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Partial purification of crude stem bromelain improves it sensitivity as a protease inhibitive assay for heavy metals

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Abstract: The protease inhibitive assay for heavy metals is a novel and robust detection method suitable for preliminary screening method for detecting heavy metals in agriculture and environment samples. Stem bromelain has been previously showed to be sensitive towards mercury and copper. However, commercial preparation is in crude form and contains possible interfering entities. Using ion exchange and gel filtration chromatography, a 5.6 fold purification was achieved. The partially purified fraction showed significantly lower IC_{50}s ($p<0.05$) for both copper and mercury than the crude fraction demonstrating that partial purification improves the sensitivity of the bromelain inhibitive assay for heavy metals.

Key words: Bromelain, partial purification, heavy metals.

INTRODUCTION

Heavy metals are toxic elements that exert their toxicity mainly via binding to sulfhydryl group of enzymes and inactivating the enzymes. Their detection in food and drinking waters are costly and have led to the development of simple inhibitive enzyme assays systems as a preliminary biomonitoring tool (Hu, 1998). Numerous enzymes have been used for inhibitive determination of heavy metal traces, e.g. peroxidase, xanthine oxidase, invertase, glucose oxidase, isocitric dehydrogenase, the proteases trypsin (S□afar□ík *et al*., 2002), and urease (Jung *et al*., 1995: Krawczyn'ski *et al*., 2000). Urease is currently being adopted by the USEPA as a biomonitoring system but its major drawback is it suffers from high interference from ammonia from the environment. More recent development on inhibitive assays to overcome the shortcoming of the urease assay is the novel Mo-reducing enzyme assay (Shukor *et al*., 2009a) and the proteases papain (Shukor *et al*., 2006), bromelain (Shukor *et al*., 2008) and trypsin (Shukor *et al*., 2009b) 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. The bromelain assay is more sensitive than the papain (Shukor *et al*., 2006) and trypsin (Shukor *et al*., 2009) assays in detecting mercury and would be the main assay for further development. Commercial bromelain (EC 3.4.22.4) preparation is a crude preparation containing the proteolytic enzymes of pineapple fruit, *Ananas comosus*. In this work, we report on even more sensitive results for the detection of mercury and copper by partially purifying the crude bromelain from the commercial preparation.

MATERIALS 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 ^{o}C unless indicated otherwise.

Preparation of Casein and bromelain solution:

Bradford Coomassie-dye binding assay was prepared according to. Casein and bromelain solution was prepared as follows. 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 5 N HCl. The resulting precipitous solution was incubated overnight with stirring at 60 ^{o}C. The casein stock solution (10 mg ml^{-1}) was initially 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 was measured using the Bradford dye-binding using crystalline bovine serine albumin (BSA, Sigma) as the standard. Bromelain (Sigma), E.C. 3.4.22.32, Lot no 118C-9002, grade 2, 2300 Units/gm was prepared at 4 ^{o}C in 50 mM sodium phosphate pH 6.5 as a 22.1 mg ml^{-1} stock solution. Bromelain (1.0 mg ml^{-1}) and casein (0.3 mg ml^{-1}) working solutions were prepared fresh daily by diluting in 100 mM phosphate buffer pH 6.5.

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Partial purification of bromelain:

Bromelain was partially purified using chromatography on the cation exchange chromatography CM-Sephadex C-50 at pH 4.5 and gel filtration on Zorbax GF-250 (Agilent). About 0.5 g of bromelain was dissolved in 5 ml of 50 mM acetate buffer pH 4.5. A column of CM-Sephadex 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 (buffer A) until the eluant had a pH of 4.5. The bromelain sample was loaded and and washed with three column volumes using buffer A and 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 7.5 and 150 mM NaCl at a flow rate of 1 ml per min. The fraction containing activity was used for inhibitive heavy metals assay.

