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Solanum aethiopicum Shum fruits can be used as a source of coagulant in cheese-making

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ABSTRACT

This study was performed to develop a sustainable process for preparing coagulant from *Solanum aethiopicum* Shum (SAS) fruits. Fruit powder of SAS was soaked at different conditions, and obtained extract was used to determine its properties in light to apply it in cheese-manufacturing. Results showed that milk-clotting enzyme was uniformly distributed in coat and seed SAS fruit, and was most active from 6 to 8 weeks maturity regardless Cameroon's agro-ecological zones. SAS extract had the greatest rennet strength with 4% NaCl used as extractant medium. The rennet strength of SAS extract was temperature, pH, calcium and enzyme concentration dependent. The milk-clotting enzyme of SAS extract was more thermosensitive, and lost its activity after wet-heating at 55°C for 10 min. The bovine casein hydrolysis by SAS extract increased hyperbolically with incubation time. SAS extract hydrolyzed essentially all k-casein after about 180 min, and the duration of non-enzymatic phase of coagulation was about 4 min. At low temperature (4°C), SAS extract can be stored without preservative for two months; and citric acid can be used as preservative for long term storage. The estimated yield of curd made with SAS extract was not significantly different from a control curd made with calf rennet.

KEYWORDS

Solanum aethiopicum;
Coagulant;
Rennet strength;
Proteolytic action;
Cheese-making.

INTRODUCTION

The conventional coagulant used in cheese-making is calf rennet, obtained from the abomasa of unweaned calves. Nowadays, the deficit of calf rennet has become chronic (70 to 80%)[1]. This shortage is accentuated in Sub-Saharan Africa, where supply solely depends on importations. Researchers worldwide have

FULL PAPER

discovered calf rennet substitutes from animals and microorganisms. However, the application of these discoveries is limited in Africa by the meat market, religion, diet, biotechnology and public health constraints. As in Roman times, natural coagulants from plant are largely used in this area for cheese production. In this regard, many cheeses are made with plant coagulants such as *Calotropis procera* in West Africa, seven *Papilionoideae* species in Austral Africa, *Cynara scolymus* in North Africa, *Solanum dobium* in East Africa and *Ongokea gore* and *Balanites aegyptiaca* in Central Africa[2-7]. Unfortunately, most of them have been found inappropriate for cheese-making due to excessive proteolysis, which lowers yield and produces bitter flavors in cheese[8]. Therefore, the search for suitable calf rennet substitute continues among local plants. Our previous trials identified the fruits of *Solanum aethiopicum* Shum (SAS) as a potential source of coagulant useful in cheese-making[9].

SAS (scarlet eggplant) is an annual and tropical plant, belonging to the Solanaceae family, and gender *Solanum*. It is a woody herb with a solid erect stem, black in colour, and about 150 to 180 cm high. The leaves are alternate and pale green in colour. Its fruits are round, grouped in clusters on the stem or branch; green in colour when unripe, while the ripe ones are red[10]. However, yellow and orange are intermediate colour. These fruits are a normal ingredient in many beverages and soups, consumed for its nutritional and therapeutic virtues. The juice of these fruits is used to separate whey from curd destined for lactose intolerant patients. The liquid extract from about ten SAS fruits is added to one liter of milk, and incubated at room temperature for 30 min, and then curd is cut, drained and given to lactose intolerant patients, while the whey is treated and given to children. To investigate the possibility of the use of SAS fruits as a source of coagulant suitable in cheese-making, the present work was carried out to examine the best conditions of coagulant preparation, its coagulating and proteolytic properties and the yield of resulting curds.

EXPERIMENTAL

Materials and chemicals

Plant material consisted of SAS fruits harvested from the environs of Ngaoundere, Adamawa Region, Cameroon; during the month of June, 2011. They were sorted, washed, disinfected, dried, hand crushed to separate seeds from coat and ground. The resulted powders were bottled and stored at room temperature until used. Milk from five free grazing *Bos indicus* (zebu cow) herds in the Ngaoundere area was collected and mixed every test day during the month of August, 2011. All analytical reagents were from sigma chemical Co (St. Louis, Mo, USA); the other reagents were of food grade. Plate Count Agar (PCA) and Potatoes Dextrose Agar (PDA) were from Liofilchem, (Roseto, Italy); *Salmonella-Shigella* (SS) agar, from Laboratorios, Conda, (Madrid, Spain); while Eosin Methylene Blue (EMB), Man Rogosa and Sharpe (MRS) and iron sulphite agar, Oxoid S. A. were form Merck, (Darmstadt, Germany).

