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Inhibition of Enzymatic Browning During the Extraction of a Milk Coagulating Protease from *Streblus asper* (Kesinai)

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Abstract

Streblus asper (Kesinai) leaf contains a protease, which can be used to coagulate milk. This extract however, has a undesirable, very dark brown colour due to enzymatic browning. Several browning inhibitors were used in this study with the objective of preventing browning during extraction of the protease. Ascorbic acid at 10 mM concentration reduced browning and polyphenol oxidase (PPO) activity by 41 percent and 10 percent. The protease specific activity and coagulation activity were 88 percent and 143 percent. Citric acid at 10 mM concentration reduced browning and PPO activity by 64 percent and 72 percent. However, it has reduced protease and milk coagulation activity by 76 percent and 65 percent, respectively. L-cysteine inhibited browning and the minimum threshold was found to be 5 mM. It improved protease activity and milk coagulation activity, but did not inhibit PPO activity. Sodium metabisulphite was found to be more effective inhibitor of *Streblus asper* browning and the minimum threshold for inhibition was 2 mM. Metabisulphate treated extract has higher protease activity, milk coagulation activity and lower PPO activity compared to extracts prepared using other chemicals and to the control.

Introduction

The leaf extract of *Streblus asper* (Kesinai) contains a milk coagulating protease (Manap *et al*., 1992) which could be used as a rennet substitute. However, homogenisation of *Streblus asper* leaves to extract the protease results in a rapid formation of a very dark brown solution. The browning of *Streblus asper* leaf extract causes an objectionable brown colour of the milk coagulum and an unpleasant taste. Enzymatic browning results from oxidation of phenolic compounds by polyphenol oxidase, which produces quinones that polymerise among themselves or react with other compounds to produce dark brown pigments (Eskin *et al*., 1971). Polyphenol oxidase (EC 1.14,18.1) is a copper-containing enzyme which catalyses the orthohydroxylation of monophenols and the subsequent oxidation of o-diphenols to o-quinones (Valero *et al*., 1991). During extraction of plant tissues, endogenous phenolics are oxidised to quinones, condensed tannins and brown pigments (Anderson, 1968). The quinones formed during extraction of plant tissues are highly reactive substances which react further with other quinones, amino acids, peptides and proteins, thus altering structural and functional properties of proteins and their nutritive value (Garcia-Carmona *et al*., 1988). The oxidation products of phenolic compounds also reduce enzyme activity (Van Driessche *et al*., 1984). Preventing oxidation of phenols to quinones protects enzymes from inactivation during extraction from tissues rich in polymerised phenols (Anderson and Rowan, 1967). Prevention of the enzymatic browning of *Streblus asper* leaf extract is important for. maintaining protease activity and improvement of colour and flavour of the resulting milk coagulum. Prevention of enzymatic browning can be achieved by inhibition of polyphenol oxidase (PPO), complexing or reduction of quinones or by exclusion of one or all of the substrates. This paper reports on the use of L-cysteine, ascorbic acid citric acid and sodium metabisulphite to prevent the enzymatic browning of *Streblus asper* leaf extract.

Materials and Methods

Streblus asper (Kesinai) leaves were obtained from several plants grown within University Putra Malaysia, research park.

Experiment 1

The effect of 10 mM L-cysteine, citric acid, ascorbic acid and sodium metabisulphite on browning of *Streblus asper* leaf extract was studied. Four different leaf extracts was prepared by homogenisation of leaves in 10 mM Tris-HCl buffer, pH 7.2 (1/10, fresh w/v) including 10 mM of each of the above chemicals. A control was prepared with buffer only. Crude leaf extract was obtained by filtration through a muslin cloth and the filtrate then centrifuged at 10,000 rpm for 30 min. at 4°C. The supernatant was used measure colour, PPO activity, protease activity, coagulation activity and protein content.

Experiment 2

The minimum threshold for inhibition of browning by cysteine and metabisulphite was studied. Leaf extract was prepared in 10 mM Tris-HCl buffer, pH 7.2 including 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 mM of L-cysteine and sodium metabisulphite. Measurement of colour was done directly using Hitachi Model U-1100 spectrophotometer (Hitachi, Japan) at 420 nm against 10 mM Tris-HCl buffer pH 7.2 as the blank and was measured as absorbance unit (AU). Protease activity was determined using azo-case (Sigma Chemicals, USA) as the substrate (0.05%, w/v

(Dosoretz *et al*., 1990). The change in absorbance was measured at 410 nm and the enzyme activity expressed as, 1.0 unit = change of 0.001 AU at 410 nm/min. Protease specific activity unit is expressed as unit/mg protein. Boiled extract was used as the enzyme control. Milk coagulation activity (MCA) was determined by measuring the time taken by the leaf extract to coagulate a 12.5 percent reconstituted milk. The sample (2.0 ml) was per-incubated at 65°C for 5 min. after which 0.2 ml leaf extract was added. The time taken to form the first visible sign of milk coagulation was recorded as milk coagulation time and the data was expressed as min/mg protein.

