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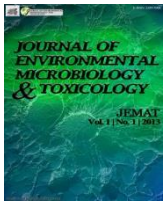

Field trials on heavy metals using alpha-chymotryopsin enzyme assay

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Abstract

An inhibitive enzyme assay using α-chymotrypsin was developed. This serine protease was assayed using Bradford-protease casein assay system. This assay is sensitive to several metals such as mercury Hg^{2+}, Zn^{2+}, and Cr^{6+}. The IC_{50} (concentration of toxicant giving 50% inhibition) of Hg^{2+}, Zn^{2+}, and Cr^{6+} is 1.34 mg/l, 2.49 mg/l and 2.19 mg/l respectively. The principle of the protein assay using casein as a substrate relies upon the inability of the Bradford dye to stain polypeptide with less than a molecular weight of 2 kDa. In the presence of heavy metals that can inhibit this enzyme, casein is not being degraded and it is stained by the Bradford dye reagent giving a blue colour. On the other hand, in the absence of inhibitors, casein is hydrolyzed to polypeptides with molecular weight of 2 kDa and below which can't be stained by Bradford dye-binding reagent. Therefore, solution remains brown in colour. The synergistic effect of combined heavy metals was studied and the results obtained shown that there were elevation of percentage of inhibition several folds. The combination of Hg^{2+} (0.3 mg/l) with Zn^{2+} (0.8 mg/l), Hg^{2+} (0.3 mg/l) with Cr^{6+} (1.8 mg/l), Zn^{2+} (0.8 mg/l) with Cr^{6+} (1.8 mg/l) increased 15.6, 73.0, 78.8 % respectively. Biomonitoring of heavy metals using an inhibitive α-chymotripsin assay was carried out. There sites for biomonitoring were Prai Industrial Areas, Bukit Tengah Industrial Area, Juru Industrial Area, Melaka River, Kuyoh River and Endau Rompin National Park. Many of the samples gave positive inhibitory effect on this enzyme. Those samples were analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) for confirmation of the presence of heavy metals that inhibited this enzyme.

INTRODUCTION

Heavy metals are any inorganic metallic compound that can exert their toxicity via binding to the thiol group of the enzyme and the disulfide bond that contribute the stability of the enzyme [1]. The metals have high affinity to the disulfide bridge between two cysteine residues in any protein compound. . Heavy metals are very dangerous to living organisms especially human as certain of them cause DNA damage and their carcinogenic effects in animals and man are probably causally related to their abilities to cause mutation [2].

The presence of the pollutants generated from industrial and agriculture activities in the waterways has been identified to produce potential harmful effect to the aquatic living organisms and the food webs [3]. Heavy metal contamination is considered to be among the most serious environmental problems nowadays.

Therefore, rapid and simple techniques are needed to detect to presence of heavy metals in the [4]. There are a few ways to detect the presence of these toxic metals by using biological agents. Monitoring of environmental toxicants by using biological means is called biomonitoring. Biosensor and bioindicator are emerging methods which provides brisk and simple measurement for the analysis of heavy metal compounds [4]. For instance, the ability of this crayfish to tolerate and accumulate toxins and heavy metals in tissues make this creature as an effective bioindicator of heavy metals pollution.

The classical methods such as atomic absorption spectroscopy, inductively coupled plasma optical emission spectrometry and their combination with chromatographic techniques are still being used to measure the exact amount of heavy metals even though these techniques require sophisticated instrumentation, skilled personnel, complicated sample pre-treatment and a long measuring period [5]. Despite this, enzyme as bioassay can provides fast, simple and less expensive method to trace the heavy metals in the environment [6]. For instance, a heavy metal enzymatic-based assay using papain was developed which can detect zinc, mercury, silver, copper, cadmium, argentums with low IC_{50} [7].

In this study, α-Chymotrypsin (EC 3.4.21.1) was used as the bioassay enzyme. α-Chymotrypsin is a digestive enzyme originated from bovine pancreas and it is a serinyl protease.

Serinyl protease consists of serine group which act as the catalytic side for the proteolytic activity. The typical function of proteases is to cleave protein to small peptides or amino acids. This enzyme is synthesized as a zymogen which is called chymotrypsinogen that is enzymatically inactive. Theoretically, tripsin which is secreted by pancreatic cells activates chymotrypsinogen to active chymotrypsin via trans proteolysis process by removing two small peptides that cover it catalytic pocket. The resulting molecule is active chymotrypsin, a three polypeptide molecule interconnected via disulfide bridge. The disulfide bridge provides stability to the enzyme.

Every enzyme is specific to its substrate, type of reaction, and the product formed. This endopeptidase is selective for peptide bonds with aromatic such as tyrosine, tryptophan and phenylalanine or large hydrophobic side chains, such as methionine and leucine which are on the carboxyl side of this bond. However it is not as specific in its cleavage site as trypsin.

2.0 MATERIALS AND METHODS

2.1 Preparation of reagent solutions

2.1.1 Preparation of buffers

Tris – HCl buffer is prepared as a medium for enzyme reaction. 0.2 L of Tris-HCl buffer was prepared as a 100mM pH8 solutions. The preparation of the buffer is as follows; 2.4228 g (100mM) of (Tris [hydro methyl] amino methane) from SIGMA was weighed. This protease needs Ca 2+ as the cofactor to enhance its activity. So, 0.2 L 10 mM calcium chloride is prepared in the ratio of 10:1 in terms of their concentration. 0.29404 g of calcium chloride is weight and being added to the solution of the stock buffer. The molecular weight of (Tris [hydro methyl] amino methane) and calcium chloride are 121.14 g/mol and 147.02 g/mol respectively. All the solutes are solubilised in the deionized water. The solution was stirred slowly and the pH value was slowly adjusted to pH 8 with 1 M HCl by using pH meter.

