

A Rapid Inhibitive Assay for the Determination of Heavy Metals Using α -Chymotrypsin; a Serine Protease

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Agricultural areas in Malaysia depend upon unpolluted source of water and hence need continuous monitoring. An enzyme-based assay for the detection of xenobiotics is a promising low cost and rapid method for monitoring. An enzymatic-heavy metal assay has been developed using α -chymotrypsin; a serine protease. This enzyme is assayed by using casein as a substrate with Coomassie dye to track the complete hydrolysis of casein. In the absence of inhibitors, casein is hydrolysed to completion and the solution is brown. Eight heavy metals were tested on the α -chymotrypsin bioassays and in the presence of Hg^{2+} , Cr^{2+} and Zn^{2+} at the final concentration of 5mg/L, hydrolysis of casein is inhibited and the solution remains blue. The assay was sensitive to Hg^{2+} , Cr^{2+} and Zn^{2+} with an IC_{50} (the concentration of toxicant giving 50% inhibition) of 1.34 mg/L, 1.974 mg/L and 2.49 mg/L, respectively. The calculated limits of quantitation (LOQ) value for Hg^{2+} , Cr^{2+} and Zn^{2+} were 0.012 mg/L, 0.324 mg/L and 0.098 mg/L, respectively. The calculated LOD values for Hg^{2+} , Cr^{2+} and Zn^{2+} were 0.003 mg/L, 0.320 mg/L and 0.074 mg/L, respectively. The results of this study indicate that this assay has the potential to be used as a tool in biosensor development for the determination of heavy metals in various samples.

Heavy metals are defined as metals that have a high atomic mass and high density. They are toxic elements that exert their toxicity mainly via binding to sulfhydryl group of enzymes and inactivating the enzymes [1]. A disturbance in enzymatic activity can seriously change the functions of the organ or tissues. As examples, mercury and arsenic both bind to certain enzymes, thereby blocking their activity [2]. Lead binds to the thiol chemical group in the enzymes and as a result, it will reduce the body's ability to synthesize enzymes necessary for respiration [3]. Their toxicity makes their presence in the environment undesirable. Since they are not biodegradable, they can accumulate in the environment and produce toxic effects in animals and plants even at low concentration [4].

Thus, a rapid and sensitive measurements of heavy metals are required in various fields such as environment, food industry and medicine because even though

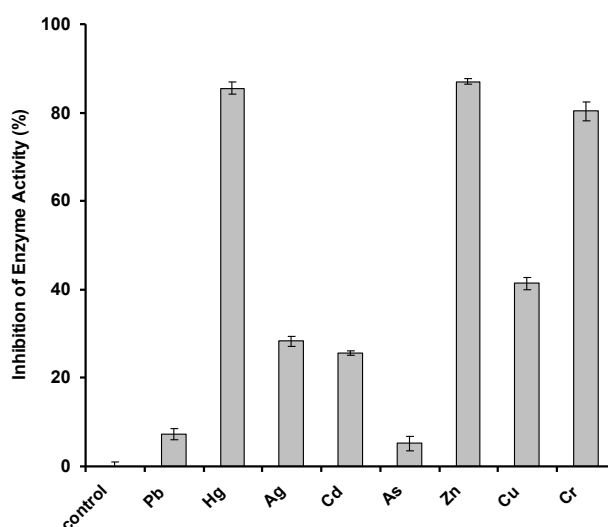


Figure 1. Screening for heavy metals towards α -chymotrypsin activity. Heavy metals were standardized at 5 mg/L in final concentration. Data is mean standard error of the mean (n=3).

the classical methods such as atomic absorption spectroscopy, inductively coupled plasma optical emission spectrometry and their combination with chromatographic techniques are in widely use, these methods need sophisticated instrumentation, skilled personnel, complicated sample pre-treatment and a long measuring period [5]. Biosensors and bioindicators provide rapid measurements without time-consuming fractionation procedures for the analysis of heavy metal compounds. Bioassay using bacteria has been developed and commercialized such as Polytox TM and Microtox TM but they are not very specific and detect both pesticides and heavy metals. Due to this, simpler and less expensive methods have been developed using enzyme-based assay based on proteases [4, 6, 7 & 8]