PPO activity was determined spectrophotometrically (Benjamin and Montgomery, 1973) by measuring the absorbance at 412 nm with Hitachi Model U-1100 spectrophotometer. The sample cuvette contained 1.0 ml of 0.2 M phosphate buffer (pH 7.0), 1.0 ml 10 mM 4-methyl catechol and 1.0 ml crude extract. The reference cuvette contained 2.0 ml of 0.2 M phosphate buffer (pH 7.0), 1.0 ml 10 mM 4-methyl catechol. Mixtures were incubated for 2 min at room temperature (26°C) and absorbance was measured. One unit of PPO activity was defined as the amount that caused a change of 0.001 AU/min. Specific activity unit expressed as unit/mg protein. Protein content was estimated using Bio-Rad protein macroassay procedure with bovine serum albumin (BSA) as standard. This method is based on the absorbance shift of Coomassie brilliant blue G-250 from 465 to 595 nm when binding to protein occurs (Bradford, 1976).

Results and Discussion

The effect of L-cysteine, citric acid, ascorbic acid and metabisulphite at 10 mM concentrations is shown in Table 1. Citric acid at a concentration of 10 mM reduced browning and PPO activity of *Streblus asper* extract to 0.329 and 0.630, respectively. However, it has largely reduced protease and milk coagulation activity to 0.550 and 76.8 min. respectively. It also reduced pH of the extract to 4.5, a value at which *Streblus asper* protease was reported to be unstable (Manap *et al*., 1992). Citric acid acts as a copper chelating agent and also as an acidulent. Both functionalities inhibit PPO (McCord and Kilara, 1983). Ascorbic acid at a concentration of 10 mM resulted in an extract that browned after a lag period with a colour reading of 0.639 and PPO specific activity units of 2.010. Protease specific activity and milk coagulation activity were 2.00 and 18.8. Ascorbic acid inhibits browning by reduction of quinones back to phenolic compounds before they can undergo further reaction to form pigments (Sapers, 1993). However, once added, ascorbic acid can be completely oxidised to DHAA by this reaction and quinones can accumulate and undergo browning. In addition, DHAA can brown as well. L-cysteine at 10 mM concentration inhibited the enzymatic browning of *Streblus asper* crude enzyme extract. However, it did not inhibit PPO activity as PRO specific activity of its extract is 1.860 units while that of the control is 2.230. The extract obtained using L-cysteine has a protease specific activity of 2.93, which is higher than that of the control. The effect of L-cysteine in inhibition of enzymatic browning was thought to be due to the formation of thiol-conjugated reaction products rather than to the inhibition of enzymatic activity (Prota, 1980). However, Valero *et al*. (1992) suggested that L-cysteine is an irreversible inhibitor of polyphenol oxidase, rendered unstable in the reaction mixture by the enzymatic catalysis. It binds to the oxy form of the enzyme, an intermediate in the catalytic turnover. In addition, it reacts with o-quinone product of enzymatic catalysis, thus preventing its appearance, leading to formation of more stable colourless products.

Sodium metabisulphite at a concentration of 10 mM in the extraction buffer, inhibited enzymatic browning, substantially reduced PPO activity and increased protease specific activity. Readings of 0.128, 0.390 and 2.95 units were recorded for colour as absorbance at 420 nm, PPO specific activity and protease specific activity, respectively. Inhibition of enzymatic browning by sulphite is suggested to be due to formation of quinone-sulphite complexes and the inactivation of PPO (Embs and Markakis,1965).