2.1.2 Preparation of α – chymotrypsin

The α – chymotrypsin working solution is prepared by solubilizing this enzyme which was in the form of crystal with 100 mM Tris – HCl buffer to 0.5mg/ml. The optimum concentration of this enzyme for screening of heavy metals and environmental samples is 0.02 mg/ml.

2.1.3 Preparation of heavy metals solution

All the heavy metal solutions that were used in the screening process are Atomic Absorption Spectrometry standard solution from MERCK, (Merck, and Darmstadt, Germany). They are chromium (vi), zinc (ii), mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii). These stock solutions were prepared at the concentration of 1000 mg/L by the manufacturer. The working solution of 10 mg/L for each of the heavy metals was prepared by diluting it with deionized water from the stock solution to the desired concentration and stored in acid-washed polypropylene containers. The concentration of heavy metals that were used for screening part was in the range of 10 mg/L to 0.1 mg/L.

2.1.4 Preparation of casein solution

Casein was prepared according to the method of Shukor et al. [7]. Two gram of casein (Sigma Chemical Co., St Louis, USA) was weighed and dissolved in 100 ml of deionised water. The solution was adjusted to pH 8.0 with 5 M of NaOH. This partially soluble solution was incubated overnight with stirring at 60°C as this temperature allows the casein to be solubilised better. The casein stock solution (10 mg/ml) was initially filtered through several layers of cheesecloth. Then, the filtrate was then centrifuged at 10,000g for 15 minutes and the protein of casein in the clear supernatant was measured using the Bradford dye-binding assay [8] using crystalline bovine serum albumin, BSA (Sigma Chemical Co., St. Louis, USA) as the standard.

Alternatively, casein from SIGMA was prepared in borate buffer pH8. About 1g of casein was weighed and diluted into 200mL borate buffer (stock solution), incubated 20 minutes at 60^{o}C and stirred for 30 minutes. The casein stock solution was centrifuged at 10,000 xg for 10 minutes and filtered through Whatman Filter Paper No. 1. The concentration of this casein stock solution is 5 mg/ml. The optimum concentration of casein for the optimum proteolytic activity of this enzyme is 0.8 mg/ml.

2.1.5 Preparation of Bradford reagent

The following experiments were carried out to prepare the Bradford assay for quantifying protein which is casein from samples. About 0.1g Coomasie Brilliant Blue G-250 from SIGMA was weighed and dissolved in mixture of 50mL ethanol 95% and 100mL phosphoric acid 85%, make up to 1000mL 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. Alternatively, commercial Bradford reagent from various manufacturers such as from BIO-RAD may be used. For this already made BIO-RAD reagent, 10mL was taken and dissolved in 40mL of distilled water which is 5 dilution factors.

2.2 Method for studying synergistic effect of combined heavy metals.

The concentrations of heavy metals that can inhibit this enzyme were manipulated to determine the inhibitory effect of combined heavy metals on α-chymotrypsin activity. Varying volume of

Table 1: Different heavy metal concentrations used to study the inhibitory effect of combined heavy metals on α-chymotrypsin activity. The enzyme was preincubated with heavy metals prior to addition of casein in the reaction mixture.

	Difference concentration of heavy metals					
	Zn(ii)	Hg(ii)	Cr(vi)	Zn(ii) and Hg(ii)	Zn(ii) and Cr(vi)	Hg(ii) and Cr(vi)
Volume of heavy metals (µl) (single and combined heavy metals)	16	6	30	22	46	36
Volume of α-chymotrypsin (µl)	8	8	8	8	8	8
Volume of casein (µl)	32	32	32	32	32	32
Volume of Tris-HCl buffer (µl)	72	77	65	69	57	62
Volume of deionised water (µl)	72	77	65	69	57	62

2.3 Collecting of environmental samples

Hundreds of water and sediments samples were obtained from several industrial outlets that release heavy metals products such as galvanized metals factories and prestine areas for comparisons. In this study, 4 states in Malaysia were targeted for the sampling works. There are Prai industrial areas (Prai industrial area 1 and Prai industrial area 2), Bukit Tengah industrial area and Juru industrial area which are located in Penang. Others sampling sites are Kuyoh river, Melaka river, and Endau Rompin National Park are located in Selangor state, Melaka state and Johor state respectively. Water samples and sediments were taken at most of the sampling points. Water samples were taken approximately 20-30 cm from the surface of the water bodies.

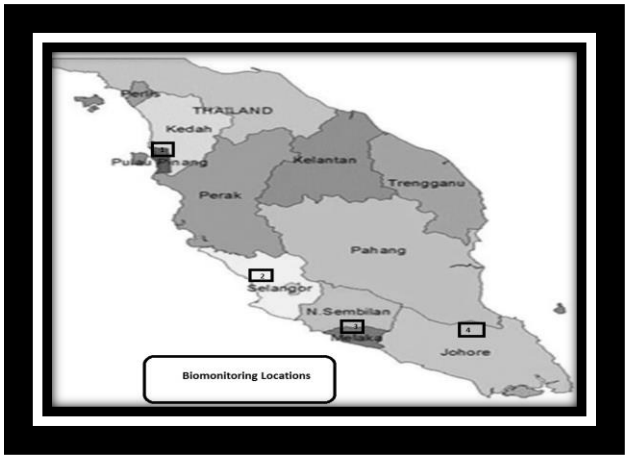

Figure 1: This figure shows biomonitoring locations in four main states in Malaysia. There are Penang, Selangor, Malacca, and Johore.

