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Factors affecting milk clotting activity of sweet leavening extract involved in coagulation of a yoghurt-like product

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The effects of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $CuCl_2$ and $FeCl_2$), milk pH (pH 6.0–6.6) and milk constituents (casein, lactose, α-lactalbumin and β-lactoglobulin) on the milk clotting activity (MCA) of sweet leavening extract, a well-known by-product from cereal wine-making, were evaluated. The MCA was improved predominantly by salts and $CaCl_2$. On the other hand, the enhancement of the MCA was also increased by casein in combination with α-lactalbumin or β-lactoglobulin, but suppressed by the increasing milk pH. Furthermore, degradation of casein (α-, β- and κ-casein) was observed during incubation with sweet leavening extract by means of sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

In addition to enzymatic coagulation of milk, there are alternative ways to develop a yoghurt-like product which caters to consumers' preference for a low-acid or non-sour tasting yoghurt. The contribution of rennet products to cheese production has recently been confirmed (Fox, 1994). Due to the high cost and reduced supply of animal rennet, microbial or plant rennet-like products may meet the requirement for milk protein coagulation. Sweet leavening extract is made from fermented cooked glutinous rice by taking advantage of the fermentation and hydrolysis potential of residues from cereal wine-making (Wei & Jong, 1983). Wang & Hesseltine (1970) have shown that glucanase, protease, and lipase activities in this extract. It has been utilized to improve the texture, flavour and taste of hard tofu, fish and meat products (Lotong, 1985). Recently, the production of a yoghurt-like milk product, characterized by a smooth, bread-like sweet, alcoholic flavour and soft curd appearance has been obtained by utilizing this sweet leavening extract and has increased interest in the mechanisms affecting milk coagulation activities (Lin *et al.*, 1996). However, the enzyme coagulation of milk products is mainly focused on cheese products and rennet (Fox & Mulvihill, 1990; Dalgleish, 1992). The contribution of microbial enzymes, and the factors affecting milk coagulation, in the process of making a yoghurt-like product are still unknown.

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The objective of this study is to determine the effect of salts, milk pH, and milk constituents on milk clotting activity (MCA) and the phenomena of protein degradation of sweet leavening extract.

MATERIALS AND METHODS

Preparation of sweet leavening extract

One kilogram of glutinous rice was washed with distilled water, drained, then soaked in 1320 ml distilled water for 12 h. After sterilizing this medium and cooling to 35°C, 5 g of wine-making dregs containing, as predominant microflora, *Rhizopus oryzae, Amylomyces rouxii* and *Saccharomycopsis fibuligera* (Hesseltine, 1983) was added, mixed completely, then incubated at 30°C for 6 days. The exudate was collected and filtered through Whatman # 1 filter paper after incubation. The filtrate was stored at 4°C for further analyses.

Determination of milk clotting activity

One ml of sweet leavening extract was added to each 10 ml of substrate. The substrates were prepared by reconstituting 12% (w/v) skim milk with warmed 10-mM phosphate buffer (pH 6.3) and allowing it to settle at 25°C for 4 h. Milk clotting time was defined as the time taken for visible coagulum to appear after the mixture was incubated at 40°C. Milk clotting activity of sweet leavening extract was calculated as MCA =

$(2400 \times F)/t$, where t represents the milk clotting time of sweet leavening extract and F represents the dilution factor of the extract. The factor of '2400' was that clotting time in sec of 1 ml (2 mg/ ml) rennet (Sigma Chem. Co., MO, USA) using reconstituted skim milk mentioned above as substrate.

Effect of milk pH on the MCA of sweet leavening extract

One ml of sweet leavening extract was added to each 10 ml of substrate. The substrate was prepared by reconstituting 12% (w/v) skim milk to various pHs (6.0, 6.1, 6.2, 6.3, 6.4, 6.5 and 6.6) with 10-mM phosphate buffer.

Determination of the MCA of sweet leavening extract affected by various salts

One ml of sweet leavening extract was added to each 10 ml of substrate. The substrate was prepared by reconstituting 12% (w/v) skim milk with 10-mM phosphate buffer (pH 6.3) containing 10 mM of one the following salts: NaCl, KCl, $CaCl_2$, $MgCl_2$, $CuCl_2$ and $FeCl_2$. Each sample was settled at 25°C for 4 h.

