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Preliminary Screening of Plant Proteases as a Potential Source for the Development of an Inhibitive Assay for Heavy Metals

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ABSTRACT

Heavy metals pollution has become a great threat to the world. Since instrumental methods are expensive and need skilled technician, a simple and fast method is needed to determine the presence of heavy metals in the environment. In this work, a preliminary study was carried out on the applicability of various local plants as a source of protease for the future development of the inhibitive enzyme assay for heavy-metals. The crude proteases preparation was assayed using casein as a substrate in conjunction with the Coomassie dye-binding assay. The crude protease from the kesinai plant was found to be the most potent plant protease. The crude enzyme exhibited broad temperature and pH ranges for activity and will be developed in the future as a potential inhibitive assay for heavy metals.

INTRODUCTION

Human activity within the last few years has resulted in worldwide toxic contamination by organic and inorganic substances [1]. The existence of the contaminants produced by commercial and agriculture activities in the streams continues to be recognized to generate likely dangerous effect to the marine living organisms and also the ecology [2–6]. These days, heavy metal toxic contamination is regarded as being among the most severe ecological issues. Heavy metals are any inorganic metallic substance that may exert their toxicity by way of binding to the thiol group of proteins and enzymes as well as to the cysteine of the disulfide bond that brings about the stability of the enzyme [7,8].

The heavy metals especially mercury, cadmium and silver possess great affinity to the disulfide bridge in proteins and enzymes between two cysteine residues. Heavy metals are very harmful to live organisms particularly humans simply because a few of the metals may cause DNA damage and cause longlasting carcinogenic effects. The Juru Industrial Estate in Malaysia, for instance, is well known for liberating elevated concentrations of heavy metals into the surrounding agricultural areas and also into the marine environment [9,10]. As a result, there have been studies of fish in the coastal areas of Malaysia being reported to be polluted by heavy metals [11]. Hence there is a need to monitor heavy metals in this area. Mining activities are also a contributor to heavy metals pollution as is reported in a copper mining area in Sabah [12,13].

Inhibitive enzyme assays have can detect toxicants such as heavy metals and their use as a preliminary screening tool is far superior to the use of classical methods such as atomic absorption spectroscopy in isolation as this will be extremely expensive, as it requires highly-trained operators, need complicated sample pre-treatment and a long measuring period [14–20]. Therefore, rapid and simple techniques are really needed to detect to the presence of heavy metals in the environment. The most recent works on the development of inhibitive enzyme assays use the plant proteases papain, bromelain and protease from garlic, tomato and coriander to detect heavy metals [21–23]. In general, a bioassay is

nonspecific towards particular heavy metal, but it can be used as an early monitoring system [24,14]. Proteases can also be obtained from plants, which includes the leaves [25], fruits [26], rhizomes [27] and may also be present in leguminous seeds [28]. As the plant proteases are recognized as a potent inhibitive assay system for the detection of heavy metals, there is a need to further screened more plant-based proteases to find a better system in terms of sensitivity to certain heavy metals. The aim of this study is to carry out a preliminary screening of plant-based proteases in terms of their casein degrading ability in comparison to commercial proteases. Plant proteases that exhibit strong casein degradation can be further developed for a sensitive assay system for heavy metals.

MATERIALS AND METHODS

Preparation of buffer solutions

All buffers were prepared according to the methods of Dawson et al., (1969) by mixing the appropriate amount of salts and acids forms of the buffer reagent. Minor adjustment of buffer was made using 5N NaOH and 5N HCl.

Bradford dye binding assay

Laboratory-prepared dye-binding Coomassie reagent has been found to have a limited linear range as the commercially available dye is impure [29]. Thus, the commercial Bradford dye-binding reagent from Bio-Rad (USA) was used according to the instruction by the manufacturer.