2.4 Digestion of environmental samples

The samples that were collected from these sites of sampling were placed in the acid-washed HDPE bottles containing several drops of 1 % v/v nitric acid, HNO3. The purpose of this step was to extract out heavy metals that bound to other compounds in the samples. The samples were filtered by using 0.45 µm syringe filter and were used for assaying process using an inhibitive α-chymotrypsin assay.100mM Tris-HCl buffer and deionised water were introduced into each of the well in the microplate with the ratio of 1:1. α-Chymotrypsin with final concentration of 0.02 mg/ml was next added into the buffered medium. The concentration of stock heavy metals solution used in this study was 5 mg/L. Heavy metals (single and combined heavy metals) with difference final concentrations each was introduced into the enzyme mixture prior to incubation of casein. This enzyme was incubated with heavy metals about one hour before the addition of casein. After one hour of incubation, casein with final concentration of 0.8 mg/ml was added to the mixture. 20 µl of aliquot was withdrawn and mixed with 200 µl of Bradford dye-binding reagent in the microplate well. The absorbance at 595nm was set to measure using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA).The inhibitory effect of single metal was compared with the inhibitory effect of combined metals on the activity of α-chymotrypsin.

2.5 Method for assaying environmental samples

Microplate was the instrument used in assaying heavy metal solution and environmental samples. First and foremost, 65 µl of Tris-HCl buffer and deionized water were introduced in each well of the microplate. This buffered medium was added with 8µl of α-chymotrypsin which its final concentration in the well was 0.02 mg/L. 30 µl of heavy metals which correspond to 3 mg/L was introduced next. This mixture was the positive control for this assay. The same volume of samples were introduced the rest of the microplate wells. The negative control for this assay was tap water. These mixtures were incubated for an hour for fully inhibition of the enzyme by the heavy metals in the digested samples. Then, 32 µl of casein from stock solution of 5 mg/L was added to the mixture of the solution. The final concentration of casein in the well was 0.8 mg/L. Next, 20 µl aliquot was withdrawn and mixed with 200 µl of Bradford dye- binding reagent in microplate well and incubated for 5 minutes to get the absorbance for time zero. After the incubation time, a 20 µl aliquot from the mixture was again taken and treated in the same manner with the aliquot at the time zero. The absorbance at 595 nm was the wavelength used to quantify protein in the samples which reflected the inhibitory effect of the heavy metals in the

samples on the activity of the enzyme. The absorbance was measured by using microplate reader (Stat Fax® 3200 Microplate reader, Awareness technology Inc., USA).

2.6 Instrumental analysis of samples

The high technology instrument that was used in this study is inductively coupled plasma-optical emission spectrometry. The heavy metals standards were prepared for measuring the exact concentration of heavy metals content in every sample. The samples were filtered first by Whatman Filter Paper No. 1 before being analyzed by ICP – OES.

2.7 Analyzing data

The results which were obtained from instrumental analysis of ICP – OES were compared with the results gained from the inhibitive α-chymotrypsin assay. The aim of the step is to compare the accuracy of this bioassay with ICP – OES.

3.0 RESULT AND DISCUSSION

Synergistic effect of combined heavy metals was carried in this project and the results indicated that combined heavy metals increased inhibition of α-chymotrypsin several fold. The next part of this project was biomonitoring of heavy metals using an inhibitive α-chymotrypsin assay. All samples that were assayed prior to instrumental analysis using inductively coupled plasma-optical emission spectrometry. Most of the samples collected from the sampling sites contained zinc and chromium which gave positive inhibitory effects on the activity of α-chymotrypsin.

3.1 Synergistic effect of combined heavy metals on α-chymotrypsin activity

The mixture of enzyme with the casein as the substrate in the Tris-HCl buffer was set as the control for this experiment. Figure 2 shows the percentage of activity of α-chymotrypsin in the presence of zinc at 0.8 mg/l, mercury at 0.3 mg/l, hexavalent chromium at 1.8 mg/l and the combination of these metals respectively. Zinc at 0.8 mg/l contributed about 0.4 % of inhibition on this enzyme activity. Mercury and hexavalent chromium caused 1.5 % and 18.7% inhibition on this enzyme at 0.3 mg/l and 1.8 mg/l respectively.

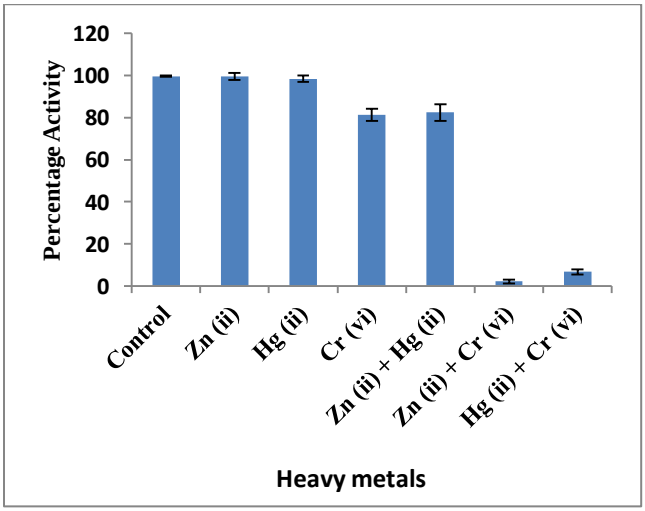

Figure 2: Synergistic effect of combined heavy metals on the activity of α-chymotrypsin activity. All values represent mean ± standard error mean (SEM), (n=3).

Theoretically, the combination of zinc (0.8 mg/l) which contributed about 0.4 % of inhibition with mercury (0.3 mg/l) which contributed about 1.5 % of inhibition will cause total decrease in activity of this enzyme about 1.9 %. However, the total percentage of inhibition on α-chymotrypsin for these combined metals was 17.6%. This result indicates that this combined metals cause increase of percent inhibition several fold. The increase of inhibition is about 15.6 %.

