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Evaluation of Inhibitive Proteases-based Enzyme Assay for Detecting Mercury Pollution in Paddy Field Waters Polluted with Artisanal Gold Mine

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ABSTRACT
Mercury contamination from artisanal and small-scale gold mining (ASGM) threatens agricultural ecosystems, and this necessitates rapid and cost-effective monitoring approaches. In this study, papain- and ficin-based inhibitive enzyme assays were evaluated for the detection of mercury in paddy-field water and soil samples collected from an ASGM-affected area in Mandailing Natal, North Sumatra, Indonesia. Protease inhibition was measured using casein as substrate and Coomassie dye binding to measure residual protein. Mercury concentrations were determined. In water samples, mercury concentrations ranged from approximately 0.03–0.18 mg/L, and in soil extracts, from 0.31–0.46 mg/L; mercury was negligible in the deionized-water control. Papain inhibition was approximately 38-63% in water samples and 94-102% in soil samples. Ficin was much more sensitive, with an inhibition of about 98-103 % in the water samples and 99-102%inhibition in the soil extracts and almost no inhibition in the control. The stronger response of ficin is consistent with its previously reported IC50 for Hg2+ of 0.017 mg/L, compared to 0.39 mg/L for papain. The findings indicate significant mercury contamination of the paddy environment under study and suggest that ficin represents a highly sensitive, rapid screening assay for detection of mercury contamination. Confirmatory instrumental analysis will be required for quantitative and metal-specific assessment.

KEYWORDS: Artisanal and Small-Scale Gold Mining (ASGM); Mercury contamination; Enzyme inhibition assay (Ficin/Papain); Paddy-field ecosystems; Rapid environmental monitoring
SDG Keywords: SDG 3: Good Health and Well-Being; SDG 6: Clean Water and Sanitation, and SDG 15: Life on Land

INTRODUCTION

The hike in the price of gold has resulted in an increase in the number of artisanal and small-scale gold mining (ASGM) in Indonesia. Approximately 5 Billion USD worth of gold is mined yearly but at a cost of the release of 5000 tons of mercury to the environment every year (Veiga & Fadina, 2020). The Mandailing Natal district is the location of an intense ASGM activity. The District Health Office reported that in the year 2012 alone there were at least 10,000 “gulundung” or gold trommel processing units using more than 200 L of mercury per day (Siregar et al., 2016). Such is the level of mercury pollution in the area that a previous study has reported mercury levels measured from the upper, middle and end parts of the river running into a village (Desa Tamiang) at 0.215, 0.072 and 0.008 mg/L mercury, respectively (Siregar et al., 2016). Significantly, mercury released during amalgamation can be entrained with mine wastewater via drainage networks and irrigation canals, leading to accumulation in soil and sediment-associated matrices of agricultural landscapes. A recent study of paddy fields in Hutabargot Sub-District, Mandailing Natal Regency reported no less than 170 amalgamation points that drain waste into local waters connected to irrigation systems. Samples of soil were taken from seven sites, and at depth intervals from 0–100 cm, in paddy fields receiving this runoff (Syahril et al., 2024). The results indicated that mercury contamination exceeded the relevant soil threshold values in the entire upper soil profile. The highest Hg concentration measured (14.26 ppm) was recorded in a field near an amalgamation site, with the Indonesian regulatory reference value for mercury-contaminated soil being 0.3 ppm and an additional benchmark of 0.5 ppm. This concentration is approximately 48 times above the threshold of 0.3 ppm and signals serious, localized contamination. Mercury concentrations increased from the surface to a maximum at around 21–30 cm depth and decreased with depth. However, Hg concentrations were above the stated threshold from 0 to 60 cm, whereas concentrations were below the threshold at depths greater than 60 cm. The values exceed the Maximum Permissible Limit for mercury in irrigation waters, which is 0.005 mg/L (Rusnam & Efrizal, 2016).

In another district with ASGM activity, the Hg concentration from cattle near ASGM areas is significantly higher (11.44 µg/g hair) than in those area farmed outside the ASGM area (2.89 µg/g hair, $p < 0.05$) (Basri et al., 2017).

Furthermore, samples of paddy rice grain collected adjacent to ASGM tailings ponds showed methyl mercury concentrations greater than 100 ng/g which is five times the permissible level for total mercury in food crops (Krisnayanti et al., 2012). To worsen the situation, an increase in birth defects, first seen in the infamous Minamata disease, started to be observed in the Mandailing Natal district, with mercury levels increasing in the drinking water wells, and mercury is blamed for the increase (Gunawan, 2019; Warjio et al., 2015). Hence, there is a need for intensive monitoring of mercury in ASGM area to safeguard the drinking and irrigation water sources. In addition, mercury can also inhibit bioremediation works, impacting remediation processes in general (Rusnam et al., 2023).