Table 1: Effect of 10 mM Ascorbic acid, Citric acid, L-cysteine and Sodium metabisulphite on browning of *Streblus asper* (Kesinai) leaf extract

Treatment	Colour (abs 420 nm)[a]	PPO Specific activity (units)[b]	Protease specific activity (units)[c]	MCA (min/mg protein)[d]
Control	1.077	2.23	2.27	27.0
Ascorbic acid	0.639	2.01	2.00	18.8
Citric acid	0.329	0.63	0.55	76.8
L-cysteine	0.124	1.86	2.93	17.8
Metabisulphite	0.128	0.39	2.95	17.5

[a]Colour measured spectrophotometrically as absorbance units at 420 nm against 10 mM Tris-HCl, pH 7.2 as the blank. AU >0350 indicate a visibly brown extract; [b]One unit is the amount that causes a change of 0.001 AU at 412 nm/min/mg protein; [c]One unit is expressed as a change of 0.001 AU at 410 nm/min/mg protein, using azo-casein (0.05%, w/v) as the substrate; [d]Time (min) taken to coagulate 2.0 ml of reconstituted milk (12.5%, w/v) at 65°C/mg protein

Table 2: Effect of L-cysteine at different concentrations on browning of *Streblus asper* (Kesinai) leaf extract

Concentration (mM)	Colour (abs 420 nm)[a]	PPO specific activity (units)[b]	Protease specific activity (units)[c]	MCA (min/mg protein)[d]
0.0	1.081	2.235	2.27	26.0
0.5	0.962	2.220	2.46	23.5
1.0	0.854	2.205	2.51	23.0
2.0	0.777	2.190	2.71	20.3
3.0	0.531	2.184	2.86	18.2
4.0	0.378	2.175	2.89	18.1
5.0	0.293	2.160	2.91	18.0
10.0	0.132	1.870	2.92	17.8

[a]Colour measured spectrophotometrically as absorbance units at 420 nm against 10 mM Tris-HCl, pH 7.2 as the blank > 0.350 indicate a visibly brown extract.; [b]One unit is the amount that causes a change of 0.001 AU at 412 nm/min/mg protein.; [c]One unit is expressed as a change of 0.001 AU at 410 nm/min/mg protein, using azo-casein (0.05%, w/v) the substrate.; [d]Time (min) taken to coagulate 2.0 ml of reconstituted milk (12.5%, w/v) at 65°C/mg protein

Table 3: Effect of sodium metabisulphite at different concentrations on browning of *Streblus asper* (Kesinai) leaf extra

Concentration (mM)	Colour (abs 420 nm)[a]	PPO specific activity (units)[b]	Protease specific activity (units)[c]	MCA (min/mg protein)[d]
0.0	1.081	2.235	2.27	26.0
0.5	0.667	2.150	2.84	19.9
1.0	0.519	1.930	2.87	19.5
2.0	0.138	1.140	2.89	18.8
3.0	0.126	1.065	2.91	18.7
4.0	0.118	0.960	2.94	17.9
5.0	0.114	0.805	2.95	17.6
10.0	0.103	0.395	2.97	17.4

[a]Colour measured spectrophotometrically as absorbance units at 420 nm against 10 mM Tris-HCl, pH 7.2 as the blank > 0.350 indicate a visibly brown extract; [b]One unit is the amount that causes a change of 0.001 AU at 412 nm/min/mg protein; [c]One unit is expressed as a change of 0.001 AU at 410 nm/min/mg protein, using azo-casein (0.05%, wlv) as substrate; [d]Time (min) taken to coagulate 2.0 ml of reconstituted milk (12.5%, w/v) at 65°C/mg protein

Sayavedra-Soto and Montgomery (1986) reported that mechanism of inhibition of enzymatic browning by sulphite is the formation of PPO-SO_3 complex and formation of a complex between quinones and sulphite. Detection of some PPO activity in metabisulphite treated *Streblus asper* extract (pH 7.2) was in agreement with Sayavedra-Soto and Montgomery (1986) who found that sulphite did not inhibit PPO at pH levels equal or greater than 7 unless the concentration was increased above 2.0 mg/ml. This suggests that inhibition of enzymatic browning of *Streblus asper* leaf extract is due to formation of quinone-sulphite complex and partial inhibition of PRO.

Comparison of L-cysteine and sodium metabisulphite at seven concentration levels in the extraction mixture (Table 2 and 3) showed that the minimum threshold for inhibition of enzymatic browning by L-cysteine is 5 mM (Table 2) while that for metabisulphite is 2 mM (Table 3). Extract prepared using metabisulphite has high protease activity compared to those prepared using L-cysteine. Beneficial effect of metabisulphite during extraction of leaf enzymes is reported by Anderson and Rowan (1967) who found that metabisulphite at 10 mM concentration gave optimal peptidase activity and appeared to inhibit polyphenoloxidase permanently. Loomis (1974) reported that addition of 10 mM metabisulphite doubled the activity of puleg reductase when extracted from peppermint leaves. It thus concluded that metabisulphite was a more effective inhibitor of the enzymatic browning of *Streblus asper* extract than L-cysteine, ascorbic acid and citric acid. It also the most effective inhibitor in maintaining *Streblus asper* (Kesinai) protease activity.

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