The development of an inhibitive determination method for zinc using a serine protease

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Abstract: A new inhibitive heavy metals determination method using trypsin 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 bioassay was able to detect zinc and mercury with IC₅₀ (concentration causing 50% inhibition) values of 5.78 and 16.38 mg l⁻¹ respectively. The limits of detection (LOD), for zinc and mercury were 0.06 mg l⁻¹ (0.05-0.07, 95% confidence interval) and 1.06 mg l⁻¹ (1.017-1.102, 95% confidence interval), respectively. The limits of quantitation (LOQ) for zinc and mercury were 0.61 mg l⁻¹ (0.51-0.74 at a 95% confidence interval) and 1.35 mg l⁻¹ (1.29-1.40 at a 95% confidence interval), respectively. The IC₅₀ value for zinc was much higher than the IC₅₀ values for papain and Rainbow trout, but was within the range of Daphnia magna and Microtox®. The IC₅₀ value for zinc was only lower than those for immobilized urease. Other toxic heavy metals, such as lead, silver, arsenic, copper and cadmium, did not inhibit the enzyme at 20 mg l⁻¹. Using this assay, we managed to detect elevated zinc concentrations in several environmental samples. Pesticides, such as carbaryl, fucytoximate, metolachlor, glyphosate, diuron, diazinon, endosulfan sulphate, atrazine, coumaraphos, imidacloprid, dicamba and parataquat, showed no effect on the activity of trypsin relative to control (One-way ANOVA, \( F_{12,24} = 0.3527, p > 0.05 \)). Of the 17 xenobiotics tested, only (sodium dodecyl sulphate) SDS gave positive interference with 150% activity higher than that of the control at 0.25% (v/v).

Key words: Trypsin, Serine protease, Inhibitive determination method

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Introduction

Human activity in the last few decades has led to global contamination by organic and inorganic compounds. This pollution has been reported and studied all across the globe (Agлас et al., 2007; Sahu et al., 2007; Ozdilek et al., 2007). The toxicity and the biogeochemical cycles of these inorganic and organic compounds have led us to classify these compounds as pollutants. Waterways are being polluted by organic and inorganic compounds, such as pesticides and heavy metals from various industries and mining activities. The infamous case of mercury poisoning in Minamata, Japan, provides a tragic example. The tragedy led to a massive outbreak of methylmercury poisoning (Tsubaki and Inukayama, 1977).

In Malaysia, heavy metal pollution often comes from waste effluents. The galvanized metal industry is among the several industries that have been found to produce highly polluted effluents. The effluents contained zinc at a concentration of several hundred parts per million (DOE, 2002). The maximum permissible limit (MPL) for zinc as outlined by the Department of Environment (DOE) is 5 mg l⁻¹ (DOE, 2002). Waterways that are contaminated with zinc have been shown to have negative effects on fish and important microorganisms (Ravikumar et al., 2007; Shukla et al., 2007). Zinc can be determined by use of atomic absorption spectrophotometry (AAS) and the colorimetric method (Clesceri et al., 1989). Although AAS is very sensitive, it requires a highly skilled technician and is not amenable to use in field trials. The results are often received several days after the sample was provided, due to logistics problems. The colorimetric method was able to quantify zinc within the permissible limits and is amenable to being carried out in the field, but requires the use of toxic cyanide (Clesceri et al., 1989). In addition, the colorimetric zinc determination method using zinc (c-carboxy-2-hydroxy-5-sulfoformazyl-benzene) requires toxic cyanide and is interfered by copper hydroxyl-5-sulfoformazyl-benzene) requires toxic cyanide and is interfered by copper requiring the addition of 1 g of thiourea for masking agent. Moreover, in the presence iron (iii), aluminum, copper (ii) and mercury (ii), zinc extraction from the mixture has to be done using dithione-carbon tetrachloride solution (Ghasemi et al., 2003). Hence, a simple, safe and field trial-friendly method is needed to monitor zinc levels in the environment. This method should allow an early warning system to be developed and a large screening program to be carried out more frequently, especially effluents from the galvanized metal and rubber industries.

