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An Inhibitive Determination Method for Heavy Metals Using Tomato Crude Proteases

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ABSTRACT

A new inhibitive heavy metals determination method using extract from *Lycopersicon esculentum* or tomato from has been developed. The enzyme was assayed using the casein-Coomassie-dye-binding method. In the absence of inhibitors, casein was hydrolysed to completion and the Coomassie-dye was unable to stain the protein and the solution became brown. In the presence of metals, the hydrolysis of casein was inhibited and the solution remained blue. The inhibitions shown by lead, chromium and zinc were, 67.9, 53.1 and 53 %, respectively. The IC_{50} (concentration causing 50% inhibition) values were 1.407, 0.835 and 0.707 mg/l, respectively. The limits of quantitation (LOQ), for zinc, chromium and lead were 0.729, 0.506 and 0.541 mg/l, respectively. The limits of detection (LOD) for zinc, chromium and lead were 0.032, 0.0317 and 0.0317 mg/l, respectively. The IC_{50} value for zinc was much lower than the IC_{50} values for papain and Rainbow trout assays. The IC_{50} value for zinc was lower than the immobilized urease assay. Other toxic heavy metals, such as silver, arsenic, copper, mercury and cadmium, did not inhibit the crude proteolytic enzyme activity. Based on the characteristics, crude protease enzyme from *L. esculentum* (tomato) can be used to detect heavy metals in various samples in conjunction with the dye-binding assay.

INTRODUCTION

Environmental pollution is one of the important global issues nowadays. Pollutants can be classified into several types such as pollutants from chemicals, pathogens and physical or sensory changes such as elevated temperature and discoloration. Heavy metals is one of the factor that leads to the environmental pollution as these toxicants are recognized to be highly toxic and can cause negative effects even at low concentrations. Metals are elements that cannot be degraded. Thus, metals remain in the environment [1]. Various types of methods can be used to quantify toxicity in contaminated samples. Classical methods such as the use of atomic absorption spectroscopy, inductively coupled plasma mass spectrophotometry and other highly sophisticated laboratory instrumentation can be used to quantify metals in contaminated samples. However, classical methods are expensive, complicated sample pretreatment, long measuring period and needs skilled personnel to operate them before the results can be obtained. Thus, a new alternative method which can detect heavy metals and at the same time can overcome the classical methods' disadvantages is really needed. This alternative method could be able to give accurate results, fast, cheap and easy to be conducted [2]. Hence, nowadays enzymatic bioassays are being used widely to quantify toxicity as they are highly selective and sensitive. Various kinds of enzymes such as papain, bromelain, trypsin and proteases from plants are being used due to their high sensitivity towards heavy metals. Proteases have excellent stability, wide range of pH and temperature for enzyme activity and tolerance towards many solvent [3]. The use of proteases in inhibitive assays for heavy metals could generate a robust bioassay kit. Plant proteases are discovered to be excellent source of bioassay for heavy metals [4-6]. In this study, the potential extract from *Lycopersicon esculentum* or tomato, as a simple inhibitive assay for heavy metals is presented for the first time.

MATERIALS AND METHODOLOGY

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

Bradford dye binding assay
One hundred milligrams of Coomassie Brilliant Blue G-250 from SIGMA (Sigma Chemical Co., St. Louis, USA) was weighed and dissolved in a mixture of 50 ml 95% ethanol and 100 ml 85% phosphoric acid. The solution was made up to 1000 ml and stirred overnight. The solution was filtered through Whatman Filter Paper No. 1 and stored in dark bottles. Alternatively, commercial Bradford dye-binding reagent from Bio-Rad (Bio-Rad, USA) was used according to manufacturer's instructions.

Preparation of casein solution
Two grams of casein (Sigma) was weighed, dissolved into 100 ml of deionised water and adjusted to pH 8.0 with 5N NaOH and 5N HCl. The resulting precipitous solution was incubated overnight in stirring condition at 60 ℃. The casein stock solution (10 mg/ml) 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 assay using crystalline BSA (Sigma) as the standard.