Instrumentation analysis for mercury is expensive due to the requirement of cold vapor techniques such as the Flow Injection Mercury System. Biomonitoring through enzyme assays emerges as a powerful approach in environmental management, providing a sensitive, cost-effective, and rapid means for detecting pollutants, which greatly benefits both public awareness and authoritative action. These assays are adept at identifying low concentrations of contaminants like heavy metals and organic compounds, facilitating the early implementation of remedial measures by authorities. Their cost efficiency and the minimal requirement for sophisticated equipment enable widespread and frequent environmental assessments, contributing to a detailed understanding of ecological health across vast areas (Amaro et al., 2011; Lopez-Roldan et al., 2012; Osimitz et al., 2015; Prabhakaran et al., 2017).

The swift processing of enzyme assays ensures timely interventions critical for preventing environmental degradation and safeguarding public health. Additionally, the simplicity and visual appeal of some enzyme assays serve as excellent resources for educational initiatives aimed at enhancing environmental consciousness among the community, thereby promoting active conservation efforts. The accurate data generated from these assays support evidence-based policymaking, enabling authorities to establish precise pollutant thresholds, assess the effectiveness of environmental protections, and make necessary adjustments. Moreover, enzyme activity indicators offer early warnings of ecological distress, allowing for interventions before visible damage occurs, thereby preventing long-term ecological damage (de Souza et al., 2018; Hayat et al., 2015; Lionetto et al., 2013; Perić & Petrović, 2011). By aligning local monitoring practices with international standards through enzyme assays, authorities can foster global collaboration in environmental protection, addressing transboundary challenges effectively. In essence, enzyme assays for biomonitoring equip both the public and decision-makers with essential tools and knowledge for more effective environmental stewardship and public health protection.

Several near-real-time monitoring methods for pollution, especially heavy metals, using enzymes from microorganisms and plants have been developed and utilized to monitor various potential and polluted sites in Malaysia, including two UNESCO sites (Halmi et al., 2013, 2014; Rahman et al., 2022; Sabullah et al., 2015; M. Shukor et al., 2013; M. Y. Shukor et al., 2014; Uba et al., 2020). In this study, we explore the feasibility of using the papain (Y. Shukor et al., 2006; Uba et al., 2020) and ficin dye binding assays previously developed for mercury biomonitoring to detect agricultural waters and soils polluted with artisanal gold mines that are generally known for using mercury to extract gold. The papain sensitivity towards heavy metals are in the form of IC_{50} (concentration of toxicant giving 50% inhibition) for Hg^{2+}, Ag^{+}, Pb^{2+} and Zn^{2+} are 0.39, 0.40, 2.16 and 2.11 mg/L (Y. Shukor et al., 2006), while the ficin inhibitive assay is sensitive to Hg^{2+}, Ag^{+} and Cu^{2+} with IC_{50} values of 0.017, 0.028 and 0.023 mg/L (Uba et al., 2020).

MATERIAL AND METHODS

Sampling

Water samples were collected from a paddy field in 2022 at 0.86542877° N, 99.54830434° E, VG8X+587, Panyabungan Jae, Mandailing Natal Regency, North Sumatra, Indonesia. Triplicate samples were collected from points located several meters apart to obtain representative field samples. Approximately 40 mL of water was collected at each sampling point using sterile 50 mL polypropylene tubes. After collection, several drops of nitric acid were added to each tube to preserve the samples and minimize changes in metal speciation or microbial activity during storage and transport. The samples were then properly sealed, labelled, and transported to the laboratory for further analysis.

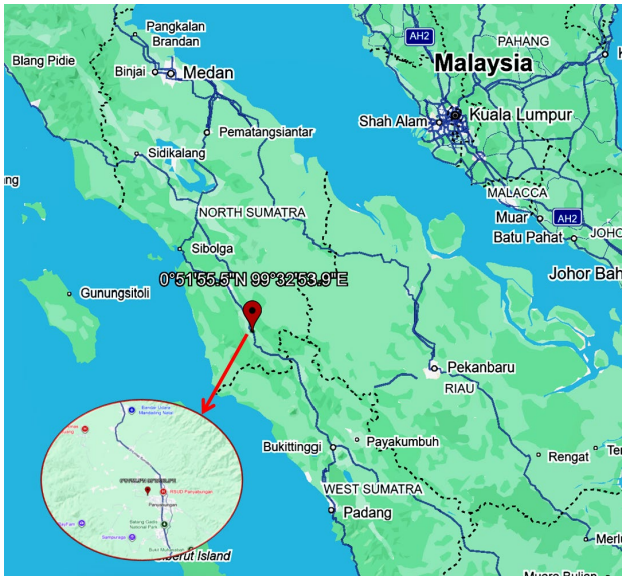


Figure 1. Location of water sampling. (Source Google Earth image).