Methods

Extract preparation process

Different amounts of SAS coats, seeds and whole fruit powders (1, 5, 10, 15, and 20 g) were macerated in 100 ml of distilled water at 4°C for 20 h. 10 g of whole fruits powder at different maturities (1, 2, 4, 6, 8, 10 and 12 weeks) from Cameroon's five agro-ecological zones (Sahel Savannah, Guinea Savannah, Sudan Savannah, Rain Forest and Mangrove Forest) was macerated in 100ml of distilled water under the same conditions of time and temperature. The same amount of whole fruits powder was macerated for 20 h in 100 ml of distilled water within the temperature ranging from 5-45°C (10°C intervals). Then, 10 g of this powder was macerated for 20 hours at 4°C in 100ml of other extractant media: 50 mM citrate (pH 4 and 5), phosphate (pH 6 and 7) and Tris-HCl (pH 8, 9 and 10) buffers; salts (NaCl; KCl and KI) ranging from 1-10% (w/v); 4% NaCl in 50 mM citrate buffer (pH 5.0) and 4% NaCl in 100 ml 50 mM Tris-HCl buffer (pH 8.0). For each experiment, the mixture was filtered through cheese cloth and centrifuged at 7500 rpm for 45 min at 5°C, and the supernatant (SAS extract) stored at 4°C until analysis.

Determination of rennet strength

Rennet strength of SAS extract was determined according to the method described by Chazarra et al.[11],

with slight modifications. Reconstituted milk (12 g of skimmed milk in 100 ml of 0.01 M $CaCl_2$) was used as substrate. The assay was performed by adding 0.1 ml of SAS extract to 1 ml of substrate. The temperature of milk sample was controlled using a water-bath at 35°C, and rennet coagulation time was determined by manually rotating the test tube at short time intervals, and checking visual gel formation. The rennet strength (RS) was defined as the fraction of volumes of coagulated milk clotted per volume of rennet in 40 min at 35°C. The RS is thus given by: RS (U) = 2400* V/ tv, where v equals volume of SAS extract (ml), V volume of milk (ml), and t the rennet coagulation time (s).

Coagulating properties of SAS extract

Enzyme concentration

Enzymatic solutions of were prepared by diluting SAS extract in NaCl solution (4% w/v). Each enzymatic solution was added to the milk sample at 1:10 ratio. The strength of SAS extract was determined as a function the inverse of protein concentration ranged from 2.4 to 23.8 $(mg/ml)^{-1}$.

pH

The pH of milk was adjusted at 35°C by the gradual addition of 0.1M lactic acid/NaOH during rapid stirring. The optimal pH of milk coagulation was determined by observing the strength of SAS extract with pH varying between 6 and 6.8 at 0.2 increments.

Calcium Chloride concentration

Milk substrates supplemented with different concentrations of calcium were prepared using $CaCl_2$: 0, 0.9, 1.8, 2.7 or 3.6 mM (at 0.9 mM intervals). The optimal concentration of $CaCl_2$ was determined by observing the strength of SAS extract with added $CaCl_2$.

Temperature

The optimal temperature of SAS extract was determined within the milk coagulating temperatures ranging from 30-55°C at 5°C intervals.

Thermal stability of extract

Study of thermal stability was performed by wet heating the SAS extract at different temperatures ranging from 35 to 55°C (at 5°C intervals). The remaining activity was determined as function of incubation time ranging from 10 to 60 min under standard assay conditions using reconstituted milk as substrate.

Proteolysis

The protease activity of SAS extract was determined according to the method described by Silva & Malcata[12], with slight modifications. It was quantified by evaluating the peptides soluble in aqueous 5% (w/v) trichloroacetic acid (TCA). 1% Casein (w/v) was subjected to hydrolysis at 30°C in 10 mM citrate buffer (pH 6.2). The hydrolysis was initiated by the addition of 120 µL of SAS extract to 3 ml of substrate solution. At selected times: 5, 10, 30, 60, 120 and 180 min, the reaction was quenched by heating at 100°C for 5 min. Aliquots of each sample were treated with 5% (w/v) TCA at a volumetric ratio of 1:2 respectively. The mixture was allowed to settle for 10 min, and then centrifuged at 3700 rpm for 20 min. The absorbance of the supernatant was measured at 280 nm.

Determination of the primary and secondary phases of coagulation

The primary and second phases of SAS extract coagulation were determined by McSweeney[13] procedure, with slight modifications. Milk substrate was cooled to 15°C and renneted with SAS extract at 10%, and mixture was maintained at 15°C for 200 min. The rennet coagulation time was determined at 30 min intervals according to procedure described by Chazarra et al.[11].