Similarly, the combination of zinc (0.8 mg/l) which contributed about 0.4 % of inhibition with hexavalent chromium (1.8 mg/l) which contributed 18.7 % of inhibition will cause total decrease in activity around 19.1%. This combination of metals causes 97.9 % of inhibition on α-chymotrypsin activity which indicates that the synergistic effect of these two metals can cause almost fully inhibition on this enzyme. The net increase of percentage of inhibition for this combined metal is about 78.7 %.

Likewise, the combination of mercury (0.3 mg/l) which contributed about 1.5 % enzyme inhibition with hexavalent chromium (1.8 mg/l) which contributed about 18.7 % enzyme inhibition lead to almost fully inhibition of this enzyme. The total percentage of inhibition for this combined metal is 93.2 % as compared to the theoretical value by summing up the percentage of inhibition of mercury and hexavalent chromium individually which is 20.2 %. This indicates that the combination of mercury with hexavalent chromium cause increase of inhibition of enzyme several fold. The net increase of percentage of inhibition is about 73 %.

Combination of mercury with the other two metals that can inhibit this enzyme will cause significant increase in percentage of inhibition on α-chymotrypsin. Mercury is possibly the key that contributes to this significant increase in percentage of inhibition when it combines with the other metals. There are two main mechanisms that have been suggested on how mercuric ion can cause inhibitive effect on the enzyme [1].

The inhibition of metal ion especially mercury is possibly due to the binding of mercuric ion at the sulfhydryl group at the active site. Sulfhydryl group or in other word, thiol group is the most reactive nucleophilic site of protein amino acids side chains at the catalytic site. In this mechanism, the mercuric ion inactivates the enzyme by binding to a single residue to form R-S-Hg-Cl. In another mechanism, mercury can also react with the disulfide bridge that hold the structure of the enzyme which leads to the cleavage of the disulfide bond forming R-S-Cl and Cl-Hg-S-R groups. The R-S-Cl moiety would then be oxidized by another mercury ion to form the compound R-S-Hg-Cl. Since the disulfide bridge of two cysteine residues provides stability to protein tertiary structure, their destruction lower the stability of proteins and eventually affecting enzyme activity [1].

3.2 Screening result of samples collected from Prai Industrial Area 1

A biomonitoring work was set up in Prai industrial Area 1, a site well known to harbour many metal-related work industries. Most of the water channels found here, empty their contents in Juru

River. 24 samples were collected in almost all water channels that can be found through out this industrial area. At each point of sample being collected, 3 replicates of sample were taken. Out of 24 samples, 12 samples gave more than 50 percent inhibitory effect on α-chymotrypsin activity. This indicates that these

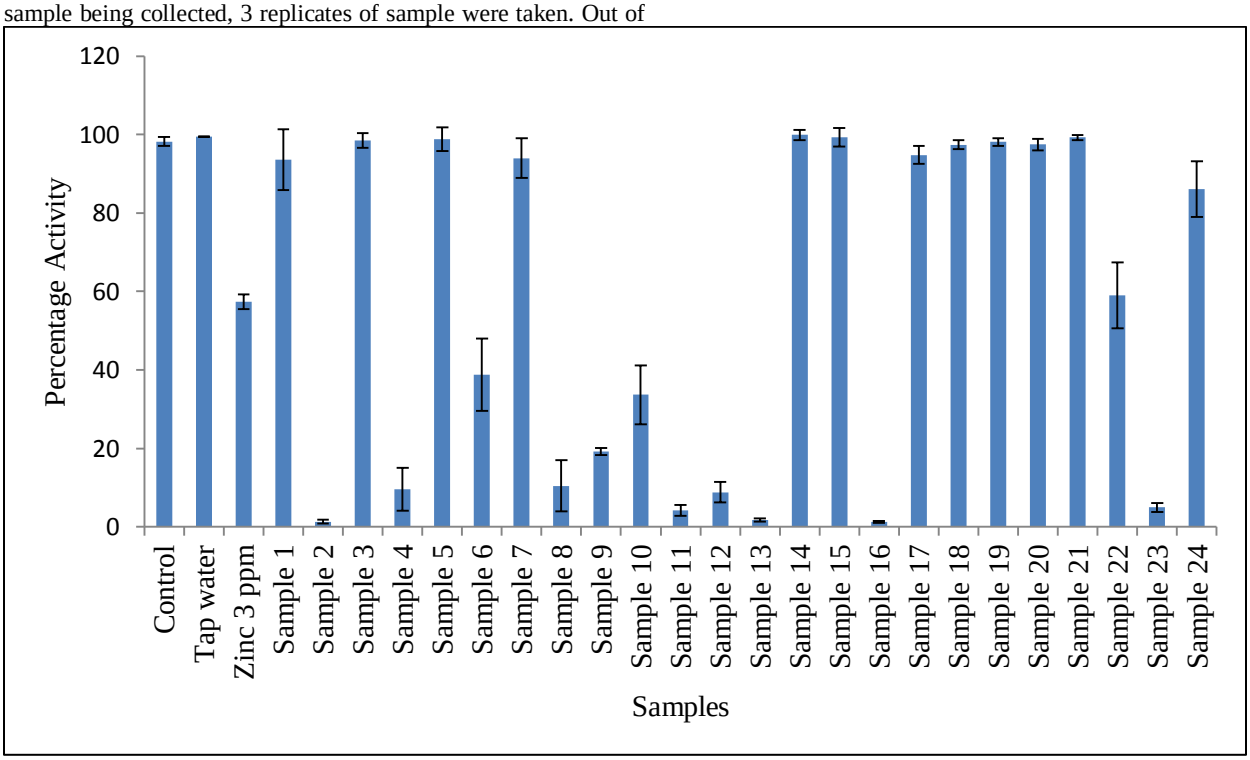

Figure 3: The inhibitory effect of the samples obtained from Prai Industrial Area 1 on the activity of α-chymotrypsin. All values represent mean ± standard error mean (SEM), (n≥3).

