BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2012. Vol. **9**(1), 239-242

Papain Partial Purification Improves its Sensitivity towards Heavy Metals Papain Partial Purification

N.A. Masdor* **and N.A.M. Said**

Biotechnology Research Centre, MARDI, P. O. Box 12301, 50774 Kuala Lumpur, Malaysia.

(Received: 25 March 2012; accepted: 03 May 2012)

The protease papain, in combination with the dye-binding coomassie protein assay of its substrate has been shown to be sensitive towards toxic heavy metals and has good potential as a preliminary screening method for detecting heavy metals in various samples. The original assay uses crude preparation and there is a possibility to improve its sensitivity towards heavy metals by removing interfering proteins. Using a combination of ion exchange and gel filtration chromatography, a 3.3 fold purification was achieved, and the partially purified fraction showed significantly lower IC_{50}s ($p<0.05$) for all heavy metals tested except for zinc, than the crude fraction. This demonstrates that partial purification improves the sensitivity of the papain inhibitive assay towards heavy metals.

Key words: Papain, Partial purification, Heavy metals.

Biomonitoring of heavy metals has been suggested as a low cost and rapid approach towards the detection of heavy metals in aquatic bodies. The USEPA has recommended both microorganism and enzyme as principal assay systems. Numerous enzymes have been used for inhibitive determination of xenobiotics and heavy metal traces, e.g. peroxidase, xanthine oxidase, invertase, glucose oxidase, isocitric dehydrogenase, urease[1,2] and acetylcholinesterase[3,4]. Urease is currently being adopted by the USEPA as a biomonitoring system[5] although it suffers from high ammonia interference from the environment leading to its immobilization as a solution[2]. More recent development on inhibitive assays to overcome the shortcoming of the urease assay is the novel Mo-reducing enzyme assay[6] and the proteases papain[7], bromelain[8] and trypsin[9] assays. The plant proteases papain, bromelain and trypsin are part of a novel inhibitive assay that utilizes the casein-dye binding coomassie system as the principal inhibitive assay. In the presence of heavy metals, these proteases are unable to digest casein and the solution remains blue after the addition of the dye binding reagent. Currently, the papain assay[7] could detect more heavy metals that the proteases bromelain[8] or trypsin[9] assays. Commercial papain (EC 3.4.22.2) preparation is a crude preparation containing the proteolytic enzymes from papaya latex. Previously we have shown that purification of bromelain improves its sensitivity towards heavy metals. In this work, we report on a similar effect of improvement of sensitivity towards heavy metals by partially purifying the crude papain from the commercial preparation.

* To whom all correspondence should be addressed.
Tel.: 03-8943 5942, 03-8945 6037
Email:azlina@mardi.gov.my/azlina.mardi@gmail.com

MATERIAL AND METHODS

All buffers were prepared by mixing the appropriate amount of salts and acids forms of the

reagent. Minor adjustment of buffer was made using 5 N NaOH and 5 N HCl. All experiments were carried out at 4 °C unless indicated otherwise.

Preparation of heavy metals solutions

Heavy metals such as mercury (ii), cadmium (ii), lead (ii), copper (ii) and silver (i) were prepared from Atomic Absorption Spectrometry standard solutions from MERCK and diluted to form working solutions (10 mg l^{-1}) using phosphate buffer pH 6.8.

Preparation of casein and papain solutions

Bradford Coomassie-dye binding assay, casein and papain stock solutions were prepared according to Shukor et al.[7]. About two grams of casein (Sigma) was weighed and dissolved into 100 ml of deionised water adjusted to pH 8.0 with 5 N NaOH and incubated overnight with stirring at 60 °C. The solution was then filtered through several layers of cheesecloth. The filtrate was then centrifuged at 10,000 g for 15 minutes and the protein concentration of casein in the clear supernatant (10 mg ml^{-1}) was measured using the Bradford dye-binding using crystalline bovine serine albumin (BSA, Sigma) as the standard. Papain (Sigma), (EC 3.4.22.2), lot no: 32K2619, crude dried papaya latex. 0.5 Units/mg) was prepared at 4 °C in 50 mM sodium phosphate pH 6.5 as a 10.0 mg ml^{-1} stock solution and stored at -20 °C for a maximum period of one month. Papain (2.0 mg ml^{-1}) and casein (0.3 mg ml^{-1}) working solutions were prepared fresh daily.