Inhibitive enzyme assay for heavy metals using enzyme is a recently emerging method. The method is generally nonspecific towards any particular heavy metal, but can permit an early monitoring
system to be developed (Jung et al., 1995). The inhibition of the assay could be due to one heavy metal at excessive concentration or multiple heavy metals with a combined toxic effect. In either case, the inhibition of the assay means that the sample contains toxic metal ions. The identity and exact concentration of each metal ion can be verified using AAS or ICP-OES or ICP-MS. An inhibitive determination of heavy metal traces using enzymes does not require a skilled technician and is amenable to use in field trial work (Jung et al., 1995). Enzymes that have been used for inhibitive determination of heavy metal traces include peroxidase, xanthine oxidase, invertase, glucose oxidase and the proteases papain (Shukor et al., 2006) and bromelain (Shukor et al., 2008). The use of the protease trypsin in the inhibitive determination of heavy metals has been previously carried out in the form of an immobilized enzyme (Safarik et al., 2002). However, the sensitivity is relatively low with a complete inhibition of trypsin achieved with silver and lead concentrations in excess of 0.1 M. Trypsin is a member of the serine protease family.

In this work, the potential of trypsin as a simple inhibitive assay for the heavy metal zinc is presented for the first time. We discovered that the limits of quantitation (LOQ) and the IC50 for zinc using this method is within the permissible limits allowed by the Malaysian authority (DOE, 2002) and the reagents used in the assay are non-toxic. This suggests that the assay is suitable as a preliminary biomonitoring tool for zinc detection, especially in the galvanized metal and rubber industries.

Materials and Methods

Bradford dye-binding assay: The followings were carried out to prepare the 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% (v/v) ethanol and 100 ml 85% (v/v) 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 bottle. Alternatively, commercial Bradford dye-binding reagent from various manufacturers such as from Bio-Rad (Bio-Rad, USA) may be used. The commercial preparation was used according to manufacturer’s instructions.

Preparation of casein and trypsin solution: Casein was prepared according to the method of Shukor et al. (2006). Two grams of casein (Sigma Chemical Co., St. Louis, USA) was weighed and dissolved into 100 ml of deionised water adjusted to pH 8.0 with 5 M 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 (10.0 mg ml\(^{-1}\)) was then 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 (Bradford, 1976) using crystalline BSA (Sigma Chemical Co., St. Louis, USA) as the standard. Trypsin from pancreas (MERCK), Lot no 936F199763, 200 FIP U g\(^{-1}\) was prepared at 4°C in 50 mM sodium phosphate pH 6.5 as a 10.0 mg ml\(^{-1}\) stock solution. Trypsin (2.0 mg ml\(^{-1}\)) and casein (0.3 mg ml\(^{-1}\)) working solutions were prepared fresh daily.

Trypsin optimization studies: Trypsin activity and optimization studies were carried out according to the method of Shukor et al., (2006). When studying the optimum concentration for enzyme, varying volumes of trypsin giving final concentrations from 0 to 0.5 mg ml\(^{-1}\) was added to 50 µl of 100 mM phosphate buffer pH 6.5. The final volume was made up to 100 ml using deionized water and the mixture was incubated for 20 minutes at room temperature. After the incubation period, 50 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 min to get the absorbance for time zero. The remaining solution was incubated at room temperature (28°C) for 30 min. After this incubation period, a 20 µl aliquot was again taken and treated in the same manner with the aliquot at time zero. The absorbance at 595 nm wavelength 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 0.1 mg ml\(^{-1}\) as the final concentration. To study optimum pH for enzyme activity, 0.3 mg ml\(^{-1}\) trypsin and 0.2 mg ml\(^{-1}\) casein was added to 50 µl of 100 mM of different buffers. The overlapping buffer system used were; citrate phosphate buffer pH 5.0 to 6.0, sodium phosphate buffer from pH 6.0 to 7.5 and Tris buffer from pH 7.2 to 9. To study the optimum temperature for enzyme activity, the reaction mixture contained 0.3 mg ml\(^{-1}\) trypsin and 0.2 mg ml\(^{-1}\) casein mixed with 50 µl of 100 mM sodium phosphate buffer pH 6.5 and the incubation temperature was varied from 25 to 80°C. 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 (Bickerstaff and Zhou, 1993).

