International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 1 (2): 131-135, 2006
ISSN 1811-9743

Factors Affecting Milk Coagulating Activities of Kesinai *(Streblus asper)* Extract

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Abstract: In the present study, a milk coagulating enzyme was extracted from kesinai (*Streblus asper* Lour) leaf with Tris-HCL buffer in Biotechnology Laboratory at University Putra Malaysia during the year 2003. The effects of extraction pH, coagulation temperature, $CaCl_2$ and enzyme concentrations on the milk coagulating activities of kesinai leaf crude extract were evaluated. The maximum enzyme activity was observed at extraction pH 7.2 and 65°C. The presence of $CaCl_2$ up to 6 mM decreased the milk clotting time. Higher salt concentrations, however, giving rise to harder coagulum-like gel. The milk coagulation activity was improved predominantly by increasing the volume of crude enzyme solutions up to 60 μL.

Key words: $CaCl_2$, enzyme, kesinai, pH, temperature

Introduction

Milk clotting is the result of two processes involving the attack on the κ-casein of the casein micelles by the proteolytic enzymes contained in rennet and the clotting of the micelles, which have been destabilized by this enzymatic attack (Picón *et al*., 1994). In the dairy industry, rennet is the main milk-clotting enzyme. The enzymes occur in animal tissues, higher plants and microorganisms. Rennet from animal sources (especially extracted from animal stomach) is relatively expensive to produce whereas rennet from microbial sources is often associated with proteolytic enzymes, which cause peptonization of curd and hence, its dissolution. The use of animal rennet is limited due to religious reasons (e.g., Judaism and Islam), diet (vegetarianism), or being genetically engineered foods (e.g., Germany and Netherlands) (Luisa *et al*., 2003). Application of plant coagulants allows target cheese production and hence on whom restrictions are imposed by use of animal rennet (Gupta and Eskin, 1977). Therefore, attention has been focused on the search of milk-clotting enzymes from plant sources for use as rennet substitutes.

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Kesinai or scientifically known as *Streblus asper* Lour inhabits various Asian countries, such as India, Southern China, the Philippines and Malaysia. Studies have shown that kesinai has been used for several pharmaceutical purposes, e.g., the bark extract has been used in fever, dysentery, relief of toothache and antigingivitis (Gaitonde *et al*., 1964). The leaf extract has been shown to possess insecticidal activity towards mosquito larvae (Kritsaneepaiboon, 1989). Taweechaisupapong *et al*., (2000) reported the antistreptococcal activity of mouth rinse containing kesinai leaf extract. In Malaysia the leaf extract of this plant is reported to contain a milk coagulating protease, which could be a potential rennet substitute (Manap *et al*., 1992).

Therefore, the present research was undertaken to investigate the effect of extraction pH, temperature, $CaCl_2$ and enzyme concentrations on milk coagulating activity of kesinai leaf extract.

Materials and Methods

Twenty gram each of kesinai leaves that were collected from Taman Pertanian University Putra Malaysia during the year 2003 were washed several times with distilled water and homogenized in a blender for 2 min at room temperature in 200 mL of buffer with different pH range (6.0 to 8.0). Buffers used were 100 mM phosphate buffer for pH 6.0 to 7.0 and 100 mM Tris-HCl buffer for pH 7.2 to 8.0. The homogenates were filtered through muslin cloth and the filtrate centrifuged at 11872 G for 30 min at 4°C using a high-speed refrigerated centrifuge (Sigma 3K 30). The supernatants were collected and used as the crude enzyme extract.

The crude leaf extract was ultrafiltered and concentrated 10 fold at room temperature with 10 KDa (43 mm) or (76 mm) disc membranes (Amicon, USA), using stirred cell Amicon 8050, for 43 mm membrane and Amicon 8400 for 76 mm membrane. Gas pressure was 8psi. Retentates (R) and Filtrates (F) were collected separately and stored at 4°C till assayed. Protein content was determined according to the method described by Bradford (1976) using bovine serum albumin as standard. The changes in color were measured directly using Hitachi Model U-1100 spectrophotometer (Hitachi, Japan) at 420 nm. For each of the crude extracts that were extracted using different buffer pH, the respective buffer solution was used as the blank.

Milk coagulating activity were measured by added 200 μL of kesinai leaf extract was added to each 2.0 mL of substrate. The substrates were prepared by reconstituting 12.5% (w/v) skim milk with distilled water and allowed it to settle at 25°C for 4 h. The pH of the milk was adjusted to 6.7 for all the experiments. Test tube was tilted at 45°C and the time taken to form the first visible sign of milk coagulation incubated at 70°C was defined as milk coagulation time. One unit milk coagulation activity is that which coagulate 1 mL milk in 1 min under the assay conditions and specific milk coagulation activity is activity unit/mg protein. Boiled enzyme that heated to 100°C was used as the control.

The effect of extraction buffer pH (6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0) of the crude extract on the coagulating activity was determined. The crude extract with optimum extraction buffer pH was then used for the other experiments. The effect of temperature on the coagulating activity of crude extract was determined by incubating the mixture of crude extract at optimum extraction buffer pH and milk sample at different temperatures ranging from 20-95°C. The milk coagulating times were determined.

The effects of activator such as $CaCl_2$ at different concentrations (1, 2, 4, 6, 8 and 10 mM), on the enzyme activity were determined by pre-incubation with the substrates for 10 min at the optimum temperature of enzyme. The crude enzyme was added and the milk coagulating activity was determined. To determine the effect of enzyme concentrations, the crude leaf extract was added in the range of 10-100 μL to the milk samples (pH 6.7) and the milk coagulation times were determined.