Determination of the MCA of sweet leavening extract incubated with various milk constituents

One ml of sweet leavening extract was added to each 10 ml of test substrate. The substrates were prepared by dispersing 12% (w/v) casein, or 6% casein + 6% lactose, or 6% casein + 6% α-lactalbumin, or 6% casein + 6% β-lactoglobulin with warm 10-mM phosphate buffer (pH 6.3).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Each sample was diluted to a protein concentration of 3.0 mg/ml, protein concentration was determined by the Folin–Lowry method (Lowry *et al.*, 1951), bovine serum albumin (Sigma Chem. Co., MO, USA) being used as the standard, mixed with 1/2 volume of 0.5-M Tris–HCl (pH 6.8) containing 14.5% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, 10% sodium dodecyl sulphate and 0.05% bromophenol blue. The mixture was boiled for 5 min before loading samples on the gels. SDS–PAGE was accomplished by the method of Laemmli (1970), using 15% polyacrylamide gels and a Hofer PS 500 XT electrophoresis unit (Hofer Co., USA) at 110 V for 3 h. After electrophoresis, the protein on each gel was stained overnight in 0.01% (w/v) Comassie brilliant blue R 250 (Bio-Rad Co., USA), then destained in methanol–glacial acetic acid-deionized water at a ratio of 4:1:5 (v:v:v).

RESULTS AND DISCUSSION

The effect of salts on the milk clotting activity of sweet leavening extract is shown in Fig. 1. They generally enhance MCA in comparison with the control. The

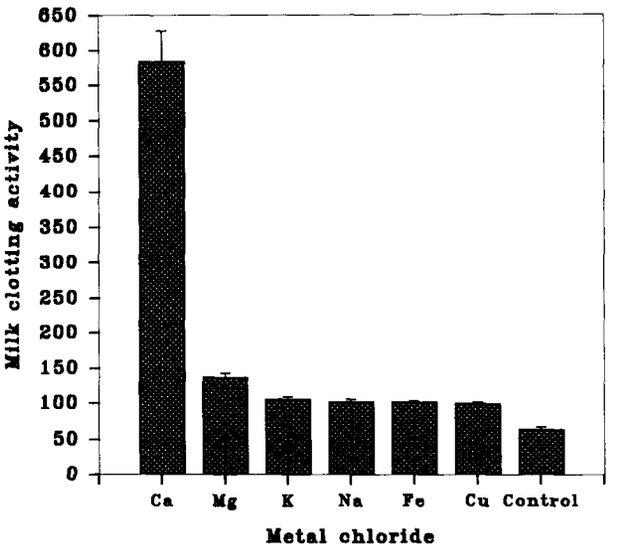

Fig. 1. Effect of salts on the milk clotting activity of sweet leavening extract.

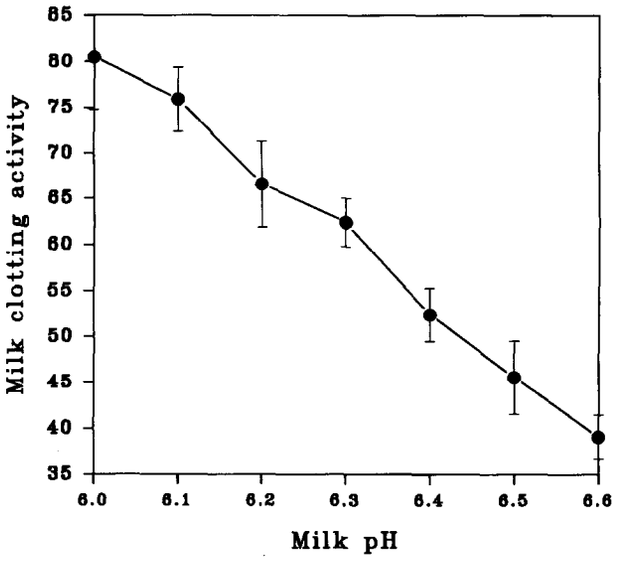

Fig. 2. Effect of milk pH on the milk clotting activity of sweet leavening extract.