Storage of SAS extract

Sterile bottles containing 5 mL of SAS extract were treated with 1% of preservatives: citric acid (v/v), acetic acid (w/v) or sorbic acid (w/v); NaCl solution (4%) was used as control and stored at 4°C and at room temperature. Samples were removed at 0, 1, 2 and 3 months to count the microorganisms. Samples were serially diluted 10^6 times, pre-reduced in tryptone water and inoculated onto a range of agars designed to be selective for predominant bacteria: total viable count on PCA at 37°C for 48 h, yeast and moulds on PDA at 25°C for 72 h, *Salmonella* spp on SS agar at 37°C for 48 h, *Escherichia coli* on EMB at 44°C for 48 h, *Lactobacillus* on MRS agar at 37°C for 48 h, and *Clostridium* spp on iron sulphite agar at 37°C for 48 h. The dishes were placed in anaerobic jars for *Clostridium* and *Lactobacilli*; and the rest were incubated aerobically.

FULL PAPER

Colony forming units (cfu) were counted in triplicates. The remaining activity was also determined with storage period and preservative treatment.

Yield curd evaluation

Yield was determined following Mercanti et al.[14] procedure, with slight modifications. Five trials of zebu milk curd were carried out on five different days. In each trial, curds were made with calf rennet and SAS extract. Prior to this process, physicochemical properties of zebu milk were determined; then it was standardized to 0.2 g/l $CaCl_2$, and the pH adjusted to 6.40±0.02 using 0.1 M lactic acid. A milk sample was used to adjust enzyme concentration to similar coagulation time. 20 g of milk was transferred into initially calibrated centrifuge tubes, placed in a water bath, and the temperature adjusted to 37°C. One percent of each coagulant was then added, coagulation stage lasted 20 min, the curd obtained was cut manually in an "H" form, syneresis lasted 40 min. The sample was then centrifuged at 3700 rpm for 30 min at 25°C for expulsion of whey. Curd was transferred onto a cheese cloth for further whey drainage. Mass and dry matter content in milk and curd were directly measured. Estimated yield1 (Ye1) was calculated following the formula of Mercanti et al.[14]:

Ye1 (%) = 100*(curd mass/milk mass), and estimated yield2 (Ye2) was determined with the dry matter (g/kg) of the curd, milk and whey was calculated following Formaggioni et al.[15] formula:

Ye2 (%) = 100* (milk dry matter – whey dry matter) / (curd dry matter – whey dry matter).

Statistical analysis

Statistical analysis of the data was carried out using Statgraphics Plus version 5.0 (Statpoint, Inc., Warrenton, VA, USA). One way ANOVA was used for testing statistical significance between different parameters, and individual pair difference was tested by means of Duncan's multiple range tests at 95% level of confidence. Results were presented as means ± standard deviation (SD). Curves were drawn with Sigmaplot version 11.0 (Systat Software Inc).

RESULTS AND DISCUSSION

Extrinsic and intrinsic conditions of extraction

The extraction of milk-clotting enzyme from plant is influenced by intrinsic (biological raw material) and extrinsic factors (initial pH, temperature of soaking, initial salt concentration). Each of these factors affected rennet strength differently, which depends on the releasing and stability of milk-clotting enzyme.

Intrinsic conditions

Effect of plant material

To test the effect of plant dosage on the activity of SAS extract, different amounts (2, 5, 10, 15 and 20%) seed, coat and whole fruits powders of SAS were soaked in distilled water at 4°C for 20 h. Figure 1i shows that the strength of SAS extract increased with plant material dosage up to 10% and remained relatively constant, irrespective of the part of fruit used. The releasing of clotting enzymes gave a hyperbolic tendency with plant material dosage. This could be due to saturation as a result of osmotic pressure[16]. There was no significance difference between the rennet strength of extracts from coat, seed and whole fruits ($P > 0.05$). It can be assumed that milk-clotting enzyme was uniformly distributed in coat and seed fruits. This observation is in contrast with findings of Yousif et al.[3] who reported that milk-clotting enzyme are more located in the seeds of similar species (*Solanum dobium*). Thus, whole fruits powder at 10% was used as plant material dosage throughout for the rest of this study.

Effect of maturity stage

Figure 1(ii) presents the effect of fruit maturity on the rennet strength for each Cameroon's agro-ecological zone. As function of these agro-ecological zones, the strength of SAS extract increased linearly with fruit maturity up to the sixth week and remained stationary till the eighth week, decreased slightly and variably from the eighth to the tenth week and remained constant until the twelfth week. There was no significance difference between the rennet strength of extracts from the five Cameroon's agro-ecological zones ($P > 0.05$). At the sixth week, the fruits were unripe (green in colour), began to ripen (yellow and orange in colour) between eight and ten weeks and finally became ripe (red in colour) from the tenth week of maturity. This suggests that unripe fruits from 6 weeks maturity of this plant are more interesting source of milk-clotting enzyme than the ripe ones, regardless Cameroon's agro-ecological

zones. However, many authors have usually targeted the ripe fruits as sources of plant rennet[17]. This study puts into evidence the maturity stage in the search for coagulant from plant.