Preparation of heavy metals solutions: Heavy metals such as manganese (ii) (MnSO\(_4\)\(\cdot\)H\(_2\)O, BDH), borate (iii) (H\(_3\)BO\(_4\), anhydrous BDH), cobalt (ii) (CoCl\(_2\)\(\cdot\)6H\(_2\)O, J.T. Baker, Phillipsburg, USA), aluminium (iii) (Al\(_2\)(SO\(_4\))\(_3\), anhydrous BHD) chromium (vi) (KCrO\(_4\)\(\cdot\)anhydrous BHD), selenium (vi) (Na\(_2\)SeO\(_4\)\(\cdot\)anhydrous BDH), nickel (ii) (NiCl\(_2\), anhydrous (Ajax Chemicals, Sydney, NSW, Australia), zinc (ii) (ZnSO\(_4\) anhydrous J.T. Baker, Phillipsburg, USA), iron (ii) (FeSO\(_4\)\(\cdot\)6H\(_2\)O, BDH), tungsten (vi) (Na\(_2\)WO\(_4\)\(\cdot\)2H\(_2\)O, BDH), tin (ii) (SnCl\(_2\)\(\cdot\)2H\(_2\)O, BDH) were prepared from commercial salts or from atomic absorption spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany) such as mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii). The heavy metals were each added into the reaction mixture to the final concentration of 20 mg l\(^{-1}\) as a preliminary screening. Heavy metals that inhibit the assay at 20 mg l\(^{-1}\) were then further screened using lower heavy metal concentrations. Common nontoxic metals such as potassium (K), calcium (Ca) and magnesium (Mg) were added in the form of KCl, CaCl\(_2\) and MgSO\(_4\) respectively into the reaction mixture at the final concentration of 50 mg l\(^{-1}\) (Botsworth, 1998).

Pesticides and xenobiotics studies: Pesticides with chemical purity > 99%, (Ehrenstorfer, Augsburg, Germany and Pestanal®, Riedel de Haen, Germany) such as carbaryl, fluencythrine, metolachlor, glyphosate, diuron, diazinon, endosulfan sulphate, atrazine, coumaphos, imidacloprid, dicamba and parquat were
Development of an inhibitive determination method for zinc

Table - 1: Comparison of trypsin bioassay to immobilized urease, MicrotoxTM, Daphnia Magna, fish bioassay (Rainbow trout) and papain assay.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Immobilized urease</th>
<th>15-min. Microtox™</th>
<th>48 hr Daphnia magna</th>
<th>96 hr Rainbow trout</th>
<th>Papainb</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn²⁺</td>
<td>14.6±3.2</td>
<td>0.27-29</td>
<td>0.54-5.1</td>
<td>0.55-2.2</td>
<td>2.11±0.56</td>
<td>4.8-6.7</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0.33±0.021</td>
<td>0.029-0.05</td>
<td>0.005-0.21</td>
<td>0.033-0.21</td>
<td>0.24-0.62</td>
<td>15.76-17.04</td>
</tr>
</tbody>
</table>

a Jung et al., 1995, b Shukor et al., 2006

Table - 2: Field trial results.

<table>
<thead>
<tr>
<th>Locations</th>
<th>Geo-positional satellite (GPS) locations</th>
<th>% Inhibition of trypsin activity¹</th>
<th>Concentrations of heavy metal (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prai Industrial Estate outlet sediment, Penang (Prai 1)</td>
<td>N 05°20.96, E 100°24.17'</td>
<td>100</td>
<td>18.46±1.34, 105.39±1.65</td>
</tr>
<tr>
<td>Galvanized metal factory outlet lagoon, Perak</td>
<td>N 04°37.59', E 101°05.18'</td>
<td>100</td>
<td>0.07±0.025, 23.63±3.67</td>
</tr>
<tr>
<td>Sungai Udang Recreational Jungle, Melaka (SURJ)</td>
<td>N 02°18.102 E 102°07.837'</td>
<td>0</td>
<td>n.d.², n.d.</td>
</tr>
<tr>
<td>Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan (UBRJ)</td>
<td>N 02°43.767' E 102°04.668'</td>
<td>0</td>
<td>n.d.², n.d.</td>
</tr>
<tr>
<td>Gunung Arong Forest Reserve, Mersing Johor (GAFR)</td>
<td>N 02°33.197' E 102°45.340'</td>
<td>0</td>
<td>n.d., n.d.</td>
</tr>
<tr>
<td>Tap water</td>
<td>University Putra Malaysia</td>
<td>0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