α -Chymotrypsin (EC 3.4.21.1) is a digestive enzyme extracted from bovine pancreas and it is one of the serine proteases that can perform proteolysis. This enzyme is selective for peptide bonds with aromatic or large hydrophobic side chains, such as tyrosine, tryptophan and phenylalanine which are on the carboxyl side of this bond. It also can catalyze the hydrolysis of the ester bond in the protein compounds particularly those with leucine-donated carboxyl [9]. Previously, similar inhibitive assay in detecting heavy metals has been developed by Shukor et al. [4, 7 & 8]. using papain, bromelain and trypsin. In this work, a potential α -chymotrypsin as another assay for heavy metals is presented for the first time.

Materials and methods

Preparation of buffer solution

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 mM NaOH and 5 mM HCl.

Preparation of Bradford reagent

0.1 g Coomassie Brilliant Blue G-250 from SIGMA was weighed and dissolved in mixture of 50 ml ethanol 95% and 100 ml phosphoric acid 85%, make up to 1000 ml after the dye complete dissolved and stirred for at least 5 hours. The solution was filtered through Whatman Filter Paper No. 1 and stored in dark bottle (Scopes, 1988). Alternatively, commercial Bradford reagent from various manufacturers such as from BIO-RAD was used. The commercial preparation was used according to manufacturer's instructions.

Preparation of Casein and α -chymotrypsin Solution

Casein was prepared according to the method of Shukor et al. [4]. 4 grams of casein (Sigma Chemical Co., St. Louis, USA) was weighed and dissolved into 400 ml of borate buffer adjusted to pH 8.0 with 5 mM of NaOH.

The resulting precipitous solution was incubated overnight with stirring at 60°C as this temperature allows the casein to be solubilised better. The casein stock solution was then centrifuged at 10,000 g for 15 minutes and filtered through Whatman Filter Paper No. 1. The protein concentration of casein in the clear super-natant was measured using the Bradford dye-binding assay [10] using crystalline BSA (Sigma Chemical Co., St. Louis, USA) as the standard. α -chymotrypsin from bovine pancreas purchased from Sigma Chemical Co., St. Louis, USA (C3142, type VII, MDL no. MFCD 00130481, 40 units/mg protein) was prepared at 40C in 100 mM, pH 8 Tris-HCl buffer with additional of 10 mM CaCl₂ because chymotrypsin needs Ca²⁺ for its cofactor as 2 mg ml⁻¹ stock solution. Enzyme (1 mg ml⁻¹) and casein (5 mg ml⁻¹) working solutions were prepared fresh daily.

Preparation of Heavy Metals Solutions

Heavy metals such as chromium (vi), zinc (ii), mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii) are Atomic Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany). From these stock solutions, working solutions at concentrations of heavy metals at 10 mg l⁻¹ and 5 mg l⁻¹ were prepared by diluting them in deionized water and stored in acid-washed polypropylene containers.

Chymotrypsin Optimization Studies

Chymotrypsin activity and optimization studies were carried out according to the method of Shukor et al. [4]. When studying the optimum concentration for enzyme, varying volumes of chymotrypsin giving final concentrations from 0 to 0.08 mg ml⁻¹ was added to 1 mg ml⁻¹ final concentration of casein in the final volume of reaction mixture was at 200 μ L 100 mM tris-HCl buffer and the mixture was incubated for 20 minutes at 30°C. After the incubation period, 20 μ L aliquot were then added with 200 μ L 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 30°C for 10 minutes. After this incubation period, a 20 μ L 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).

To study the optimum concentration of substrate casein, enzyme concentration was fixed at 0.02 mg ml⁻¹ as the final concentration. To study optimum pH for enzyme activity, 0.02 mg ml⁻¹ chymotrypsin and 0.8 mg ml⁻¹ casein was added to 200 μ L of 100 mM of different buffers. The overlapping buffer system used were; acetate buffer from pH 4.0 to pH 6.0, sodium phosphate buffer from pH 6.0 to pH 8.0 and Tris-HCl buffer from pH 8.0 to pH 9.0. To study the optimum temperature for