Preparation of casein, papain, and ficin solutions

A casein stock solution was prepared by dissolving 2 g of casein from Sigma in 100 mL of deionized water. The pH of the suspension was adjusted to 8.0 using 5 N NaOH or 5 N HCl, as required. The mixture was stirred continuously at 60 °C overnight to improve solubilization. Any undissolved material was removed by passing the solution through several layers of cheesecloth, followed by centrifugation at 10,000 × g at 4 °C to obtain a clear supernatant. The protein concentration of the clarified casein solution was measured using the Bradford assay, with crystalline bovine serum albumin from Sigma used as the calibration standard. The prepared casein solution was kept at 4 °C when used immediately or stored at −20 °C for longer-term preservation.

Ficin and papain from Sigma-Aldrich, St. Louis, USA, prepared from crude dried fig tree and papain latex, respectively, were dissolved in chilled 20 mM sodium phosphate buffer at pH 6.77. Papain and ficin stock concentrations of 10.0 mg/mL were prepared at 4 °C. Fresh working solutions were prepared daily

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from the stock solution, with papain and ficin diluted to 2.0 mg/mL and casein diluted to 10 mg/mL before use in the assay.

Ficin and papain inhibition studies

Ficin and papain inhibition by mercury was assessed using a reaction mixture containing ficin (or papain), mercury solution, and casein substrate. For the positive inhibition assay, 50 µL of ficin solution was mixed with 50 µL of mercury solution to give final concentrations of 0.6 mg/mL ficin and 0.040 mg/L mercury. The ficin was prepared in 20 mM phosphate buffer at pH 6.77, a condition previously optimized using a Central Composite Design, as reported by Uba et al. (2020). The ficin–mercury mixture was incubated at 30 °C for 10 min to allow enzyme–metal interaction. For the uninhibited control, the mercury solution was replaced with an equal volume of 20 mM phosphate buffer at pH 6.77. After pre-incubation, 50 µL of casein solution was added to each reaction mixture to produce a final casein concentration of 2.36 mg/mL.

The mixture was then mixed thoroughly. Immediately after substrate addition, 20 µL of the reaction mixture was transferred into 200 µL of Bradford dye-binding reagent. The mixture was allowed to react for 5 min at room temperature, and the absorbance was measured at 595 nm. This reading was taken as the initial absorbance. The remaining reaction mixture was further incubated at 30 °C for 30 min. After incubation, another 20 µL aliquot was withdrawn and mixed with 200 µL of Bradford reagent using the same procedure. Absorbance was again measured at 595 nm after 5 min of colour development. The change in absorbance between the initial and final readings was used to evaluate ficin activity and the inhibitory effect of mercury.

Determination of Mercury in Paddy Plant Soil Samples and waters

Mercury determination was conducted using water and sedimentary soil paddy plant samples collected from a paddy field located at 0.86542877° N, 99.54830434° E, VG8X+587, Panyabungan Jae, Mandailing Natal Regency, North Sumatra, Indonesia. Sampling was carried out in 2022. Water samples were collected in triplicate from points located several meters apart to obtain representative field samples. Approximately 40 mL of water was collected at each sampling point using pre-cleaned 50 mL polypropylene tubes. Several drops of nitric acid were added immediately after collection to preserve the samples and minimize changes in metal speciation during storage and transport. A 2 g sample of homogenized soil samples taken from the depth of between 5 and 10 cm was digested with HNO_3:HCl ratio of 3:1 (aqua regia) with controlled heating and addition of hydrogen peroxide to oxidize residual organic matter until a clear digest was obtained.

The acidic extract was allowed to cool and then neutralized with a validated laboratory procedure and adjusted to about pH 6.5 to simulate the phosphate-buffered conditions of the ficin assay (Uba et al., 2020). Precipitates were not observed in this study. Where required, samples were diluted to reduce residual acid, salt, or matrix interference, and the dilution factor was incorporated in subsequent calculations. Appropriate controls were employed to differentiate Hg^{2+} inhibition from matrix effects. These were a free ficin control, a digestion blank, a sample blank without ficin, and a matrix-spiked sample with a known concentration of Hg^{2+}. Samples were analyzed using matrix-matched mercury standards. The prepared extract was then subjected to ficin inhibitive assay as described by (Uba et al., 2020). Ficin was preincubated with the sample prior to addition of casein substrate and residual casein was quantified by Coomassie dye binding at 595 nm. Hg^{2+} inhibition of ficin causes decreased casein hydrolysis with increased residual casein and increased absorbance. The enzyme inhibition was calculated compared to the control without inhibitor. Only extracts that were within the validated assay pH range and exhibited acceptable blank and spike-recovery performance were used for mercury determination. For confirmatory analysis, mercury concentrations in water samples and in the separate root, stem, and leaf digests were determined using a PerkinElmer ICP-OES. This approach enabled field-based screening of mercury in paddy field water and laboratory-based confirmation of mercury accumulation in different paddy plant tissues.