¹ 20% Inhibition is considered significant toxicity, ² n.d. = not detected

prepared by dissolving the pesticides in the appropriate solvent or used directly from the liquid solutions. Stocks solutions at 30 mg l⁻¹ were prepared in deionized water and added into the reaction mixture to final concentrations of 5 mg l⁻¹. The xenobiotics tested are as follows: acetoniitrile (MERCK), acetone (MERCK), ethylene glycol (SIGMA), ethyl acetate (MERCK), ethanol (BDH), DMSO (SIGMA), isopropanol (BDH), methanol (BDH), pyridine (SIGMA), triethanolamine (SIGMA), acrylic acid (SIGMA), polyethylene glycol (PEG) 600 (SIGMA), PEG 400 (SIGMA), diethylamine (SIGMA), triethanolamine (SIGMA), Triton-X-100 (SIGMA), Nonidet-P40 (SIGMA) and ethanalamine (SIGMA). These xenobiotics were prepared as a 2% (v/v) and (w/v) solutions in deionized water and added into the reaction mixture to a final concentration of 0.25% (v/v) with the exception of toluene where it was prepared as a 0.01% (v/v) solution to account for its limited solubility in water. Toluene was added into the reaction mixture to a final concentration of 0.005% (v/v).

Trypsin inhibition studies: In an Eppendorf tube, 5 µl of trypsin from the stock solution was added to 50 µl of 100 mM phosphate buffer pH 6.5 followed by the liquid solutions. Stocks solutions at 30 mg l⁻¹ were prepared in deionized water and added into the reaction mixture to final concentrations of 5 mg l⁻¹. The xenobiotics tested are as follows: acetoniitrile (MERCK), acetone (MERCK), ethylene glycol (SIGMA), ethyl acetate (MERCK), ethanol (BDH), DMSO (SIGMA), isopropanol (BDH), methanol (BDH), pyridine (SIGMA), triethanolamine (SIGMA), acrylic acid (SIGMA), polyethylene glycol (PEG) 600 (SIGMA), PEG 400 (SIGMA), diethylamine (SIGMA), triethanolamine (SIGMA), Triton-X-100 (SIGMA), Nonidet-P40 (SIGMA) and ethanalamine (SIGMA). These xenobiotics were prepared as a 2% (v/v) and (w/v) solutions in deionized water and added into the reaction mixture to a final concentration of 0.25% (v/v) with the exception of toluene where it was prepared as a 0.01% (v/v) solution to account for its limited solubility in water. Toluene was added into the reaction mixture to a final concentration of 0.005% (v/v).

incorporated with the Microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA). Regression curves are generated using the PRISM non-linear regression analysis available from www.graphpad.com (Motulsky, 2002). Means and standard errors were determined according to at least three independent experimental replicates.

Field trials: Water and sediment samples were obtained from several industrial outlets that release heavy metals products such as galvanized metal factories and several pristine areas such as recreational jungle and forest reserve areas for comparisons. Water samples were collected in acid-washed HDPE bottles containing several drops of 1% v/v HNO₃. The samples were filtered with 0.45 µm syringe filter and 45 µl of the clear filtrate was mixed with 50 µl of 100 mM phosphate buffer pH 6.5 followed by the addition of 5 µl of trypsin in an eppendorf tube and again mixed thoroughly. The mixture was incubated for 20 min at room temperature and then assayed according to the procedure outlined previously. Since two of the outlets from industrial effluent sites do not contain much water at the time of sampling, sedimentary materials were collected instead of water samples. Aqua regia digestion of the sediment samples was performed in 250-ml glass beakers covered with watch glasses. A 0.5g of the sediment samples was digested in 12 ml of aqua regia (one part HNO₃ and three parts HCl) on a hotplate for 3 hr at 110°C. After evaporation to near dryness, the sample was diluted with 20 ml of 2% (v/v with H₂O) nitric acid and filtered through Whatman No. 42 paper (Chen and Ma, 2001). The diluted samples were neutralized using NaOH and then diluted to 100 ml with deionized water in a 100-ml volumetric flask and used for the inhibitive assay immediately. Heavy metals in the samples were determined by atomic emission spectrometry on a Perkin Elmer Optima 3000 ICP-AES. All experiments were performed in triplicate.

Statistical analysis: Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a
Student’s t-test or a one-way analysis of variance with post hoc analysis by Tukey’s test (Miller and Miller, 2000). p < 0.05 was considered statistically significant.