Partial purification of papain

Papain was partially purified using chromatography on CM-Sepharose at pH 4.5 and gel filtration on Zorbax GF-250 (Agilent). About 0.5 g of papain was dissolved in 5 ml of 50 mM acetate buffer pH 4.5. A column of CM-Sepharose having the dimension of 1 cm x 10 cm with a bed volume height of 5 cm was preequilibrated with 50 mM acetate buffer pH 4.5. Papain was loaded and washed with three column volumes of the same buffer until no traces of protein in the eluant was detected. Papain was eluted with 300 ml of a linear 1 M NaCl gradient in the same buffer at a flow rate of 2 ml per min. The fraction containing activity was pooled and concentrated (Amicon) and chromatographed on Zorbax GF-250 using 50 mM phosphate buffer pH 6.5 and 150 mM NaCl at a flow rate of 1 ml per min. The fraction containing activity was used for inhibitive heavy metals assay.

Papain Assay

Papain inhibition studies were carried out according to Shukor et al.[7]. One unit of papain activity is defined as the amount of casein (in mg) hydrolyzed per minute by 1 mg of protease under the specified assay conditions[10]. The inhibitive assay for heavy metals assay is as follows. In an Eppendorf tube, 20 µl of papain from the stock solution was mixed with 100 µl of phosphate buffer pH 6.8. The mixture was incubated for 15 minutes at room temperature. This was followed the addition of 50 µl of casein to. The final volume was made up to 200 µl using deionized water. About 20 ml aliquot was withdrawn and mixed with 200 µl of Bradford dye-binding reagent in a microplate well and incubated for 5 minutes. The remaining solution was incubated at 40 °C for 30 minutes. After this incubation period, a 20 ml aliquot was again taken and treated in the same manner with the aliquot at time zero. The effect of heavy metals was studied by replacing 100 µl of phosphate buffer pH 6.8 in the reaction mixture with various concentrations of heavy metals. The absorbance at 595 nm was measured using a microplate reader (BioRad model 680). PRISM non-linear regression analysis software from www.graphpad.com was used to calculate the IC_{50} using regression analysis for sigmoidal and hyperbolic dose-response curves. The conditions employed in this section such as pH, temperature, concentrations of substrate and enzymes are optimum conditions resulted from papain optimization studies were from Shukor et al.[7]. Protein (casein) was assayed according to the dye-binding method[11]. Means and standard errors were determined according to at least three independent experimental replicates.

Statistical analysis

Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05 available from www.graphpad.com. 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. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows that the partial purification was successful and a 3 fold purification was

achieved. Proteolytic activity was highest in fraction between 40 and 42 that eluted at approximately 0.175 M NaCl and several smaller peaks showed significant but weak proteolytic activity. The fractions with the highest activity was pooled, concentrated and run on gel filtration. A major peak with high proteolytic activity (Results not shown) was pooled and used in the inhibitive assay as partially purified fraction. The IC_{50} value for silver, lead and mercury showed significantly ($p<0.05$) lower values than the crude fraction while there was no significant difference ($p>0.05$) in the IC_{50} values for zinc between partially purified and crude fraction (Table 2).

Table 1. Purification table of the partial purification of papain

Enzyme	Protein (mg)	Total Activity(U)	Specific activityU/mg)	Purification Fold	Yield
Preparation					
Crude extract	450	5,040	11.2	1	100
Ion exchange	190	4,560	24	2.1	90.5
Gel filtration	38	1,398	36.8	3.3	27.7

Table 2. Comparison of IC_{50}s (mg l^{-1}) of crude and partially purified papain (95% Confidence Interval)

Heavy metals	Regression model	Crude papain (Shukor *et al.,* 2006)	R^2	Partially purified Papain (this work)	R^2
Hg	Four-parameter logistic	0.24-0.62	1.00	0.16-0.20	0.978
Pb	One phase binding	1.63-2.69	0.968	0.89-1.23	0.979
Ag	Four-parameter logistic	0.33-0.49	0.987	0.21-0.29	0.991
Zn	One phase binding	1.55-2.67	0.998	1.51-2.63	0.993

DISCUSSION

The protease-based assay for heavy metals is more robust than other enzyme assays for heavy metals such as as the protease assay has very broad optimal pH and temperature for activity and stability, and are also solvent stable[7]. Couple with sensitivity for heavy metals makes the protease assay a prime candidate for biomonitoring of heavy metals. Of the entire protease-based assay studied so far, only papain shows sensitivity towards numerous heavy metals. Papain consists of a single 212 amino-acid chain protein containing seven cysteine residues with six of the residues forming three disulphide bonds while the remaining cysteine-25 providing the active site for the thiol group[12]. Heavy metals such as silver and mercury have been shown to bind to sulfhidryl groups[13].