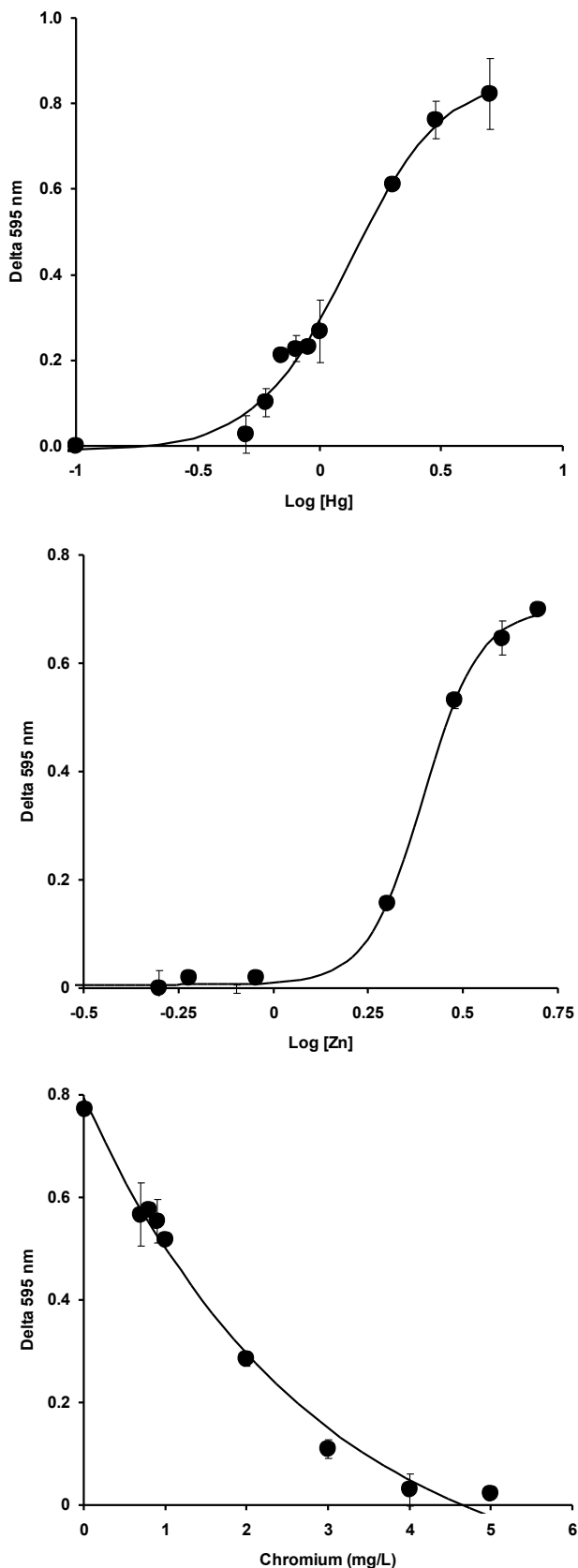


Figure 2. Inhibition of proteolytic activity of α -chymotrypsin on substrate casein by mercury (A), zinc (B) and chromium (C) as measured using the Coomassie-brilliant blue assay. Data is generated using the PRISM non-linear regression analysis for four-parameter logistic model (A and B) and for one-phase exponential decay model (C) using the GraphPad software. Data is mean standard error of the mean (n=3).

enzyme activity, the reaction mixture contains 0.02 mg ml⁻¹ chymotrypsin and 0.8 mg ml⁻¹ casein mixed in 200 μ L of 100 mM tris-HCl buffer pH 8 and the incubation temperature was varied from 15 to 45°C. The optimum incubation time for protease was determined by assaying the enzyme from time 0 to 40 minutes with time elapse of 5 minutes together with the optimum temperature previously determined. One unit of proteolytic activity is defined as the amount of casein (in mg) hydrolyzed per minute by one milligram of protease under the specified assay conditions [11].

Chymotrypsin Inhibition Studies

Protease activity was assayed according to the method of Shukor et al. [4] which are modified method of Buroker-kilgore and Wang. [11]. In an Eppendorf tube, 4 μ L of protease from the working solution was added with 10 to 100 μ L of heavy metals solution. As a control, heavy metals were replaced with deionized water. The mixture was incubated for 60 minutes at room temperature. After the incubation period, 32 μ L of casein from a working solution was added to a final concentration of 0.8 mg ml⁻¹ and mixed thoroughly and the final volume was made up to 200 μ L using deionized water. Initially a 20 μ L aliquot was withdrawn and mixed with 200 μ L 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 30°C for 20 minutes. After this incubation period, a 20 μ L 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). After this incubation period, a 20 μ L aliquot was again taken and treated in the same manner.