Results and Discussion

The assay relies upon the inability of the Bradford dye-binding reagent to stain polypeptide (digestion products of casein by trypsin) with a molecular weight of less than 2 KD. The addition of the dye binding reagent after degradation of casein results in minimal or no color changes. In the presence of heavy metals that inhibit trypsin activity, casein remains undigested and the color after incubation was blue. Aside from casein and azocasein, trypsin will also hydrolyze ester and amide linkages of synthetic derivatives of amino acids, such as benzoyl L-arginine ethyl ester (BAEE), p-toluenesulfonfyl-L-arginine methyl ester (TAME), tosyl-L-arginine methyl ester, Nα-benzoyl-L-arginine p-nitroanilide (BAPNA) and L-lysyl-p-nitroanilide (Buck et al., 1962; Barman, 1969; Keil, 1971). Even the Bradford dye-binding reagent can be replaced with Biuret-Lowry (Lowry et al., 1951) and Folin Ciocalteu (Bensadoun and Weinstei, 1976) and Bichinconic (Smith et al., 1985). The optimization studies showed that trypsin activity was stable at a pH between 5 and 8.5, and the optimum temperature was at 40 °C. The results are similar to those of trypsin using BAEE as a substrate (Fernandez et al., 2005). The optimum combination of enzyme and casein as a substrate giving the maximum difference in absorbance was 0.1 mg ml⁻¹ trypsin and 0.1 mg ml⁻¹ of casein. The results are similar to those of the papain assay with the best combination of enzyme and casein in the latter is 0.1 mg ml⁻¹ for both enzyme and casein (Shukor et al., 2006). On the other hand, in the bromelain assay, the best combination is 0.11 mg ml⁻¹ bromelain and 0.25 mg ml⁻¹ casein, respectively (Shukor et al., 2008).

Inhibition of trypsin by heavy metals: Of all the heavy metals, only mercury and zinc showed significant inhibition of trypsin activity at 54 and 98% (p<0.05), respectively, when tested at 20 mg l⁻¹ (Fig. 1). Metals, such as K, Ca, Mg and Al, commonly found in the environment were non-toxic at 50 mg l⁻¹ (p>0.05). The inhibition profile of zinc exhibited a nonlinear one-site binding hyperbolic curve (Fig. 2) with a correlation coefficient of 0.98 and a calculated IC₅₀ value for zinc at 5.78 mg l⁻¹ (the 95% confidence interval was from 4.8 to 6.7 mg l⁻¹). Mercury showed a sigmoidal dose-response curve (Fig. 3) with a correlation coefficient of 0.99 and a calculated IC₅₀ value of 16.38 mg l⁻¹ (the 95% confidence interval was 15.76 to 17.00 mg l⁻¹).

Fig. 1: Screening for heavy metals inhibition of trypsin activity

Fig. 2: Inhibition of proteolytic activity of trypsin on substrate casein by zinc as measured using the Coomassie brilliant blue assay. Data is generated using the PRISM non-linear regression analysis for one site binding (hyperbola) model using the GraphPad software available from www.graphpad.com

Fig. 3: Inhibition of proteolytic activity of trypsin on substrate casein by mercury as measured using the Coomassie brilliant blue assay. Data is generated using the PRISM non-linear regression analysis for sigmoidal dose-response model using the GraphPad software available from www.graphpad.com

Fig. 4: The effects of pesticides on trypsin activity. Data is mean ± standard error of the mean (n=3)

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The limits of detection (LOD), usually assigned as three times the standard deviation of the blank for the y-intercept (Armbruster et al., 1994) for zinc and mercury, were 0.06 mg l$^{-1}$ (0.05-0.07, 95% confidence interval) and 1.06 mg l$^{-1}$ (1.017-1.102 at a 95% confidence interval), respectively. The Limits of Quantitation (LOQ) is the concentration at which quantitative results can be reported with a high degree of confidence. The LOQ for zinc and mercury were 0.61 mg l$^{-1}$ (0.51-0.74 at a 95% confidence interval) and 1.35 mg l$^{-1}$ (1.29-1.40 at a 95% confidence interval), respectively. The IC$_{50}$ value for zinc is much higher than the IC$_{50}$ values for papain and Rainbow trout, but is within the range of Daphnia magna and Microtox™. The IC$_{50}$ value for zinc is only lower than those for immobilized urease (Table 1).