Unlike other enzyme-based inhibitive assays, the protease assay has a unique advantage. In the absence of heavy metals the colour produced is brownish, whereas in the presence of heavy metals, the colour of the reaction mixture is blue. In the original assay for protease, sulfhydryl group protective agents such as DTT and 2-mercaptoethanol were added to ensure maximum activity[10]. However, these agents will alleviate the binding of heavy metals to the active sites of the proteases, reducing their sensitivity towards heavy metals. Once they were removed, the proteolytic activity was reduced but the sensitivity of the system towards heavy metals is increased several fold[7-9].

Partial purification was achieved as evident from the increase in purification fold. Additional chromatographic steps are probably detrimental towards enzyme yield and stability and were not pursued further as judged by the low yield obtained after gel filtration (Table 1).

The significant improvement in sensitivity is probably due to the removal of potential mercury and silver binding proteins and compounds from the crude preparation[5]. In conclusion, partial purification of commercial crude preparation of enzyme in this works successfully increased the sensitivity of the system towards heavy metals and other works using crude preparation in inhibitive assays could benefit from the results of this work.

ACKNOWLEDGEMENTS

This work was supported by the research grant from the Ministry of Science, Technology and Innovation (MOSTI) under the Science Fund grant no: 02-03-08-SF0061.

REFERENCES

1. Jung, K., Bitton, G., Koopman B. Assessment of urease inhibition assays for measuring toxicity of environmental samples. *Water Res.*, 1995; **29**(8): 1929-1933.
2. Krawczyn´ski, T., Moszczyn´ska, M.G., Trojanowicz, M. . Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. *Biosens. Bioelectron.*, 2000; **15**: 681-691.
3. Villatte, F., Marcel, V., Estrada-Mondac, S., Fournier, D. Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. *Biosens. Bioelectron.*, 1998; **13**: 157-162.
4. Tham, L.G., Perumal, N., Syed, M.A., Shamaan, N.A., Shukor, M.Y. Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticide. *J. Environ Biol.,* 2009; **30**: 135–138.
5. Masdor, N.A., Said, N.A.M. Partial purification of crude stem bromelain improves it sensitivity as a protease inhibitive assay for heavy metals. *Aust. J. Basic Appl. Sci.*, 2011; **5**(10): 1295-1298.
6. Shukor, M.Y., Bakar, N.A., Othman, A.R., Yunus, I., Shamaan, N.A., Syed, M.A. Development of an inhibitive enzyme assay for copper. *J. Environ Biol.,* 2009; **30**(1): 39-44.
7. Shukor, Y., Baharom, N.A., Rahman, F.A., Abdullah, M.P., Shamaan, N.A., Syed, M.A. Development of a heavy metals enzymatic-based assay using papain. *Anal. Chim. Acta*, 2006; **566**(2): 283-289.
8. Shukor, M.Y., Masdor, N., Baharom, N.A., Jamal, J.A., Abdullah, M.P.A.,Shamaan, N.A., Syed, M.A. An inhibitive determination method for heavy metals using bromelain, a cysteine protease. *Appl. Biochem. Biotechnol.*, 2008;**144**(3): 283-291.
9. Shukor, M.Y., Baharom, N.A., Masdor, N.A., Abdullah, M.P.A., Shamaan, N.A., Jamal, J.A., Syed, M.A. The development of an inhibitive determination method for zinc using a serine protease. *J. Environ Biol.,* 2009; **30**(1): 17-22.
10. Burokerkilgore, M., Wang, K.K.W. A coomassie brilliant blue G-250-based colorimetric assay for measuring activity of calpain and other proteases. *Anal. Biochem.*, 1993; **208**(2): 387-392.
11. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein, utilising the principle of protein-dye binding. *Anal. Biochem.*, 1976; **72**: 248-254.
12. Lowe, G. The cysteine proteinases. *Tetrahedron*, 1976; **32**(3): 291-302.
13. Herblin, W.F., Ritt, P.E. The binding of heavy metal ions by papain. *Biochim. Biophys. Acta,* 1964; **85**: 489-490.