Results and Discussion

The results indicate that the milk coagulating activity retained its activity at pH 7.2, but the activity decreased outside this pH range. The first visible sign was recorded as milk coagulating time. The clotting time decreased because the higher level of proteolysis of κ-casein (Lopes *et al*., 1998). Plant coagulants share many features with chymosin; they are aspartic proteinases, they hydrolyze the Phe_{105}-Met_{106} bond of κ-casein and have a similar catalytic coefficient (K_{cat}/K_m) towards κ-casein (Macedo *et al*., 1993). The optimum milk coagulating activity was found at pH 7.2 with 1.32 unit/mg. The milk coagulating activity decreased 62 and 61% when the protease was extracted at pH 8.0 and 6.0, respectively (Table 1). This result agreed with Daviau *et al*. (2000) who found an interactive effect between pH and ionic strength on coagulation parameters.

The color changed of color was measured at 400 nm by a spectrofotometric procedure. Table 2 shows the color of crude extract increased by 75.38% with the increasing of extraction buffer pH up to 8.0. This result was agreed with Vamos-Vigyazo (1981) which stated that polymerization of *o*-quinones to high molecular weight complexes is higher in the alkaline region as the darker the color the higher the molecular mass of the complexes.

The milk coagulating activity was optimum by 0.7 unit/mg at 70°C. Milk coagulating activity decreased by 95.71%, for 30 and 85.71°C, respectively (Table 3). Generally, this data is in agreement with that reported by Hashem (2000) where optimum temperatures of milk-clotting enzyme produced by *Penicillium oxalicum* are in the range of 65 to 75°C. These optimum temperatures are higher than that of calf rennet, which is 42°C.

Table 1: Effect of extraction buffer pH on specific milk coagulating activity of the crude extract

Extraction buffer pH	Specific milk coagulating activity (Unit/mg)	SD
6.0	0.52	±0.09
6.2	0.82	±0.12
6.4	0.84	±0.06
6.6	0.87	±0.08
6.8	0.96	±0.11
7.0	1.17	±0.09
7.2	1.32	±0.03
7.4	1.10	±0.10
7.6	0.95	±0.08
7.8	0.56	±0.13
8.0	0.50	±0.07

Values are mean±SD of triplicate measurements, *One unit milk coagulating activity is that which coagulate 1 mL milk in 1 min under the standard assay conditions and specific milk coagulation activity is activity unit/mg protein

Table 2: Effect of extraction buffer pH on color of the crude extract

Extraction buffer pH	Color (OD)	SD
6.0	0.65	±0.01
6.2	0.71	±0.01
6.4	0.74	±0.02
6.6	0.76	±0.03
6.8	0.79	±0.05
7.0	0.81	±0.05
7.2	0.85	±0.02
7.4	0.96	±0.04
7.6	1.11	±0.01
7.8	1.13	±0.03
8.0	1.14	±0.04

Values are mean±SD of triplicate measurements

Table 3: Milk coagulating activity was assayed at various temperatures (20-95°C). Milk coagulation activity was assayed using fresh milk (pH 6.7)

Temperature (°C)	Milk coagulating activity (Unit/mg)	SD
20	0.00	±0.00
30	0.03	±0.04
40	0.05	±0.03
50	0.20	±0.03
60	0.60	±0.03
65	0.65	±0.04
70	0.70	±0.02
75	0.53	±0.03
80	0.20	±0.03
85	0.10	±0.05
90	0.00	±0.00
95	0.00	±0.00

Values are mean±SD of triplicate measurements, *One unit milk coagulating activity is that which coagulate 1 mL milk in 1 min under the standard assay conditions and specific milk coagulation activity is activity unit/mg protein

Table 4: Effect of added calcium chloride concentration on milk coagulation time of crude extract

Calcium Chloride (mM)	Milk coagulating time (min)	SD
0	13.00	±0.3
2	10.00	±0.2
4	7.50	±0.43
6	5.00	±0.27
8	4.50	±0.45
10	4.00	±0.33

Values are mean±SD of triplicate measurements, *The first visible sign of coagulation has recorded as milk coagulating time

Table 5: Effect of crude extract concentration on milk

Crude extract concentration (μL)	Milk coagulating time (min)	SD
10	150.00	±0.54
20	70.00	±0.34
40	40.50	±0.23
60	29.50	±0.12
80	16.00	±0.12
100	10.50	±0.12

Values are mean±SD of triplicate measurements, * The first visible sign of coagulation has recorded as milk coagulating time

Generally milk-coagulating activity is enhanced in comparison with the control. The milk coagulating time in the presence of 2.0, 4.0, 6.0, 8.0 and 10 mM calcium chloride concentrations was 23.08, 42.31, 61.54, 65.38 and 69.23% reduced than with salt, respectively (Table 4). It appears that the addition of calcium chloride concentration binds to casein micelles and reduces the repulsive forces between them when kesinai extract was used for milk coagulation. Addition of calcium chloride was also reported to decrease the milk clotting time of calf rennet, rennet-pepsin and *Mucor miehei* rennet (Kowalchyk and Olson, 1979). Ca^{2+} generally associated with casein phosphate and carboxyl groups and increased the H^{+} concentration required to initiate the coagulation of casein. Furthermore, the hydration forces between casein micelles were reduced and allowed attractive hydration forces to cause casein coagulation (Lopes *et al*., 1998).

The result in Table 5 shows a progressive decrease in milk coagulating time with increase in concentration of the crude extract used. The coagulating time decreased with 93% by the increasing of enzyme concentration up to 100 μL. Generally, these data are in agreement with that reported by Ogundiwin and Oke (1983) for Sodom apple extract and De Sa and Barbosa (1972) for cardoon extract

on the reduction of coagulation time with the increase in the enzyme concentration used. Moreover, coagulum firmness increased progressively as the concentration of enzyme increased (Lopes *et al*., 1998).

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