The LOQ value permits the detection of zinc, since the MPL for zinc is at 5 mg l$^{-1}$ as outlined by the Department of Environment (DOE) of Malaysia. This suggests that this assay is suitable for use as a rapid preliminary screening method for detection of elevated zinc levels in the environment. The IC$_{50}$ for mercury was higher than those of the selected assays and bioassays shown in Table 1. The LOQ value for mercury is, however, much higher than the MPL for mercury in irrigation water as outlined by the DOE of Malaysia (2002) at 0.001 mg l$^{-1}$. This suggests that the trypsin assay is not suitable for use in monitoring mercury levels in the environment. The assay is not specific for zinc, as an elevated concentration of mercury (>10 mg l$^{-1}$) would also show some inhibition towards the assay. Other heavy metals will not caused inhibition if their concentrations were below 20 mg l$^{-1}$. However, the inhibition of the assay registered by other heavy metals would also appear in subsequent AAS works if their levels were excessively high. At these levels, zinc toxicity is of less concern since the toxicity of the other heavy metal ions would be severe. This means that, although the system is intended for zinc, it could also be used to detect other heavy metals albeit at concentrations far more toxic and far likely less to be reported, except in cases of toxic spills. Repeated measurement of trypsin inhibition by zinc suggests that the assay is reproducible with a CV (coefficient of variation) of the replicated data ranging from 3 to 13%.

### Effects of pesticides and miscellaneous xenobiotics on trypsin activity

The influence of foreign species on trypsin activity was investigated. Since the method was developed for the assay of heavy metals, the inhibitory or stimulatory effects of foreign species on trypsin could lead to false negative or positive results. Toxicants commonly found in Malaysian agriculture and industrial effluents, such as pesticides and solvents, were tested on trypsin activity. Pesticides, such as carbaryl, flucythrinate, metolachlor, glyphosate, diuron, diazinon, endosulfan sulphate, atrazine, coumaphos, imidacloprid, dicamba and paraquat, showed no effect on the activity of trypsin relative to control (One-way ANOVA, $F_{12,28} = 0.3527$, $p>0.05$) (Fig. 4). Pesticides were tested at the final concentrations of 5 mg ml$^{-1}$. The concentration of pesticide chosen in this work is generally much higher than what is normally found in natural water and also limited by the solubility of pesticide in water. Of the remaining 17 xenobiotics tested, only SDS gave positive interference with activity 150% greater than that of the control (100%) at 0.25% (v/v) (Fig. 5). SDS also interferes with the bromelain (Shukor et al., 2008) and the papain assay (Shukor et al., 2006) due to the similar Coomassie dye-binding assay employed. SDS interferes with the Bradford Coomassie dye-binding assay and its concentration should not be more than 0.1% (v/v) in order to not interfere with the assay (Stoscheck, 1990). The concentration of SDS in aquatic bodies is usually not found at this high level (Schott and Jones, 2000) and is not a great concern in the bioassay method. Trypsin has been known to retain most of its activity in 2.0 M urea, 2.0 M guanidine HCl, or 0.1% SDS (w/v) (Smith, 1988) reflecting excellent trypsin stability in the presence of a wide range of xenobiotics. Samples can

![Fig. 5: The effects of xenobiotics on trypsin activity. Data is mean ± standard error of the mean (n=3)](image_url)
be easily tested for the presence of excessive detergents by mixing the sample with the dye-binding reagent and observing any increase in blue intensity. Detergents and solvents are common constituents of industrial effluents. Bioassays that are performed to detect selected xenobiotics in the effluent of industrial discharge must not be affected by non-target xenobiotics, such as detergents. For a trypsin bioassay system that is intended to detect zinc in the environment, non-interference by detergents and solvents is an important criterion.

Field trial: The field trial results (Table 2) showed that two industrial sites gave positive toxicity results with 100% inhibition of trypsin activity. The concentrations of zinc and mercury as measured using atomic spectroscopy exceed the MPL limit at 5.0 and 0.001 mg/l, respectively (DOE, 2002). As expected, waters from tap water, recreational and forest reserves were free from heavy metals. The results from Table 1 indicates that the waters from the Prai Industrial Estate would contribute to the inhibitory effect on the assay as it contains elevated mercury levels, far in excess to the maximum permissible limit at 0.001. The inhibitory effect seen from the galvanized metal factory, however, would be solely due to zinc as its concentration was very high.

We are currently using different substrates for trypsin such as BAEE, TAME, BAPNA using the same conditions obtained in this work. We also anticipated that by replacing the Bradford dye-binding assay with other protein assays, we could prepare or eliminate the effect of interference compounds in samples to certain protein assays. We are also currently screening other potential protease candidates for the inhibitive determination of heavy metals. The usage of this simple colorimetric enzyme assay for the detection of zinc could be simplified down to the use of colour chart to qualitatively detect toxicants or microplate format for quantitative analysis.

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