Preparation of casein solution

Preparation of casein was carried out according to a previous method [30]. Briefly, a 100 ml solution of deionised water mixed with two grams of casein (Sigma) was adjusted to pH 8.0 to aid solubility and stirred overnight at 60 ◦C. Casein was first filtered through several layers of cheesecloth followed up by centrifugation of the filtrate at 10,000×*g* for 10 min. The clear supernatant was used and forms the stock solution. The protein content of this stock was measured using the Bradford dye-binding assay using crystalline BSA (Sigma) as the standard. A working stock solution of casein at 10.0 mg/ml was prepared fresh daily.

Preparation of heavy metals solutions

Heavy metals are either from the Atomic Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany) or prepared from their salts, preferably nitrate salts. Working solutions at from 0.5 to 10 mg l^{-1} were prepared by diluting them in deionized water, and all of them were stored in acid-washed polypropylene containers. These solutions were prepared fresh daily.

Protein micro assay procedure

Bovine serum albumin (BSA) from SIGMA at 1 mg ml^{-1} was added into the reaction mixture from 5 to 20 µL. Each reaction mixture composition was made up to 100 µl using deionized water. Bradford reagent was added (1000 µl) and the mixture was incubated at room temperature for 10-20 minutes before the absorbance was read at 595 nm. The first sample which did not contain BSA was set as the control.

Extraction of plant protease

The method of Jiang et al. [31] was utilized for the extraction of plant proteases. Briefly, chopped plant tissues were first immersed for two days in the chiller in a solution containing 50 mM of sodium phosphate buffer pH 7. Samples were blended in a blender using a homogenization buffer (1 ratio of the plant, 3 ratios of chilled buffer). The blending of the mixture was carried out for 20 s followed by a 10 min cooling period. A high-speed setting was utilized, and ten cycles of blending and cooling were utilized. The homogenized sample was first sieved and then centrifuged at 10,000 × g for 15 minutes at 4 ℃. Proteases activity was checked for both pellet and supernatant fractions.

Protease activity

Protease assay was carried out according to the method of Shukor *et al.* [30] where protective reagents such as EDTA and DTT are removed. This is to enhance the sensitivity to heavy metals in future studies. In an Eppendorf tube, 100 µl of plant proteases from appropriate stock solutions were added to 20 µl of 50 mM sodium carbonate buffer at pH 6.5. The plant protease papain, ficin and bromelain (Sigma-Aldrich, USA) were utilized as a positive control. 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 minutes to get the absorbance for time zero. The best absorbance for time 0 min should be between 0.9 and 1.0 taking into account the linearity of the Coomassie dye binding assay.

The mixture was then incubated for 20 min at room temperature (27 ºC). After the incubation period, 60 µl of casein from a stock solution of 5.0 mg ml^{-1} was added and mixed thoroughly. The remaining solution was incubated at room temperature for 20 min. After this incubation period, a 20 µl aliquot was again taken and mixed with the Coomassie dye reagent 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). Three independent experimental replicates were utilized to generate the means and standard deviation. Percentage of casein degradation activity was carried out according to the following equation;

$$\%Casein\ degradation = \frac{A595nm(initial) - A595nm\ (final)}{A595nm(initial} x100\%$$

Optimization of proteolytic activity

Temperatures and pH are two important parameters for optimization of the plant protease inhibitive assay. The optimization of pH was carried out between pH 4 and 8 using a 50 mM citrate-phosphate buffer system than can span within this range. Optimization of temperature was carried out between the room and 60 ºC.

RESULTS AND DISCUSSION

Screening of plant protease for casein degradation capacity

Recognition of the plant source that provides the best enzyme activity is extremely essential in because the protease inhibitive assay is simply practical for an enzyme that exhibits substantial proteolytic activity. This is due to the fact that substantial color changes of the Bradford reagent are only visible if the protease has high activity. **Table 1** shows the percentage activity between plant sources. Of all the new plant samples tested, the kesinai plant gave the best enzyme activity. The activity is just below that from the coriander [22]. Kesinai is known to harbour proteolytic activity [25]. Commercial plant-based proteases all gave activity higher than 90% (**Table 1**) indicating that they are potent proteases.