Table 2: Table of percentage activity (%) of α-chymotrypsin and the concentration of heavy metals contained in each Prai Industrial Area 1 sample as determined by ICP-OES. All values represent mean ± standard error mean (SEM), (n=3).

Prai industrial area 1 samples	GPS location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)		
			Zinc	Mercury	Hexavalent chromium
Sample 1	N 05° 20.87' E 100° 24.692'	93.59	0.04 ± 0.05	N.D.	N.D
Sample 2	N 05° 20.87' E 100° 24.692'	1.34	125.30 ± 0.33	N.D.	0.75 ± 0.01
Sample 3	N 05° 20.862' E100° 24.674''	98.54	0.21 ± 0.12	N.D.	0.01 ± 0.00
Sample 4	N 05° 20.836' E 100° 25.177'	9.54	7.63 ± 0.05	N.D.	0.26 ± 0.00
Sample 5	N 05° 20.224' E 100° 26.302'	98.80	0.06 ± 0.03	N.D.	N.D.
Sample 6	N 05° 21.983' E 100° 24.023'	38.80	0.89 ± 0.01	N.D.	0.03 ± 0.00
Sample 7	N 05° 21.967' E 100° 24.044'	93.98	0.14 ± 0.00	N.D.	N.D.
Sample 8	N 05° 20.87' E 100° 24.692'	10.46	14.54 ± 0.13	N.D.	0.36 ± 0.01
Sample 9	N 05° 20.87' E 100° 24.692'	19.21	3.97 ± 0.04	N.D.	0.13 ± 0.00
Sample 10	N 05° 20.862' E 100° 24.674'	33.66	3.50 ± 0.02	N.D.	0.01 ± 0.00

Sample 11	N 05° 19.699' E 100° 26.129'	4.21	8.74 ± 0.09	N.D.	0.30 ± 0.00
Sample 12	N 05° 19.699' E 100° 26.129'	8.82	12.00 ± 0.02	N.D.	0.62 ± 0.00
Sample 13	N 05° 19.699 E 100° 26.129	1.77	15.80 ± 0.13	N.D.	0.58 ± 0.01
Sample 14	N 05° 20.263' E 100° 25.774'	99.94	0.02 ± 0.00	N.D.	N.D
Sample 15	N 05° 20.263' E 100° 25.774'	99.39	0.03 ± 0.00	N.D.	N.D.
Sample 16	N 05° 20.135' E 100° 26.925'	1.39	38.65 ± 0.18	N.D.	0.86 ± 0.00
Sample 17	N 05° 21.153' E 100° 26.073'	94.81	0.02 ± 0.00	N.D.	N.D.
Sample 18	N 05° 21.153' E 100° 26.073'	97.42	0.09 ± 0.00	N.D.	0.08 ± 0.00
Sample 19	N 05° 21.153' E 100° 26.073'	98.11	N.D.	N.D.	N.D
Sample 20	N 05° 20.091' E 100° 25.269'	97.46	0.06 ± 0.02	N.D.	N.D.
Sample 21	N 05° 21.132' E 100° 25.081'	99.24	0.71 ± 0.01	N.D.	0.17 ± 0.00
Sample 22	N 05° 20.472' E 100° 26.891'	59.05	10.84 ± 0.06	N.D.	0.26 ± 0.00
Sample 23	N 05° 20.387' E 100° 24.429'	4.95	0.05 ± 0.00	N.D.	N.D.
Sample 24	N 05° 20.263' E 100° 25.774'	86.09	12.69 ± 0.03	N.D.	0.18 ± 0.00

n.d., not detected.

samples were highly polluted with heavy metals. The almost full activity (100%) of tap water form a negative control and 57.4 percent of inhibition by 3 mg/l zinc forms a positive control. Most of the samples collected in this industrial area contain extremely high concentration of zinc up to 125.3 mg/l. This exceeds the maximum permissible limit (MPL) for zinc as outlined by the department of Environment (DOE) which is 5 mg/l.

3.3 Screening result of samples collected from Prai Industrial Area 2

Prai Industrial Area 2 is located beside Prai Industrial Area 1 and there are a lot of metal-based factories can be found here. Twelve samples were collected from this sampling site. All the samples were taken from the water channels found in this area. Some of the samples were assayed at the site of interest to check the ability of this bioassay. The rest of the samples were assayed in laboratory and being analysed by ICP-OES machine for confirmation the presence of heavy metals plus the exact concentration of them. Out of 12 samples, 7 samples show high inhibitory effects on the activity of α-chymotrypsin. This indicates that all the six samples are highly polluted with heavy metals. Zinc was again found to be the cause of the inhibitory effect of this enzyme activity. The highest concentration of zinc found in these samples was 219.96 mg/l. The result shows that this industrial area is highly polluted with zinc.

3.4 Screening result of samples collected from Bukit Tengah Industrial Area

Galvanised metal industries are among the several industries that can be found in Bukit Tengah Industrial Area. The results (Fig. 5) show that out 5 samples taken from this industrial area, 2 samples gave positive inhibitory result on α-chymotrypsin activity while the rest of the samples gave negative inhibitory result for the presence of toxic heavy metals studied. The heavy metal that found in these samples was zinc. Among the 5 samples, the last sample contained high concentration of hexavalent chromium which was 1.565 mg/l. The exact concentration of zinc was measured by the aid of inductively coupled plasma-optical emission spectrometry (ICP-OES). The results from this bioassay were parallel with the results obtained from ICP-OES. This indicated that α-chymotrypsin bioassay can give accurate result for the presence of heavy metals studied.