When studying the inhibition curves of the heavy metals which gave sigmoidal and hyperbolic dose-response curves, the values for the IC₅₀ calculated using four parameter logistics and one-phase binding models, respectively, available from PRISM nonlinear regression analysis software from www.graphpad.com. Protein was assayed according to the dye-binding method [10]. Means and standard errors were determined according to at least three independent experimental replicates.

Water samples were collected in acid-washed HDPE bottles containing several drops of 1% v/v HNO₃. The samples were filtered with 0.45 μ m syringe filter. Fourty five microliters of the clear filtrate was mixed with 50 μ l of 100 mM phosphate buffer pH 7.5 followed by the addition of 4 μ l of chymotrypsin in an eppendorf tube and again mixed thoroughly. The mixture was incubated for twenty minutes at room temperature and then was assayed according to the procedure outlined above.

Results and Discussion

The basis of the protein assay using casein as a substrate relies upon the inability of the Bradford reagent to stain polypeptide with less than a molecular weight of 2 k Dalton. Addition of Bradford reagent after casein is hydrolysed to completion and the solution remains brown while undigested casein in the reaction mixture makes the solution become blue after the addition of the reagent. The Bradford reagent can be replaced with Biuret-Lowry [12], Folin Ciocalteu [13] and bicinchoninic acid [14] but they employ the basic Copper-Biuret reaction, where copper is involved as a main reagent and thus produce other contaminants. In addition, the Bradford assay is better system in terms of rapidity, simplicity and sensitivity compared to the other biuret-copper based system for the use as a protease assay [15].

Optimize Conditions for Chymotrypsin

Chymotrypsin activity was found to be stable in between the pH of 7 and 9 and the optimum temperature was in between 30-35°C with no significant differences ($p > 0.05$) in activity at temperatures within the range. The optimum combination of enzyme and casein as a substrate giving the maximum difference in absorbance was 0.02 and 0.8 mg ml⁻¹ of chymotrypsin and casein, respectively. The optimum incubation time was 15 minutes (Data not shown). The optimum pH for maximal activity was similar to other protease-based inhibitive assays previously developed [4, 7 & 8] but the most promising results is the optimal temperature profile. The broad range temperature for optimal activity coinciding with tropical climate range is lower than all of the other protease assays with papain having the highest requirement for optimal activity of between 40 and 60°C [4]. This would allow for ambient temperature monitoring of heavy metals without the need for costly incubators. The optimum concentration of chymotrypsin giving the maximum difference in absorbance translated as maximal proteolytic activity is the lowest compared to 0.1 mg ml⁻¹ for the papain [4] and trypsin assays [8] and 0.11 mg ml⁻¹ for the bromelain inhibitive assay [7].

Inhibition of Chymotrypsin by Heavy Metals

Three heavy metals; mercury, zinc and chromium show strong inhibition of proteolytic activity of α -chymotrypsin using a standardized heavy metals concentration at 5 mg/L. Dark blue colour indicates that substrate casein is not fully degraded by enzyme. Amongst the three heavy metals, zinc gives the strongest inhibition towards α -chymotrypsin activity followed by mercury and chromium (Fig. 1).

In terms of sensitivity, the IC₅₀ (concentration that can give 50% inhibition) values are often used as a comparison between established assays [16]. The graphs of inhibition and IC₅₀ values for each of the heavy metals detected by α -chymotrypsin are shown in Figs. 2-4. The

profile of the inhibition shows a variety of shape ranging from sigmoidal curve to saturation to linear. Mercury and zinc showed sigmoidal profile curve whereas chromium showed saturation curve. In the sigmoidal profile, the concentration of the heavy metals must be transformed first into log scale and statistical software such as GraphPad Prism version 5 is used in this work to calculate the IC₅₀ values for the heavy metals. Means and standard errors were determined according to at least three independent experimental replicates. The IC₅₀ values for sigmoidal curves are halfway from the bottom and the top plateau of the curves. The calculated IC₅₀ for mercury and zinc were 1.34 mg/L and 2.49 mg/L, respectively. For saturation model as seen in the inhibition of α -chymotrypsin by chromium, a one-phase exponential decay is used and the calculation for the IC₅₀ value is equivalent to the Km values normally calculated for simple enzyme showing Michaelis-Menten Kinetics. The calculated IC₅₀ value for chromium was 1.974 mg/L.