This is likely due to the fact that they are much purer than the crude preparation from this work. Even though high enzyme activity does not associate with sensitivity towards heavy metals, the protease from this plant source will be for inhibitive

assay works. To get a good protease inhibitive assay for heavy metals, the difference in absorbance ought to be greater than 0.2 after a maximum of one h of incubation for practical reasons [30]. Other plant-based proteases that have shown good potential in past works include the crude proteases from coriander [22], garlic [21] and tomato [23]. It is important to note that the protease(s) in the crude fraction can consist of more than one fraction and their further purification is imperative to obtain greater sensitivity towards heavy metals [32,33].

Table 1: Proteolytic activity of different plant crude extracts. Data are represented as the mean ± standard deviation of triplicates.

Samples	Percentage of activity (%)
Negative control	0 ± 0.5
Starfruit*	10. ± 1.0
Sweet potato*	12 ± 0.5
Brinjal*	15 ± 1.5
Mango*	14 ± 0.5
Cucumber*	49 ± 1.0
Coriander*	95 ± 2.0
Curry leaf*	32 ± 1.0
Positive control	100 ± 0
Lime*	21 ± 0.5
Kesinai leaves (*Streblus asper*)	89.2 ± 0.4
Moringa oleifera leaves	45.2 ± 0.2
Noni leaves (*Morinda citrifolia*)	34.0 ± 0.3
Rambutan leaves (*Nephelium lappaceum*)	23.7 ± 0.4
Serai leaves (*Cymbopogon citratus*)	77.8 ± 0.6
Sirih leaves (*Piper betle*)	88.2 ± 0.3
Centella asiatica leaves	43.2 ± 0.1
Papain	99.2 ± 0.4
Bromelain	95.3 ± 0.5
Ficin	98.4 ± 0.3

Note *from [23]

The most suitable pH for protease from the kesinai leaves was between 5.5 and 7.5 (**Fig. 1**) indicating a broad range of activity. In contrast, the protease from garlic showed a sharp optimal pH at pH 6.0 [21] while the protease from coriander leaves shows a broad pH range of-of 8 and 9.5 in sodium carbonate buffer [22]. The catalytic activity of an enzyme is pH and temperature delicate because intermolecular bonds that maintain proteins in their secondary and tertiary constructions are disturbed by alterations in temperature and pH. The most beneficial pH value will be the point at which the enzyme is most active (optimum pH).

In the course of field works, the samples could have diverse pH according to in which the samples are gathered. A little alteration in pH will modify the system, and this will provide an incorrect outcome in the course of the monitoring process. Consequently, the use of a buffer in the bioassay system is very important to avoid the pH of the samples from impacting the enzyme activity.

The proteases exhibited high protease activity in a broad range of temperature which was between 25-45°C (**Fig. 2**). The negative impact on enzymes by temperatures greater than 45 °C is likely to be brought on by a number of factors such as tertiary and quaternary protein denaturation by means of thermal vibration resulting in the possible loss of cofactors and sped up the oxidation of sulfhydryl groups at greater temperatures.

A broad temperature range for protease activity is definitely an edge for field site monitoring in a warm country, and the optimized temperature is equivalent to other proteases like bromelain, papain chymotrypsin and trypsin [30,34,35,35]. Temperature stability is one of the main problems for on-site biomonitoring because some of the commercial toxicity bioassay kit work sub-optimally at tropical climates as they were meant for temperate conditions including the Microtox™-based *Vibrio fischery* that works only at 15°C, which makes it unsuitable for tropical climate [10]. The stability of this protease at a higher temperature would make this bioassay system applicable in hotter climate countries such as in the middle East.