MCA in the presence of calcium chloride was 4–5 times greater than with salts. Mg^{2+} and 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 (Bringe & Kinsella, 1991). The effect of milk pH on the MCA of the sweet leavening extract is shown in Fig. 2. The MCA significantly ($P < 0.05$) decreased with increasing milk pH; nevertheless, wheying-off was found in the milk with pH 6.0–6.2 after incubation. Protease activity was related to the substrate pH and the proteolysis of the milk protein was also accelerated by the decreasing substrate pH (Okigbo & Richardson, 1985) and might be attributable to the increase of hydrophobicity of casein, the dissociation of casein micelles, followed by aggregation or coagulation (Dalgleish &

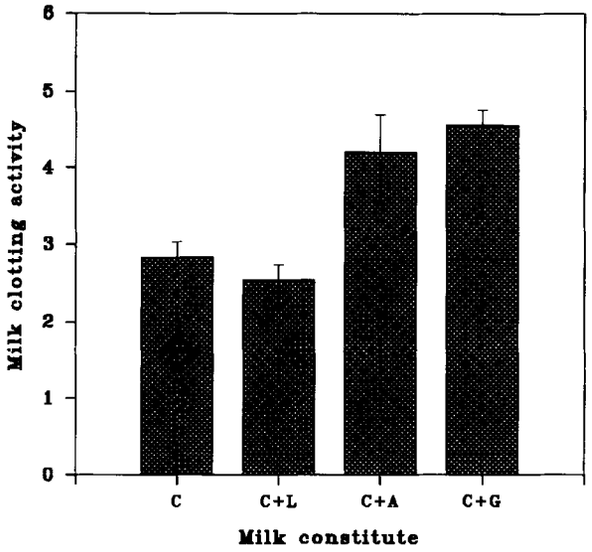

Fig. 3. Effect of milk constituents on the milk clotting activity of sweet leavening extract. C: casein; C + L: casein + lactose; C + A: casein + α-lactalbumin; C + G: casein + β-lactoglobulin.

Law, 1988). The phenomenon of wheying-off might result from increasing the random or partial aggregation of casein, which then forms an unstable coagulum (Rüegg & Moor, 1984). Hence, we suggest that the milk pH for preparing the yoghurt-like product with sweet leavening extract should not have a pH less than 6.3.

The effects of various milk constituents on the MCA of sweet leavening extract are shown in Fig. 3. No significant difference of MCA was observed between sweet leavening extract incubated with casein alone and with lactose. However, the MCA of the extract was significantly ($P < 0.05$) enhanced in the presence of α-lactalbumin or β-lactoglobulin. Enhancement of MCA might result from the stabilization effect that α-lactalbumin and β-lactoglobulin have on certain protease structures (Elfagm & Wheelock, 1978).

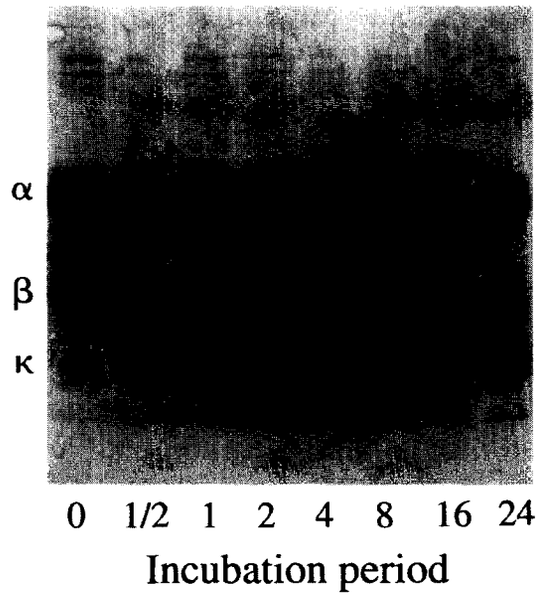

Fig. 4. Electrophoretogram of milk protein incubated with sweet leavening extract. α:α-casein; β: β-casein; κ:κ-casein.

The effect of sweet leavening extract on the casein is shown in Fig. 4. Three types of casein, (α-, β- and κ-casein), were degraded after 1/2 h incubation. The extents of α-casein and β-casein proteolysis increased with incubation and were associated with decrease in density of the protein band on the gel.

On the basis of these results, we suggest that milk coagulation and milk protein hydrolysis activity of the sweet leavening extract might result from the contribution of protease. The isolation and purification of the proteases will be attempted in the future.

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