Preparation of *L. esculentum* (tomato) crude enzyme extract
Tomato was purchased from a local market at Seri Kembangan, Selangor. Tomato was weighed (114 g) and kept in the chiller for 24 h. Then, the tomato was cut into smaller pieces and blended with cold deionized water (228 ml) with ratio 1:2 for 30-60 s. Blended homogenate tomato solution was then filtered via two layers of cheese cloth. The supernatant was collected in a beaker and kept in the ice bath. The pellets from the cheese cloth were taken out and diluted with 100 ml cold deionized water. The crude extract was then centrifuged at 10000 x g at 2-4 ℃ for 15 min. After centrifugation, the supernatant was collected and stored in -4 ℃. The crude extract was assayed to analyze the protein content. From the experiment, supernatant from the pellet of the homogenate was used as crude enzyme extract as it shows higher amount of protein content which is 9.618 mg/ml.

Preparation of heavy metals solutions
Heavy metals such as chromium (vi) ($K2Cr_2O_7$, BDH), tin (ii) ($SnCl2{\cdot}2H_2O$,BDH), cobalt(ii) ($CoCl_2{\cdot}6H_2O$, J.T. Baker) and aluminium (iii) [$Al_2(SO4)_3$, anhydrous, BHD] were prepared from commercial salts while mercury (ii), arsenic(v), cadmium (ii), lead (ii), copper (ii) and silver(ii) were obtained from atomic absorption spectrometry standard solutions from Merck. 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.

Protein micro assay procedure
BSA (SIGMA) was used as protein standard. 10 mg of BSA was weighed and dissolved in 10 ml deionized water (1 mg/ml). Vary the BSA stock solution volume from 0, 5, 10, 15 and 20 µl and make up to 100 µl using deionized water. This 100 µl protein solution was added into 1000 µl of Bradford reagent, incubated for 10-20 min and the absorbance were checked at 595 nm. The first sample contained no BSA is set as control.

Crude enzyme optimization
A modified method of Shukor et al [4] was used. Any amounts and proportion of crude enzymes and substrate may form the reaction mixture, adjusted to final volume by deionized water. A varying range amount of crude enzyme was added to 5 mg/ml final concentration of casein. Working solution of casein as a substrate was diluted with deionized water according to the enzymes' concentration. Crude enzyme and casein working solution must be prepared fresh daily. A final volume of 10 ml of these mixtures containing 9 mL enzyme, 0.5 ml of deionized water and 0.5 ml casein was incubated for 20 min at room temperature. Since enzyme and casein were prepared in the same buffer, increasing the enzyme concentration can be done simply by replacing the buffer with enzyme solutions. About 20 µl of this mixture were then added with 2 ml of Bradford reagent, incubated for 10 min to allow the reaction to take place and the absorbance at 595 nm were taken. All tests were performed in triplicate. First samples without an enzyme were set as control.

Casein optimization
A modified method of Shukor et al [4] was used. Casein at different volumes ranging from 0 to 2 ml giving a final concentrations ranging from 0 to 1 mg/ml were added into crude enzymes in a final volume of 10 ml, adjusted by deionized water. These mixtures were incubated for 20 min at room temperature and 20 µl of this mixture were then added with 2 ml of Bradford reagent, incubated for 10 min and the absorbance at 595 nm were taken.

Time optimization
A modified method of Shukor et al was used [4]. Enzymes was mixed with casein, both were prepared at their optimum concentration in a final volume of 10 ml. These mixtures were incubated at room temperature for different incubation time ranging from 0 to 60 min. For each incubation time, 20 µl of this mixture were taking out and was added with 2 ml of Bradford reagent, incubated for 10 min and the absorbance at 595 nm was taken. Sample with 0 min incubation time was referred as control.

Temperature optimization
A modified method of Shukor et al [4] was used. Enzymes was mixed with casein, adjusted by deionized water to a final volume of 10 ml, each was incubated for optimum incubation time at different temperature in water bath ranging from 10 ℃ to 70 ℃. After incubation time, 20 µl was taking out from each sample and was added with 2 ml of Bradford reagent, incubated for 10 min and the absorbances at 595 nm were taken.

pH optimization
A modified method of Shukor et al [4] was used. Enzymes was mixed with casein in a final volume of 10 ml, each was incubated for optimum conditions. Crude enzymes and casein were prepared in different pH of deionized water, ranging from pH 2.0 to pH 9.0 for crude enzyme. After incubation time, 20 µl was taken out from each sample and was added with 2 ml of Bradford reagent, incubated for 10 min and the absorbance were noted at wavelength 595 nm.