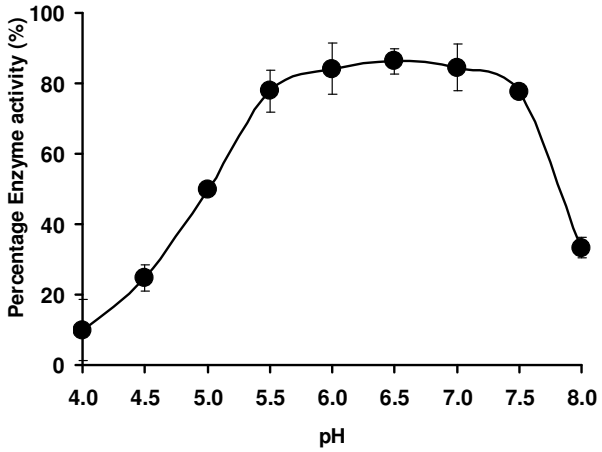

Fig. 1. Effect of pH on the activity of proteases from kesinai. Data were the mean ± standard deviation (n=3).

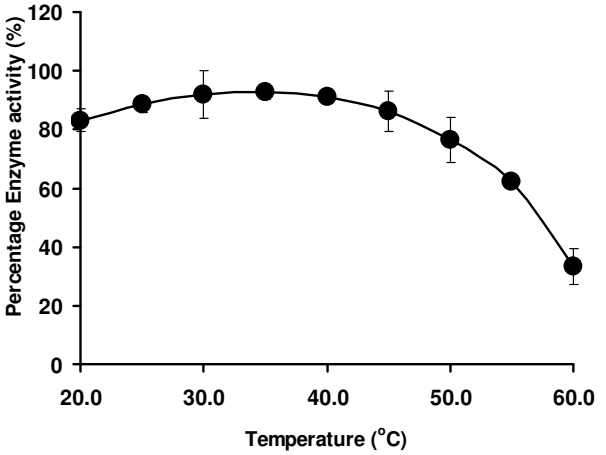

Fig. 2. Effect of temperature on the activity of proteases from kesinai. Data were the mean± standard deviation (n=3).

The optimal incubation time for this enzyme was at least 50 min with temperatures above this show no significant difference ($p>0.05$) as analyzed by ANOVA (**Fig. 3**). However, this system is considered a fast bioassay because it will have near real time applicability. Further purification of this crude enzyme will not only make the reaction reaching optimality faster but more sensitive to heavy metals.

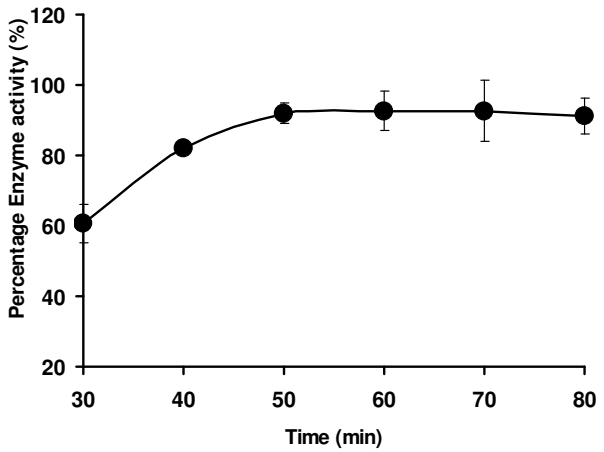

Fig. 3. Effect of incubation time on the activity of protease from kesinai. Data were the mean ± standard deviation (n=3).

CONCLUSION

Plant proteases are an excellent assay for the inhibitive assay of heavy metals coupled with the dye-binding Coomassie assay. A challenge for research in this area is obtaining a cheaper alternative to plant proteases in an application today. The plant extract from the kesinai plant has shown a very good proteolytic activity and can offer a cheaper alternative. It shows broad pH and temperature for optimal activity rendering it highly suitable for biomonitoring works in the tropical climate. In the future, the sensitivity of this enzyme towards heavy metals will be tested, and purification of the enzyme is expected to increase the sensitivity of heavy metals towards the inhibitive assay by increasing the bioavailability of heavy metals.

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