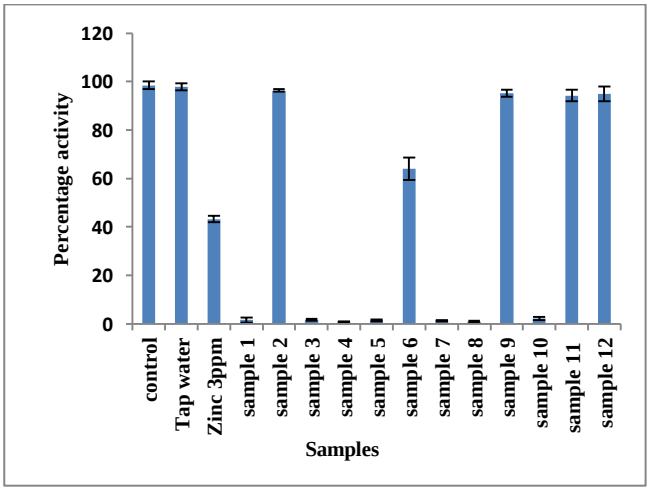

Figure 4: The inhibitory effect of samples collected from Prai Industrial Area 2 on the activity of α- chymotrypsin activity. All values represent mean ± standard error mean (SEM), (n=3).

Table 3: Table of percentage activity (%) of α-chymotrypsin and the concentration of heavy metals contained in each Prai Industrial Area 2 samples as determined by ICP-OES. All values represent mean ± standard error mean (SEM), (n=3).

Juru industrial area 2 samples	GPS location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)		
			Zinc	Mercury	Hexavalent chromium
Sample 1	N 05^0 21.599' E 100^0 24.282'	1.52	33.02 ± 0.12	N.D.	1.18 ± 0.00
Sample 2	N 05^0 21.629' E 100^0 24.249'	96.36	0.19 ± 0.00	N.D.	N.D.
Sample 3	N 05^0 21.244' E 100^0 24.178'	1.50	28.10 ± 0.03	N.D.	0.83 ± 0.00
Sample 4	N 05^0 21.599' E 100^0 24.006'	0.76	219.55 ± 0.89	N.D.	2.92 ± 0.02
Sample 5	N 05^0 21.205' E 100^0 24.975'	1.46	20.33 ± 0.07	N.D.	1.96 ± 0.00
Sample 6	N 05^0 21.068' E 100^0 23.864'	64.03	0.67 ± 0.00	N.D.	0.24 ± 0.00
Sample 7	N 05^0 21.111' E 100^0 23.780'	1.28	27.80 ± 0.17	N.D.	5.50 ± 0.03
Sample 8	N 05^0 21.106' E 100^0 23.779'	0.95	91.55 ± 0.34	N.D.	0.53 ± 0.00
Sample 9	N 05^0 20.962' E 100^0 23.819'	95.24	0.22 ± 0.00	N.D.	N.D.
Sample 10	N 05^0 20.954' E 100^0 25.164'	2.16	11.30 ± 0.00	N.D.	0.10 ± 0.00
Sample 11	N 05^0 21.828' E 100^0 24.389'	94.23	0.84 ± 0.01	N.D.	N.D.
Sample 12	N 05^0 21.462' E 100^0 24.555'	94.93	0.05 ± 0.01	N.D.	N.D.

n.d., not detected.

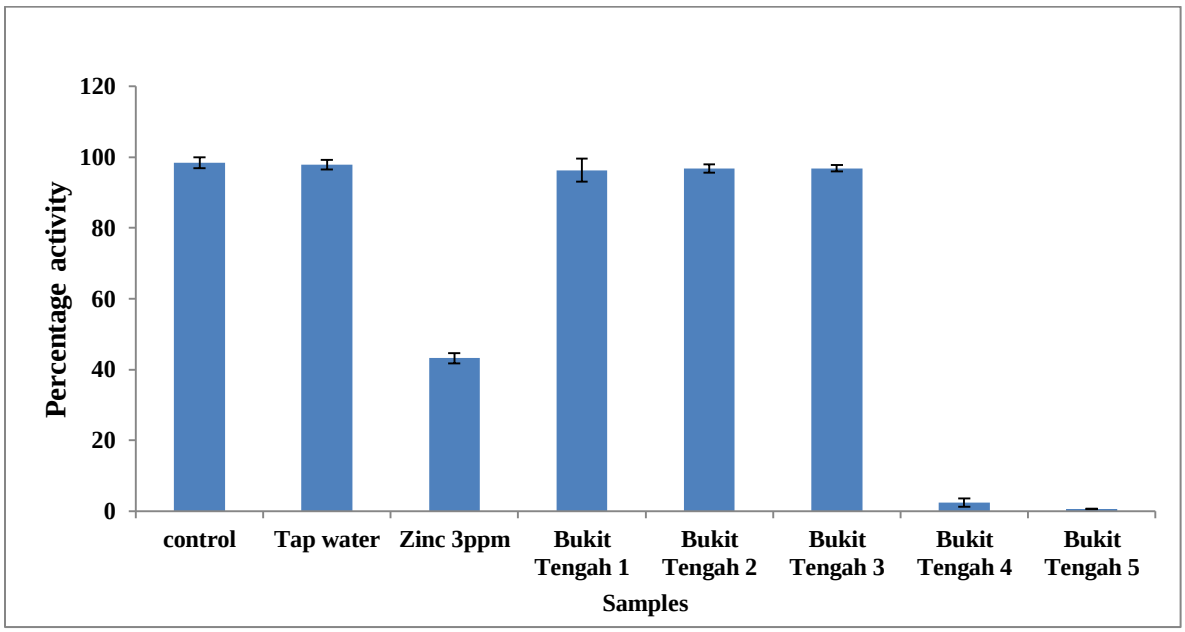

Figure 5: Inhibitory effect of samples from Bukit Tengah Industrial Area on α-chymotrypsin activity. All values represent mean ± standard error mean (SEM), (n=3).