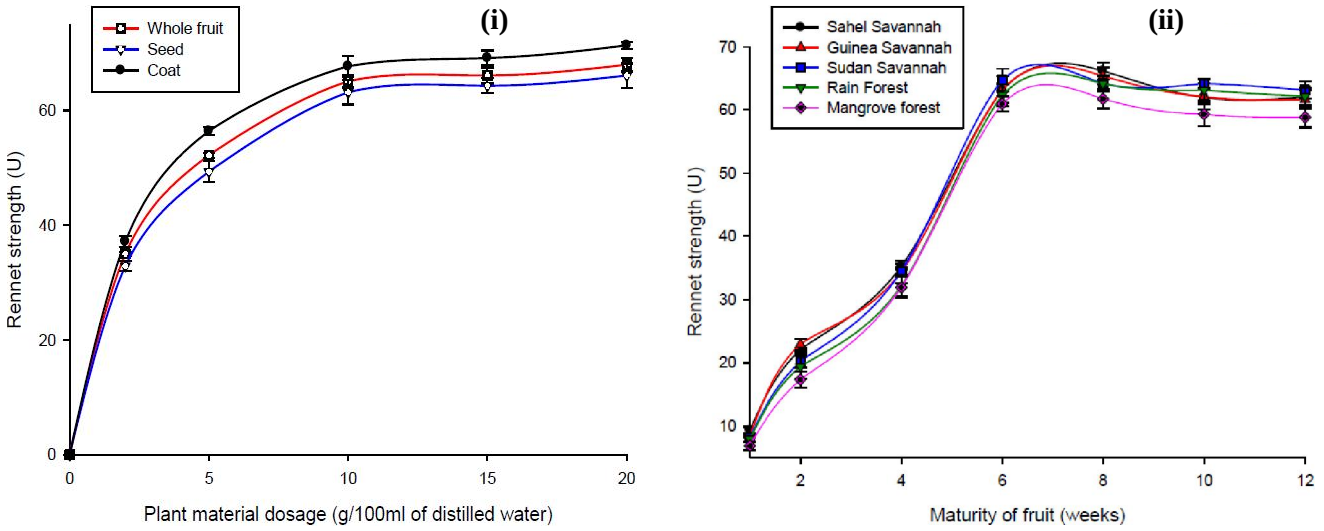

Figure 1 : Variation of the rennet strength of SAS extract with intrinsic extraction conditions: Plant material (i), maturity of fruits for each Cameroon's agro-ecological zone (ii)). SAS fruits powder was soaked in distillated water at 4°C for 20 hours. Rennet strength was determined by adding 100µL of crude extract to 1mL of reconstituted milk at 35°C. Each experiment was repeated five times, and error bar indicating standard deviation.

Extrinsic conditions

Effect of soaking temperature

Extraction of milk-clotting enzymes from plant is often carried out at low temperature[18]. The strength of SAS extract varied with extraction temperature; it was highest at 5°C and then decreased with increasing temperature (Figure 2(i)). The decrease in activity with extraction temperature could be explained either by autolysis[19] or by thermo-sensitivity of milk-clotting enzyme. This suggests that the milk-clotting enzyme of SAS extract was stable and less active at 5°C. However, the remaining activity was up to 70% after 20 h of macerating at 35°C. For practical reasons therefore, extraction can be carried out at room temperature.

Effect of initial salts concentration

Salts are largely used in the agro-food industry because they preserve and impact flavour. For the extraction of milk-clotting enzymes from plant; salts are used to increase ionic strength[16]. Figure 2 (ii) presents the effect of initial concentration of salts (NaCl, KCl and KI) on the strength of SAS extract. The three salts affected the rennet strength of this extract. The greatest strength of SAS extract was observed using 4% NaCl, and milk-clotting enzyme was completely inhibited with KI from 8%. As with other proteases, those from SAS extract are also susceptible to both activation and inhibition by chemicals[20]. Solution of NaCl at 4% enhanced more extractability, stability and activity of milk-clotting enzymes from SAS fruits. Mohamed Ahmed et al.[18] reported the similar merits of this salt in the extraction of milk-clotting enzymes from closely related species (*Solanum dubium*). In contrast, these authors found the best activity with NaCl at high concentration (11.7%). Various salts concentrations have been used for the extraction of milk-clotting enzymes: 0.9%[21], 5%[3,17]. High intake of sodium is harmful. For this reason, KCl can be replaced NaCl in both coagulant and cheese manufacture. In the other hand, Iodine deficiency disorder (IDD) till exists in developing countries. For this reason, 3% KI could be employed in extract preparations for special applications since more than 60% of activity was remained at this concentration.