Enzymes inhibition studies

Enzyme activity was assayed according to the assay developed by Shukor et al [4]. Exactly 140 µl of crude enzyme was added to 20 µl of deionized water followed by the addition of 2 µl to 20 µl of heavy metals from stock solutions. 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 min at room temperature. After the incubation period, 40 µl of casein from a stock solution of 1 mg/ml was added and mixed thoroughly. Initially a 20 µl aliquot was withdrawn and mixed with 200 µl of Bradford dye-binding reagent in a well of microplate 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 20 µl aliquot was again collected 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).

RESULTS AND DISCUSSION

The optimization studies showed that crude activity was stable at a pH between 4 and 6, and the optimum temperature was at 30 ℃. The optimum combination of enzyme and casein as a substrate gave the maximum difference in absorbance which was 9.618 mg/ml of crude and 0.7 mg/ml of casein. The results are not similar to those of papain assay with the best combination of enzyme and casein in the latter is 0.1 mg/ml for both enzyme and casein [4]. On the other hand, in the bromelain assay, the best combination is 0.11 mg/ml bromelain and 0.25 mg/ml casein, respectively [5]. This crude from tomato can be assayed by variety of methods ranging from casein and azocasein to artificial substrates such as Nα-benzoyl-L-arginine-p-nitroanilide (BAPNA) 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 [4-6]. 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 weightless than 2 kDa [4]. Casein is a big protein with varying molecular sizes ranging from monomer to octamer on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Casein is stained by the Bradford dye binding reagent giving a blue solution. Crude 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 crude activity, casein would remain undigested, and the color would remain blue. This is the advantage of using the Bradford dye-binding–crude–casein system as an assay for heavy metals [4]. A simple field trial test for the presence or absence of heavy metals would be visibly detected as blue or brown colors, respectively.

Inhibition of protease by heavy metals

Among the 6 metals tested, 3 exhibit inhibition of crude activity at 1 mg/l (Figure 1). The inhibitions shown by lead, chromium and zinc were, 67.9, 53.1 and 53 %, respectively. Cadmium, arsenic and silver do not show a significant inhibition. Zinc, chromium and lead show one phase binding hyperbola curve. The calculated IC_{50} for zinc, chromium and lead were 1.407, 0.835 and 0.707 mg/l, respectively. The calculated IC_{50} for mercury determined using nonlinear regresssion with one site binding hyperbola model available from graphpad (www.graphpad.com). IC_{50} value is referring to the concentration of heavy metals that inhibits the enzyme activity by 50%. The LOD (limits of detection), which is three times the standard deviation of the blank for zinc, chromium and lead were 0.032, 0.0317 and 0.0317 mg/l, respectively. The LOQ (limits of quantitation) for zinc, chromium and lead were 0.729, 0.506 and 0.541 mg/l, respectively (Table1). The LOQ is ten times the standard deviation of the blank. The comparison of different enzymes on the effect of heavy metals (Table 1) shows that the tomato crude enzyme gives lower IC_{50} for Zinc compared to the papain and trypsin assays. For lead, the tomato crude enzyme also gives lower IC_{50} value compared to papain which is at 0.707 mg/l. The tomato crude protease also was inhibited by chromium with an IC_{50} value of 0.835 mg/l. The previous proteases are not inhibited by chromium.

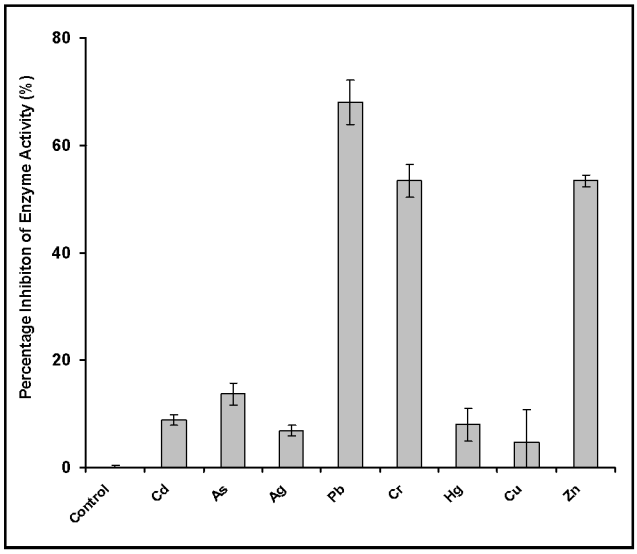

Fig. 1: Percentage of inhibition for crude tomato enzyme at 2 mg/l of heavy metals. Data is mean ± standard error of the mean (n=3).