Data and Statistical Analysis

The per cent inhibition was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Test activity of sample} - \text{test activity of control x } 100}{\text{Test activity of control}}$$

RESULTS AND DISCUSSION

The mercury concentrations and inhibition responses of papain and ficin in paddy-field water and soil samples are shown in Figures 2 and 3, demonstrating that all four water samples and all four soil samples contained mercury. The control, which is deionized water, exhibited essentially no detectable Hg. The water samples contained about 0.03–0.18 mg/L Hg, while the soil extracts contained significantly higher concentrations, about 0.31–0.46 mg/L. The highest concentration was found in Soil Sample 1 (~ 0.46 mg/L), then Soil Sample 4 (~0.45 mg/L), suggesting significant Hg contamination at the sampling site and consistent with reports of mercury contamination associated with artisanal and small-scale gold mining (ASGM) in Mandailing Natal. Previous studies reported Hg contamination in local surface and well waters (Siregar et al., 2016) and significant Hg accumulation in paddy soils around amalgamation sites (Syahril et al., 2024). The results shown in this study in relevance to the presence of mercury in agricultural environments impacted by ASGM are of particular concern because Hg released during gold processing can enter irrigation networks and subsequently accumulate in paddy soils and crops (Krisnayanti et al., 2012; Veiga and Fadina, 2020).

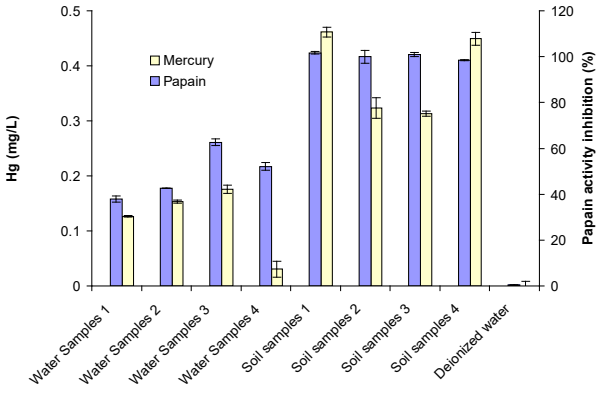


Figure 2. Papain activity inhibition (%) and mercury concentrations (Hg, mg/L) in paddy-field water samples (Water Samples 1–4), soil extracts (Soil Samples 1–4) and deionized-water control. Bars are mean values and error bars represent experimental variation.

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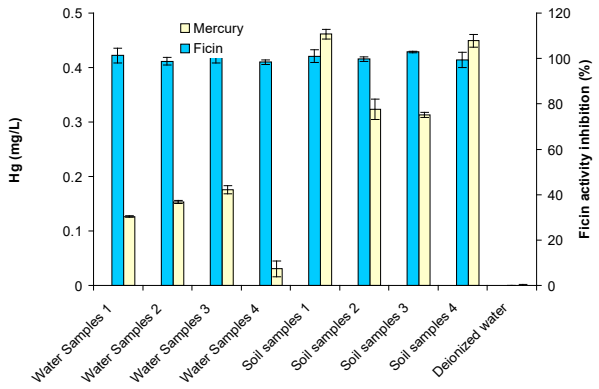


Figure 3. Mercury concentration (Hg, mg/L) and ficin activity inhibition (%) in paddy-field water samples (Water Samples 1–4), soil extracts (Soil Samples 1–4) and deionized-water control. The bars are mean values with error bars representing the experimental variation.

The response of the environmental samples to papain was evident (**Figure 2**). Inhibition varied from approx. 38-63% for water samples and 94-102% for soil samples, generally increasing with the increased Hg contamination of soil extracts. Deionized water showed essentially no inhibition, which is consistent with the interpretation that the inhibition observed in environmental samples was caused by inhibitory components and not by the assay medium itself. Papain is a cysteine protease. Its activity is strongly affected by Hg^{2+} and other thiol-reactive metals. Shukor et al. (2006) reported a Hg^{2+} IC_{50} of ~0.39 mg/L for the papain assay previously. The relatively strong responses obtained here, especially for the soil samples, therefore demonstrate the usefulness of protease inhibition as an expedient screening approach for contaminated environmental matrices. Enzyme assays offer rapid and relatively inexpensive measures of biologically available toxicants, and this makes them suitable for biomonitoring (Halmi et al., 2014; Rahman et al., 2022).