Effect of initial pH

Solubility, extractability as well as the conformation of the active site of enzyme are pH dependent. pH consideration is of prime importance in the extraction of milk-clotting enzymes from plants[11,16]. Figure 2(iii) shows the effect of initial extraction pH on the strength of SAS extract. Extraction efficiency was higher at neutral and alkaline media (50mM phosphate buffer, pH 7,

FULL PAPER

50mM Tris-HCl buffer, pH 8-9) than in acidic media (50mM citrate buffer, pH 4-6). The best strength was observed when SAS extract was prepared in 50mM Tris-HCl buffer, pH 8.0. However, this strength was lower than that of SAS extract obtained in 4% NaCl. To maximize the extraction process, extraction factors were combined.

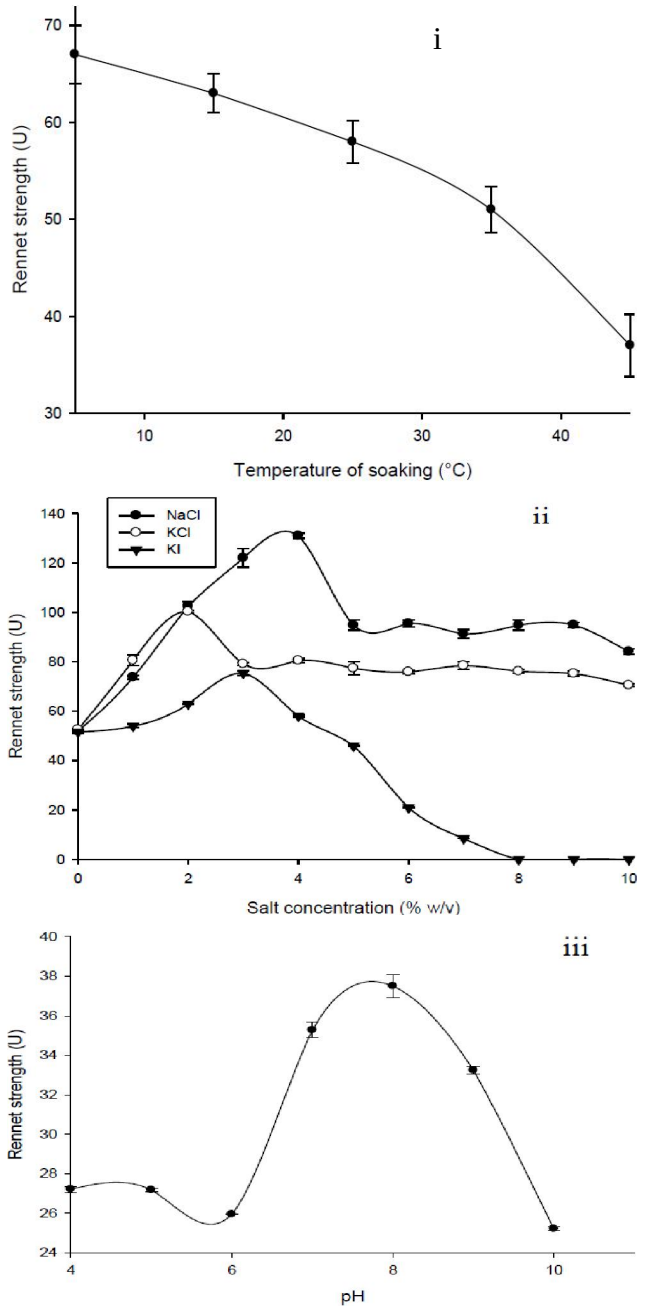

Figure 2 : Variation of the rennet strength of SAS extract with extrinsic extraction conditions: Soaking temperature (i), initial salt concentration (ii) and initial pH (iii). Rennet strength was determined by adding 100µL of SAS extract to 1mL of reconstituted milk at 35°C. Each experiment was repeated five times, and error bar indicating standard

Combined effect of extraction factors

In order to determine the combined effect of extraction factors, the SAS whole fruit powder from Guinea Savannah, NaCl concentration, temperature, maturity were used at 10%, 0 and 4%, 5°C, and from 6 weeks respectively in acidic (pH 5.0) and alkaline (pH 8.0) media. As shown in TABLE 1, the SAS extract strength was significantly different between the extractant media ($P < 0.05$). In the presence of NaCl (4%), SAS extract strength was significantly higher in acidic medium than in alkaline medium ($P < 0.05$). The same observation has been reported in other species of *Solanum*[17]. However, interaction between NaCl and pH was less efficient than 4% NaCl in distilled water used individually. NaCl in distilled water was used as extractant medium throughout the rest of this study. Since NaCl is readily available in Africa, it is an opportunity to develop a sustainable coagulant from SAS fruits. Further studies using response surface methodology would be necessary to optimize coagulant preparation process.