The comparison of different bioassay systems on the effect of selected heavy metals indicated is presented in Table 2. The value for the IC_{50} of chromium was lower than all of the other assays with the exception of *Daphnia magna*. The value for the IC_{50} of lead was lower than all of the other assays while the value for the IC_{50} of zinc was lower than all of the other assays with the exception of *Daphnia magna*. The value for the IC_{50} of zinc is within the range of the Microtox, *Daphnia magna* and rainbow trout and lower than immobilized urease.

Table 1: Comparison of the IC_{50} and LOQ values of the tomato crude protease to previous proteases. Data is mean ± standard error of the mean (n=3).

Protease	Heavy metals	Regression model	R^2	IC_{50} (mg/l)	LOQ(mg/l)
Tomato crude	Zn	One-phase binding	0.968	1.407	0.729
enzyme	Cr	One-phase binding	0.982	0.835	0.506
	Pb	One-phase binding	0.992	0.707	0.541
Papain[a]	Hg	Logistics	0.999	0.391	-
	Zn	Logistics	0.988	2.11	-
	Ag	Logistics	0.987	0.4	-
	Pb	One-phase binding	0.968	2.16	-
	Cu	Linear	0.999	-	0.004
	Cd	Linear	0.962	-	0.1
Bromelain[b]	Hg	Logistics	0.973	1.31	-
	Cu	Linear	0.999	-	0.23
Trypsin[c]	Hg	Logistics	0.99	16.38	1.35
	Zn	Logistics	0.986	5.78	0.61

[a][4].
[b][5].
[c][6].

Table 2: Sensitivity of the tomato crude protease enzyme assay to heavy metals in comparisons to Microtox[tm], *Daphnia magna* and fish bioassay (*Rainbow Trout*).

	IC_{50} (mg/l)				
Metals	Immobilized urease[a]	15-min. Microtox[TMa]	48 h *Daphnia magna*[b]	96 h *Rainbow trout*[b]	Tomato crude protease
Cd	1.59±0.26	19-220	0.041-1.2	0.15-2.5	n.d
Cr (III)	36.1±2.5	13	0.10-1.8	11	0.835
Cu	0.41±0.14	0.076-3.8	0.020-0.093	0.25	n.d
Pb	>250	1.7-3.0	3.6	8.0	0.707
Hg	0.33±0.021	0.029-0.05	0.0052-0.21	0.033-0.21	n.d
Ni	1.52±0.12	23	7.6	36	n.d
Zn	14.6±3.2	0.27-29	0.54-5.1	0.55-2.2	1.407

[a][7].
[b][8].

CONCLUSION

From this study, crude protease enzyme from *L. esculentum* (tomato) was found to be quite sensitive in detection of heavy metals. The IC_{50} values was under the range of 0.5-1.5 mg/L. Crude tomato protease enzyme was sensitive to chromium while the other proteases from previous study did not inhibited by chromium. The activities of the protease studied in this project have a broad pH range for activity and ensuring matrix-interference-free system. Thus, interference cause by the physical and chemical changes of the environment give less effect to the enzymes. Moreover, compared to the other bioassay such as Immobilized urease bioassay, Microtox™ bioassay, *Daphnia magna* bioassay and *Rainbow trout* bioassay, crude protease enzyme from tomato is comparable and even gives lower IC_{50} value for some metals. In the crude tomato, the presence of more than one protease contribute to the error of the results obtained since if one protease was inhibited by heavy metals, the other proteases still degrade casein. This affects the value of the IC_{50}. As a conclusion, crude protease enzyme from *L. esculentum* (tomato) can be used to detect heavy metals.

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