Table 1. The summary of the IC₅₀ values and LOQ for the proteases.

Protease	Heavy metals	Regression model	R ²	IC ₅₀	LOQ (mg/L)
α -chymotrypsin	Zn	Logistics	0.996	2.49	0.324
	Cr	One-phase decay	0.989	1.974	0.098
	Hg	Logistics	0.983	1.34	0.012
Papain ^b	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 ^a	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 [7], ^b [4], ^c [8].

Instead of using IC₅₀, the Limits of Quantification (LOQ) value also can be used which is equal to ten times the standard deviation of the blank to determine its sensitivity towards heavy metals. The calculated limits of quantitation (LOQ) value for mercury, zinc and chromium were 0.012 mg/L, 0.324 mg/L and 0.098 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 [17].

Figure 2. Table 2. Sensitivity of α -chymotrypsin assay to heavy metals in comparisons to immobilized urease assay, Microtox™, *Daphnia Magna* and fish bioassays (*Rainbow trout*).

Metals	Immobilized urease ^a	15-min. Microtox™ ^b	96 hours Rainbow trout ^c	48 hours <i>Daphnia Magna</i> ^a	papain ^c	chymotrypsin
Hg	0.330.021	0.029-0.05	0.033-0.21	0.0052-0.21	0.39	1.34
Zn	14.63.2	0.27-29	0.55-2.2	0.54-5.1	2.11	2.19
Cr	36.12.5	13	11	0.10-1.8	-	1.974

^a [6], ^b [18], ^c [4].

The calculated LOD values for mercury, zinc and chromium were 0.003 mg L⁻¹, 0.320 mg L⁻¹ and 0.074 mg L⁻¹, respectively.

Table 1 shows the comparison of the IC₅₀ and LOQ values between α -chymotrypsin with other published proteases by Shukor et al. [4, 8] Referring to the table, this inhibitive determination method for detecting heavy metals by using α -chymotrypsin was a novel method because it was the first protease that show positive result on the presence of chromium whilst the other protease can not do so. Based on the IC₅₀ and LOQ values of zinc, α -chymotrypsin has similar sensitivity like papain and much more sensitive compared to trypsin. For mercury, it has a similar sensitivity like bromelain, much more sensitive compared to trypsin but less sensitive compared to papain. Mercury, zinc and chromium offer good sensitivity with each LOQ value for α -chymotrypsin was much lower than the maximum permissible limit allowed in The Environmental Quality (Sewage and Industrial Effluents) Regulations 1979 by the Department of Environmental of Malaysia.

Table 2 shows the comparison of different bioassay system between chymotrypsin with immobilised urease, Microtox™, *Daphnia Magna*, fish assays (*Rainbow trout*) and papain on the effect of selected heavy metals. Some of the heavy metals such as cadmium, copper, lead and nickel were not detected using α -chymotrypsin studied in this work. Generally, this enzyme is less sensitive compared to other bioassay systems but still the advantage of the protease bioassay compared to other bioassay lies on its rapidity, economic, simple, stability in severe conditions such as pH and temperature as well as relatively interference free from detergents, solvents and pesticides. The development of more specific bioassay system is important so that the potential toxicant can be detected rapidly and bioremediation works can be employed.

Conclusion

In this works, a novel bioassay method using an inhibitive-enzyme system was developed using α -chymotrypsin. The enzyme was optimized using the Bradford-protease-casein system as the principal assay. The Bradford-casein assay for protease has been proven to be simple, reproducible and rapid with good sensitivity. The bioassay system was tested with selected toxic heavy metals and show positive result towards mercury, zinc and chromium. Future studies would include other proteases in the field trials so the bioassay method by using enzyme has a wide variation of heavy metals detection capabilities.

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