Effect of SAS extract on the milk coagulating properties

Enzyme concentration

As with other proteases, the amount of rennet affected SAS extract strength (Figure 3a). The strength of SAS extract decreased appreciably with the inverse of SAS extract concentration up to 7.9 ± 0.1 $(mg/ml)^{-1}$, and adopted an asymptotic allure. A similar tendency had been observed with enzymatic extract of *Cynara cardunculus* on the ovine and caprine casein[12]. This tendency could be explained by either the lack of speci-

TABLE 1 : Rennet strength of SAS fruits extracted under different conditions

Condition of extraction	Rennet strength (U)
0% NaCl in distilled water	67.2 ± 1.8^{c}
0% NaCl in 50 mM citrate buffer (pH 5.0)	27.2 ± 0.1^{a}
0% NaCl in 50 mM Tris-HCl buffer (pH 8.0)	37.5 ± 0.6^{b}
4% NaCl in distilled water	130.9 ± 1.1^{f}
4% NaCl in 50 mM citrate buffer (pH 5.0)	82.8 ± 0.6^{e}
4% NaCl in 50 mM Tris-HCl buffer (pH 8.0)	72.6 ± 0.7^{d}

Values within the same column bearing different letters are significantly different at 5% level

ficity of milk-clotting enzyme from SAS extract for Phe_{105}-Met_{106} bond or proteolytic activity of other proteases present in the extract[3]. In addition, these proteases also may have initiated general proteolysis which can counteract the aggregation process of milk. Therefore, to use this coagulant in cheese-making, it would be important to understand the effect of other coagulating factors such as pH, calcium chloride concentration and temperature.

Milk pH

The sensitivity to pH depends on the rennet used[22]. These authors have reported the effect of milk pH on rennet coagulation time. According to recent findings, reducing the milk pH significantly increases the rennet strength[11]. The strength of SAS extract followed this tendency (Figure 3b). This can be explained by the fact that the lowering of milk pH favoured solubilization of colloidal calcium phosphate[23]; reduced charge repulsion between micelles and increased extract activity[24]. However, at milk pH below 6.2, solubilization of colloidal calcium phosphate could decrease tension in curd and reduce firmness[25].