Table 4: Table of percentage activity (%) of α-chymotrypsin and the concentration of heavy metals contained in each Bukit Tengah Industrial Area samples as determined by ICP-OES. All values represent mean ± standard error mean (SEM), (n=3).

Bukit tengah industrial area samples	Gps location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)		
			Zinc	Mercury	Hexavalent chromium
Bukit Tengah 1	N 05^0 20.447' E 100^0 26.403'	96.39	0.12 ± 0.00	N.D.	N.D.
Bukit Tengah 2	N 05^0 20.665' E 100^0 26.364'	96.84	0.08 ± 0.00	N.D	N.D.
Bukit Tengah 3	N 05^0 20.601' E 100^0 26.427'	96.87	0.06 ± 0.00	N.D.	N.D.
Bukit Tengah 4	N 05^0 20.640' E 100^0 26.470'	2.47	24.05 ± 0.09	N.D.	0.36 ± 0.00
Bukit Tengah 5	N 05^0 18.947' E 100^0 26.348'	0.70	91.27 ± 0.66	N.D.	1.57 ± 0.02

n.d., not detected.

3.5 Screening result of samples collected from Juru Industrial Area

There were a few samples collected from Juru Industrial Area especially several water channels that empty their contents into the Juru River. However, only 5 samples were taken randomly and assayed in the laboratory. The water samples were taken about 1 meter from the surface of the river. Juru 2 and Juru 3 samples were water samples taken from the surface of the water channels which were near to main Juru River whereas Juru 1, Juru 4, and Juru 5 were the sediments from these water channels. Sediment from water channels within Juru are contaminated by Zn and Pb [9]. Figure 6 shows those 3 samples which were Juru 1, Juru 4, and Juru 5 gave highly inhibitory effect on α-chymotrypsin activity. These samples were highly polluted with heavy metal which was zinc. This also shows that the sediments from this river contain high concentration of metals as compared with the water samples taken from the surface of the river. The metals containing wastes which are channelled to this river will keep on accumulating at the base of the river. This is another reason why the heavy metal content in the sediments are much higher than the water samples taken from the surface of the river. This result supports the earlier finding on the heavy metal status in Juru area.

5.6 Screening result of samples collected from Malacca River

The water samples were taken along Melaka River. Three replicates of water samples were taken from each of the point of sampling. 10 samples were taken along this river and these samples have no inhibitory effect on the α-chymotrypsin activity. This is possibly due to the absence of the toxic metals studied. The result from the ICP-OES proves that these water samples are free from zinc, mercury and hexavalent chromium which can inhibit this enzyme.

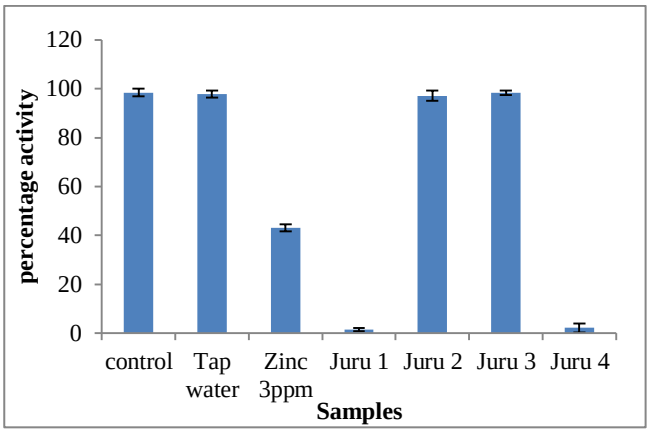

Figure 6: Inhibitory effect of samples from Juru Industrial Area on the activity of α-chymotrypsin activity. All values represent mean ± standard error mean (SEM), (n=3).

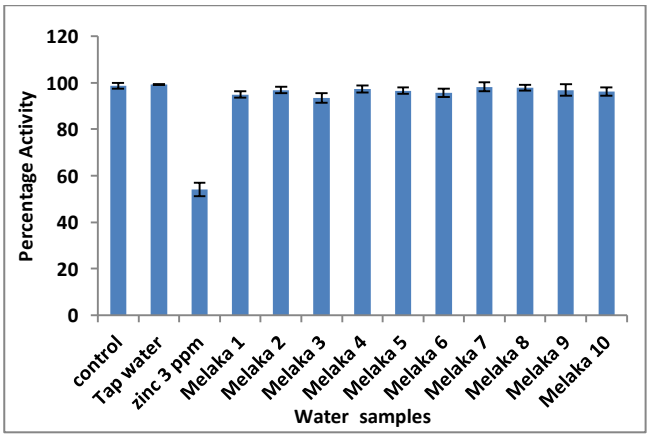

Figure 7: The inhibitory effect of Melaka River samples on the activity of α-chymotrypsin. The enzyme was preincubated with the samples prior to addition of casein as described in section 3.5. All values represent mean ± standard error mean (SEM), (n=3).

Table 6: Table of percentage activity (%) of α-chymotrypsin and the concentration of heavy metals contained in each Melaka River samples as determined by ICP-OES. The enzyme was preincubated with samples prior to addition of casein as described in section 3.5.

Melaka river samples	GPS location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)		
			Zinc	Mercury	Hexavalent chromium
MELAKA 1	N 2^0 12.466' E 102^0 15.096'	94.89	N.D.	N.D.	N.D.
MELAKA 2	N 2^0 12.414' E 102^0 15.075'	96.78	N.D.	N.D.	N.D.
MELAKA 3	N 2^0 12.388' E 102^0 15.022'	93.41	N.D.	N.D.	N.D.
MELAKA 4	N 2^0 12.361' E 102^0 15.056'	97.21	N.D.	N.D.	N.D.
MELAKA 5	N 2^0 12.360' E 102^0 15.061'	96.59	N.D.	N.D.	N.D.
MELAKA 6	N 2^0 12.283' E 102^0 15.078'	95.57	N.D.	N.D.	N.D.
MELAKA 7	N 2^0 12.257' E 102^0 15.084'	98.23	N.D.	N.D.	N.D.
MELAKA 8	N 2^0 12.214' E 102^0 15.096'	97.85	N.D.	N.D.	N.D.
MELAKA 9	N 2^0 12.179' E 102^0 15.109'	96.87	N.D.	N.D.	N.D.
MELAKA 10	N 2^0 12.123' E 102^0 15.101'	96.18	N.D.	N.D.	N.D.

n.d., not detected.

