].

The sensitivity of proteases toward mercury, copper, cadmium, nickel and chromium was determined using determination of IC_{50}, LOD and LOQ generated from PRISM (Prism version 5.00 for Windows), a non-linear regression analysis software available from GraphPad, (GraphPad Software Inc., San Diego, CA). The IC_{50} values for each of the heavy metals are summarized in **Table** 1. The corresponding correlation coefficient values of 0.95 above in all of the curves obtained suggesting a good relationship of data with theoretical model calculations. The calculated IC_{50} for mercury, copper, cadmium, nickel and chromium were 0.0590 mg/L, 0.6398 mg/L, 1.291 mg/L, 0.9865 mg/L and 1.871 mg/L, respectively. The Limits of Quantification (LOQ) value also can be used which is equal to ten times the standard deviation of the blank. The calculated limits of quantitation (LOQ) value for mercury, copper, cadmium, nickel and chromium were 0.0188 mg/L, 0.025 mg/L, 0.50 mg/L, 0.10 mg/L and 0.5 mg/L, respectively. In addition, the limit of detection (LOD) value which is equal to three times of the standard deviation of the blank is to determine the lowest concentration of the heavy metals that can be detected by the enzyme. The calculated LOD values for mercury, copper, cadmium, nickel and chromium were 0.0002 mg/L, 0.006 mg/L, 0.05 mg/L, 0.02mg/L and 0.1 mg/L, respectively

Table 2 summarized the IC_{50} values of different proteases for comparison. Gunasekaran et al. [24] reported the application proteases from *Coriandrum sativum* for detection of mercury with IC_{50} 3.21 mg/L, which is less sensitive than *Allium sativum* which has IC_{50} 0.0590 mg/L. Comparing with other proteases, protease from *Allium sativum* offers the best sensitivity with its LOD value of mercury and copper much lower than the maximum permissible limit allowed in The Environmental Quality (Sewage and Industrial Effluents) Regulations 1979 at 1 mg l^{-1}, Furthermore, the IC_{50} cadmium, nickel and chromium was lower compared to other plants proteases assay. Therefore, proteases from *Allium sativum* have potential in biomonitoring of mercury, copper, cadmium, nickel and chromium in the environment.

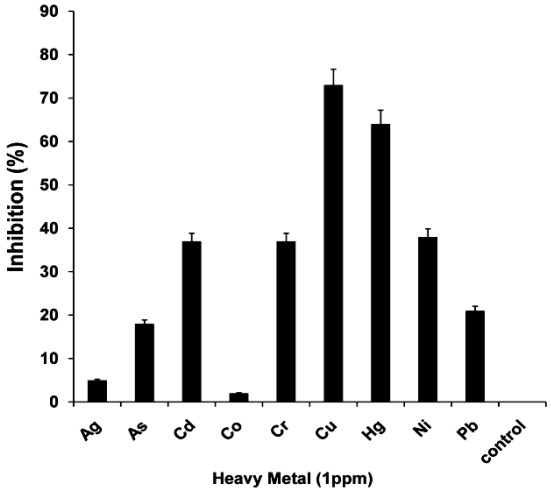

Fig. 6: Effect of heavy metals on proteases activities. Data is mean ± standard deviation of mean (n=3).

Field trial

Juru River and Prai Industrial Estate have been reported by Shukor et al, [9] as the location polluted with heavy metals. Therefore, four different locations were identified for field trials studies. All the sites were identified by GPS as shown in **Table** 3. Two controls: one positive, 1 mg l−1 copper, and one negative, tap water, were employed. The results show that one of the location at the Prai Industrial Estate and Juru River gave positive toxicity results with copper concentration exceeding the Malaysian Department of Environmental maximum permissible limit for Class II at 0.02 mg/L. Thus, The capability of proteases

from garlic as a tool detection of heavy metals in environment had been proven in this study.

Table 1. The summary of the IC_{50}, LOD and LOQ values for the three heavy metals that inhibits proteases from *Allium sativum.*

Heavy metals	Regression model	R^2	IC_{50}	LOD (mg/L)	LOQ (mg/L)
Hg	One site binding hyperbola	0.9801	0.0590	0.0002	0.0188
Cu	One site binding hyperbola	0.9933	0.6398	0.006	0.025
Ni	One site binding hyperbola	0.9819	1.291	0.05	0.5
Cd	One site binding hyperbola	0.9875	0.9865	0.02	0.1
Cr	One site binding hyperbola	0.9712	1.871	0.1	0.5

Table 2. The summary of the IC50 value in comparison to other protease assays.

Heavy Metals	*Allium sativum*	*Coriandrum Sativum* [24]	Bromelain [14]	Papain [9]	Trypsin [15]
Hg	0.0590	3.21	1.31	0.391	0.9
Cu	0.6398	-	0.23	-	-
Ni	1.291	-	-	-	-
Cd	0.9865	-	-	-	-
Cr	1.871	-	-	-	-

Table 3. Field trial results

Sample	Percentage Inhibition 50 % denotes samples is highly toxic	Heavy Metals detected
Prai Industrial estate 1 N 05° 21.599, E 100° 24.282	98	Copper (21.04ppm)
Prai Industrial estate 2 N 05° 21.162, E 100° 24.006	0	n.d
Juru River 1 N 05° 20.946, E 100° 24.745	70	Copper (0.5ppm)
Juru River 2 N 05° 21.201, E 100° 24.739	0	n.d
Tab Water	0	n.d

CONCLUSION

In this works, the application of proteases from garlic as tool for detection of heavy metals had been proven. The assay system were tested with selected toxic heavy metals and were found to be able to detect some heavy metals at the sensitivity required for routine heavy metals detection in the field. This assay has been shown to be simple, reproducible and rapid with good sensitivity.

ACKNOWLEDGEMENT

This project was supported by funds from The Research University Grant Scheme (RUGS), Project No: 05-01-09-0750RU/F1.

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