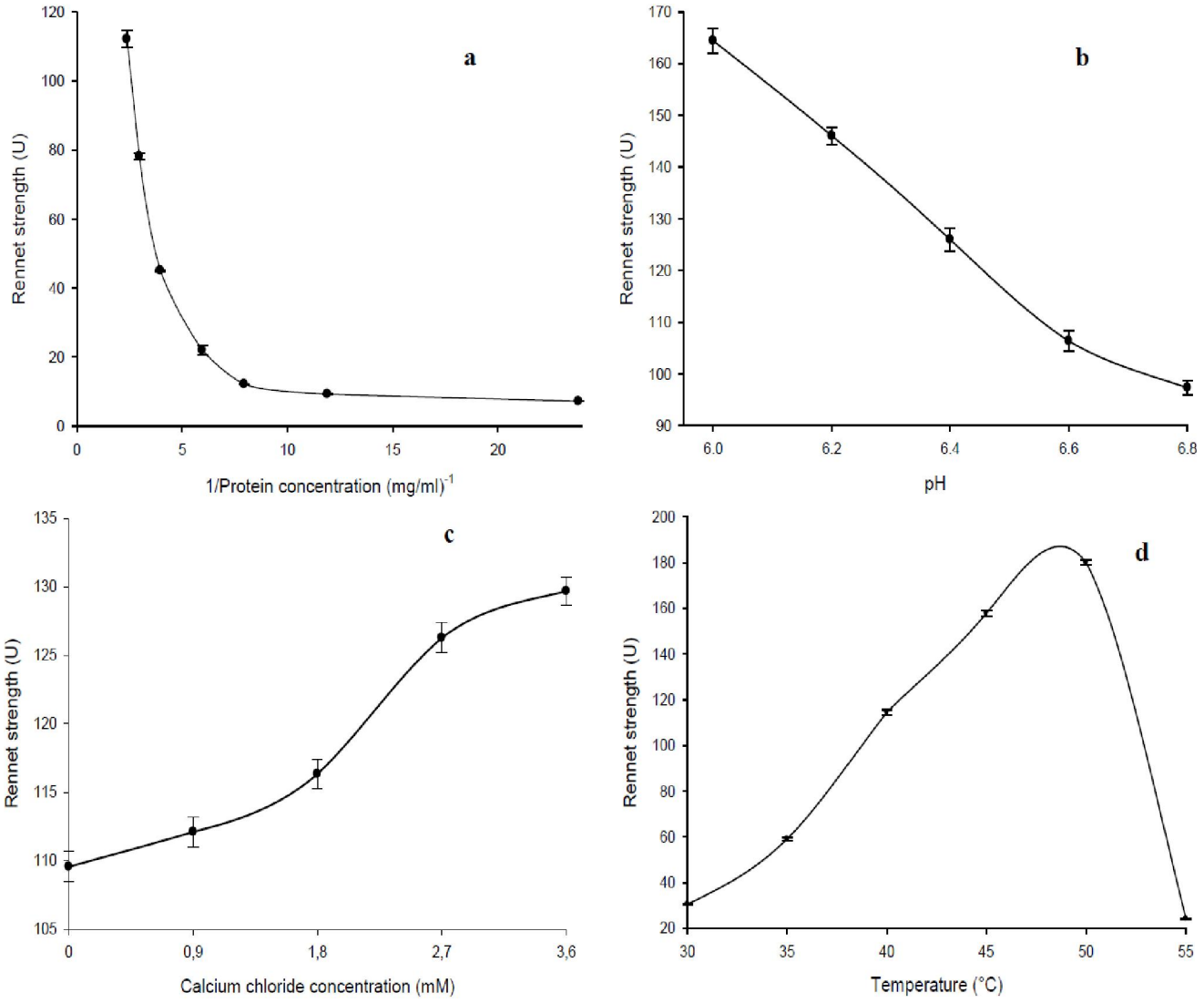

Figure 3 : Effect of various factors on the SAS extract strength: enzyme concentration (a), milk pH (b), calcium chloride concentration (c) and milk temperature (d). Experiments were carried out by adding 100µl of coagulant to 1mL of reconstituted milk. Each experiment was repeated six times, and the error bars represent the standard deviation.

Calcium chloride concentration ($CaCl_2$)

The addition of $CaCl_2$ to milk, which is common practice, promotes rennet coagulation via three beneficial changes: an increase in soluble calcium, colloidal

FULL PAPER

calcium phosphate, and a concomitant decrease in pH[26]. Figure 3c depicts the variation of rennet strength of SAS extract with calcium chloride concentration. With pH adjusted at 6.4, rennet strength increased with calcium chloride: the increase was low between 0 and 1.8 mM; high between 1.8 and 2.7 mM, and became stationary at 3.6 mM. Addition of calcium has positive effect on the firmness and yield of cheese[27].

Milk temperature

The set temperature influences the milk-clotting activity of rennet, and it is of major importance in determining the conditions in cheese-making. The strength of SAS extract varied with setting temperature of milk (Figure 3d). The SAS extract strength increased linearly from 30°C to about 50°C, and decreased drastically with temperatures above 50°C. The principal effect of set temperature is on the secondary, non-enzymatic phase of coagulation, which does not occur at temperatures below around 18°C[13]. A progressive increase in rennet strength as temperature increases from 20 to 40°C has been previously reported[11,28]. Beside the possible denaturation of proteins, another factor responsible for the decreased rennet strength of heated milk is the complex formed between κ-casein and β-lactoglobulin or α-lactalbumin[29].

Thermal stability of SAS extract

Thermal stability of rennet is important when the whey is to be used in food processing[22]. As shown in Figure 4, the strength of SAS extract was affected by temperature and time of wet heating. The milk-clotting enzyme remained fully active when exposed for 60 min at 35°C. For all the other temperatures, after 10 min of incubation, strength decreased. It decreased to 90, 75, 22 and 18% after only 10 min of incubation at 40, 45, 50 and 55°C respectively. SAS coagulant was more thermosensitive than other vegetable coagulants such as *Solanum dobium* extract ($t_{1/2}$ = 10 min at 60°C)[3], *Jacaratia corumbensis* O. Kuntze ($t_{1/2}$ = 15 min at 55°C)[21]. However, it possesses a great technological advantage because whey obtained with this plant extract could be used effectively in food processing.