The ficin assay gave an even more dramatic response (Figure 3), with inhibition approaching ~98–103% in water samples and ~99–102% in soil samples, while deionized water again gave essentially no inhibition. The strong response is in agreement with the significantly higher sensitivity of ficin toward Hg^{2+}. Uba et al. (2020) reported an optimized Hg^{2+} IC_{50} of 0.017 mg/L and a LOD of ~0.002 mg/L, compared to an IC_{50} of 0.39 mg/L reported for papain (Shukor et al., 2006). Thus, the high ficin inhibition observed in water and soil samples is consistent with its reported analytical sensitivity.

In classical and current methods for heavy metal detection, batch processing of samples is usually employed, where samples need to be collected and then transported to a laboratory for analysis (Hsieh et al., 2004; Jung et al., 1995). This process not only takes time but incurs an increase in cost and may not reflect the immediate changes in pollutant levels. To address these limitations, bioassays based on plants, microorganisms and enzymatic reactions have been shown to be promising for prompt environmental assessment (Bhat et al., 2017; Eom et al., 2020; Polyakova et al., 2020). Enzyme assays, for example as shown in this study, are nearly rapid, and the whole process from sampling to detection can be performed within an hour by means of portable spectrophotometry, which makes them suitable for on-site analysis. The need to implement rapid, near real-time biomonitoring of water systems in drinking and agricultural waters is multi-pronged but largely driven by the need to protect public health. Monitoring of mercury using papain and ficin assays help to identify mercury contaminant immediately and provides an important early warning system to avoid exposure of humans and animals to hazardous substances and reduce health risks.

Ficin and papain are cysteine proteases and Hg^{2+} has a high affinity for their thiol groups and can inhibit proteolysis due to the sulfhydryl groups being crucial for the catalytic activity of these enzymes. This inhibition leaves more undegraded casein available for action to the Coomassie dye binding (Uba et al., 2020). The near-complete inhibition of ficin in samples with widely differing Hg concentrations is an indicator of classic response saturation since the enzyme is completely inhibited at the threshold lower concentrations. A caveat to this study is ficin is also very sensitive to Ag^{+} and Cu^{2+}, therefore, without confirmation by chemical analysis for the presence of these metal ions, the inhibition should not be considered Hg-specific (Uba et al., 2020). Overall, ficin is more sensitive than papain based on the results of this study, and has great potential as a rapid screening tool for Hg pollution associated with ASGM-affected paddy environments.

CONCLUSION

This study demonstrates the feasibility of using protease-based inhibitive assays as rapid screening tools for mercury contamination in paddy-field environments affected by artisanal and small-scale gold mining. The reactions of both papain and ficin to contaminated water and soil extracts were demonstrated. Ficin always exerted a superior inhibitory effect to papain, confirming the higher sensitivity to mercury and suitability for the detection of contamination at environmentally relevant levels. However, the strong inhibition across samples suggests that response saturation may prevent direct quantitative discrimination between samples with high contamination. Thus, it should be used in conjunction with appropriate dilution, matrix controls and calibration. Additionally, other thiol-reactive metals can also inhibit ficin, so the assay should be used primarily as a quick preliminary tool for biomonitoring and not as a metal-specific analytical method. Confirmatory instrumental analysis is still relevant for definitive mercury quantification and environmental risk assessment.

CONFLICT OF INTERESTS

The authors have no competing financial interests or personal relationships to disclose.

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AUTHORS' CONTRIBUTIONS

RM contributed to conceptualization, and as project manager, FIN carried out the sampling; MM carried out instrumental analysis and manuscript write-up, and MIE carried out manuscript write-up and data analysis.

DATA AND MATERIALS AVAILABILITY

Data available upon request.

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AI STATEMENT

The authors used generative artificial intelligence (AI) tools solely in the preparation of this work for language editing and refinement. All authors reviewed and revised the text. The contents of the publication are the responsibility of the authors.

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Abbreviations:

ASGM	Artisanal and Small-Scale Gold Mining
IC_{50}	Half-Maximal Inhibitory Concentration; concentration causing 50% enzyme inhibition
LOD	Limit of Detection
ICP-OES	Inductively Coupled Plasma–Optical Emission Spectrometry
MPL	Maximum Permissible Limit

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