3.7 Screening result of samples collected from Kuyoh River

The water samples were taken along Kuyoh River and at each of the point where the sample was taken, three replicates of water samples were collected. There were 9 water samples collected from this river and most of the samples show no significant inhibitory effect on this enzyme. This is possibly due to the absence or very low concentration of heavy metals that can inhibit this enzyme. These samples were analysed by ICP-OES and the result obtained proved that these sample contained no heavy metals that inhibit this enzyme.

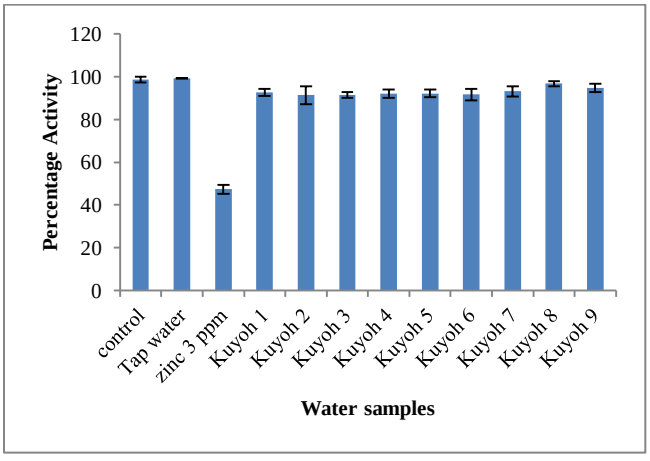

Figure 8: The inhibitory effect of Kuyoh River samples on the activity of α-chymotrypsin activity. All values represent mean ± standard error mean (SEM), (n=3).

3.7 Screening result of samples collected in Endau Rompin National Park

Endau Rompin is the second largest national park which is a major conservation area due to the diversity of flora and fauna found within it. Four distinct samples were taken from this area and the results show that there was no significant inhibitory effect on α-chymotrypsin activity. This result also indicates that these samples are not polluted with studied heavy metals which are zinc, mercury, and hexavalent chromium. The result obtained from ICP-OES also proves that the result from this bioassay is accurate.

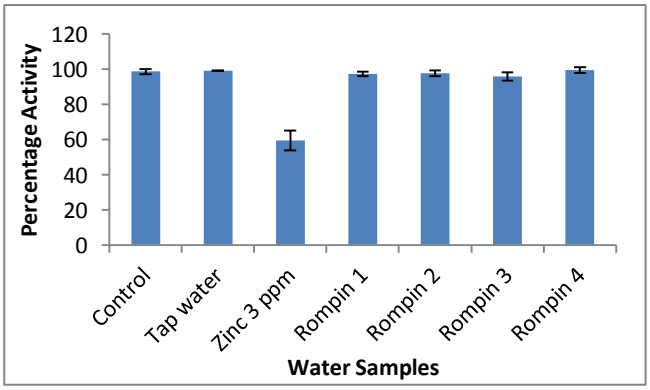

Figure 8: Inhibitory effect of Endau Rompin samples in the activity of α-chymotrypsin. The graph was generated using Microsoft Office software. All values represent mean ± standard error mean (SEM), (n=3).

CONCLUSION

A novel assay for several heavy metals such as mercury, zinc, and hexavalent chromium using inhibitive α-chymotrypsin system was developed and proven to be applicable at the sites of monitoring. Most of the samples obtained from the sampling sites contained very high concentration of zinc up to 219.996 mg/l which exceed the permissible limit outlined by Department of Environment (DOE), 5mg/l. Besides, some of the samples contained high amount of hexavalent chromium and the highest concentration of this metal was found in sample (N 05^0 21.111' E 100^0 23.780') Prai Industrial Area 2.

Water samples obtained from Endau Rompin National Park were assayed using inhibitive α-chymotrypsin system and these samples were free from zinc, mercury and hexavalent chromium. All the samples obtained from the sampling sites were analyzed by inductively coupled plasma-optical emission spectrometry for measuring the exact concentration of heavy metals contained in them. The results obtained from the bioassay proved that it is effective and able to give the accurate detection of heavy metals in the samples.

A new finding was obtained from this study where combination of heavy metals that inhibit this enzyme can increase the percentage of inhibition several fold. This is possibly one of the reasons why even the concentration of heavy metals in the sample is low; the percentage of inhibition is relatively higher compare to the theoretical value. In conclusion, this bioassay is applicable and gives promising results.

Table 8: Table of percentage activity of α-chymotrypsin and the concentration of heavy metals contained in each Kuyoh River sample. Table was generated using Microsoft Office software. All values represent mean ± standard error mean (SEM), (n=3).

Endau rompin National park samples	Gps location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)		
			Zinc	Mercury	Hexavalent chromium
Endau Rompin 1	N 02^0 30.674' E 103^0 21.387'	97.49	N.D.	N.D.	N.D.
Endau Rompin 2	N 02^0 30.802' E 103^0 21.086'	97.68	N.D.	N.D.	N.D.
Endau Rompin 3	N 02^0 30.783' E 103^0 21.140'	95.93	N.D.	N.D.	N.D.
Endau Rompin 4	N 02^0 30.784' E 103^0 21.02'	99.53	N.D.	N.D.	N.D.

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