Bromelain Assay:

Bromelain inhibition studies were carried out as follows. The inhibition assay is based on the assay developed by Shukor *et al*. (2006) which is modified from Burokerkilgore and Wang (1993). One unit of bromelain activity is defined as the amount of casein (in mg) hydrolyzed per minute by 1 mg of protease under the specified assay conditions Burokerkilgore and Wang (1993). The inhibitive heavy metals assay is as follows. In an Eppendorf tube, 20 □l of bromelain (final concentration 0.11 mg ml^{-1}) from the stock solution was added with 10 to 100 □l of heavy metals or xenobiotics from stock solutions initially dissolved in phosphate buffer pH 6.8. The mixture was incubated for 15 minutes at room temperature. This was followed the addition of 50 □l of casein to a final concentration of 0.25 mg ml^{-1}. The final volume was made up to 200 □l using deionized water. About 20 □l aliquot was withdrawn and mixed with 200 □l of Bradford dye-binding reagent in a microplate well and incubated for 5 minutes. The remaining solution was incubated at 40 ^{o}C for 30 minutes. After this incubation period, a 20 □l aliquot was again taken and treated in the same manner with the aliquot at time zero. 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 bromelain optimization studies were from Shukor *et al*., 2008. Protein (casein) was assayed according to the dye-binding method (Bradford, 1976). Means and standard errors were determined according to at least three independent experimental replicates.

RESULTS AND DISCUSSION

Table 1 shows that the partial purification was successful and a 5.6 fold purification was achieved. Proteolytic activity was highest in fraction between 30 and 40 that eluted at approximately 0.125 M NaCl and several smaller peaks showed significant but weak proteolytic activity (Figure 1). 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 both copper and mercury in the previously published work (Shukor et al., 2008) and a repeat of the same experiment showed good agreement with no significant difference between the IC_{50} values ($p>0.05$). The partially purified fraction showed significantly lower IC_{50}s ($p<0.05$).

Table 1: Purification table of the partial purification of bromelain.

Enzyme Preparation	Protein (mg)	Total Activity (U)	Specific activity U/mg)	Purification Fold	Yield
Crude extract	500	18,000	36	1.0	100.0
CM-Sephadex	120	8,280	69	1.9	46.0
GF-250	24	4,800	200	5.6	26.7

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

Heavy metals	Regression model	R^2	Crude bromelain (Shukor et al., 2008)	Crude Bromelain (this work)	Partially purified Bromelain (this work)
Hg	Four-parameter logistic	0.973-0.999	0.13 to 0.16	0.132 to 0.164	0.09 to 0.115
Cu	One phase binding	0.997-0.999	0.1631 to 0.3048	0.172 to 0.322	0.07 to 0.112

Discussion:

An advantage of the inhibition determination of heavy metal using papain or bromelain as an enzyme with coomassie blue detection of inhibition is that 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 as described by Bickerstaff and Zhou (1993) and Burokerkilgore and Wang (1993) for papain and bromelain,

chelants such as EDTA and EGTA as well as sulfhydryl group reducing agents such as DTT and 2-mercaptoethanol were added to ensure maximum activity. However, these need to be removed as these agents are known to chelate heavy metals and preventing access of heavy metals to the active sites of the proteases. Although the proteolytic activity was reduced, the sensitivity of the system towards heavy metals is increased several fold (Shukor *et al*., 2006; 2008;2009). The purification profile showed several proteolytic activities in agreement with previous works that show the presence of several cysteine proteinases in stem bromelain (Rowan *et al*., 1990).

The significant improvement in sensitivity is probably due to the removal of potential copper and mercury binding proteins and compounds from the crude preparation. 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.

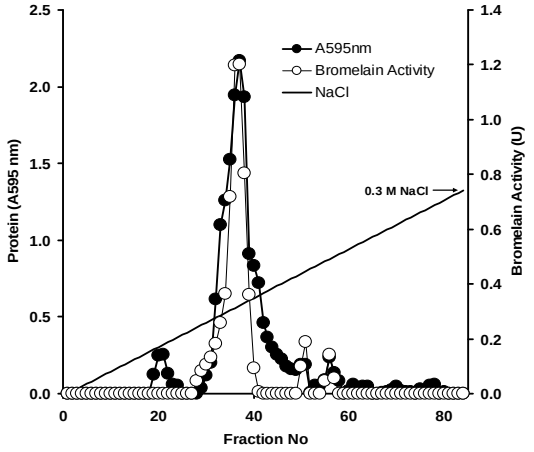

Fig. 1: Elution profile of bromelain on CM-cellulose.

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