Proteolytic action of SAS extract

Milk-clotting enzyme preparations are known to be always associated with proteolytic activity. However, for cheese-making, it is necessary to use coagulant possessing strong milk-clotting activity and least proteolytic action to avoid undesirable final products: poor yield, acid and bitter flavour and crumbly texture. Figure 5 shows the kinetics of hydrolysis of bovine casein as shown by the production of 5% (w/v) TCA-soluble peptides for a period of 3 h. The rate of hydrolysis increased appreciably with incubation time up to 1 h, and became stationary. The proteolytic action of SAS extract increased hyperbolically with incubation time. This observation is similar to these reported on the hydrolysis of ovine and caprine casein by *Cynara cardunculus* extract, and bovine casein by *Cynara scolymus*[7,12].

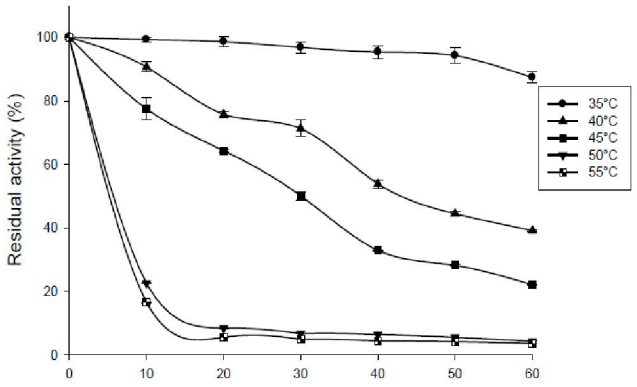

Figure 4 : Thermal stability of SAS extract. Extract was incubated at different temperatures, ranging from 35 - 55°C, and residual activity determined at intervals of 10 min for 1 hour. Each experiment was carried out in triplicate. Values were presented as mean and error bars represent the standard deviation.

Effect of SAS extract on the primary and secondary phases of coagulation

There are two phases in the rennet coagulation process: the enzymatic cleavage of κ-casein and the aggregation of the renneted micelles to form a gel[22]. These phases of the rennet coagulation can be separated by exploiting the fact that the second phase (gel assembly) effectively does not occur below 18°C, while the first phase progresses slowly at low temperatures[13]. Figure 6 shows rennet coagulation time (RCT) as a function of pre-incubation time at 15°C, indicating the duration of the two phases of SAS extract coagulation. The decrease in RCT was low during the first 60 min of pre-incubation, became drastic up to 120 min and tended towards constant from 150 min. However, in the ideal

conditions using chymosin, RCT versus pre-incubation time at low temperature give a hyperbolic decay tendency[13]. SAS extract did not follow exactly this tendency, suggesting that as with other plant coagulants, SAS extract was associated with less specific action on Phe_{105}-Met_{106} bond of k-casein. Since the primary phase of coagulation refers to the time needed to hydrolyze at least 85% of k-casein[22], SAS extract hydrolyzed essentially all the k-casein after about 180 min. The duration of secondary phase was about 241 seconds.

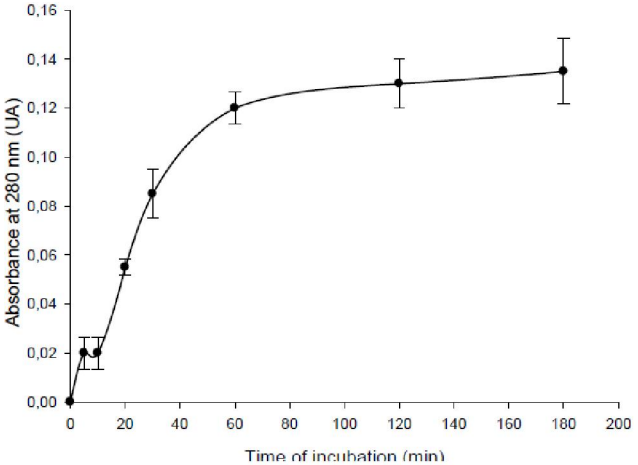

Figure 5 : Variation of absorbance at 280 nm with incubation time. Proteolytic activity was determined by adding 120 µL of SAS extract to 3 mL of 1% Casein (w/v) at 30°C. The experiment was followed for 3 h. Results were presented as mean of three replicates, and error bars represent standard deviation.

Activity and microbial changes during SAS extract storage

The activity and microbiological characteristics of coagulant varied during storage. The use of stored coagulant may cause additional microbial contamination of milk[30]. As presented in TABLE 2, the variation of microbial populations and residual activity of SAS extract was examined during three months of storage using preservatives at 4°C. The number of microorganisms per milliliter in fresh SAS extract (0 months storage) were 7.2±0.1×10² (cfu/mL) of total viable count, 0.31±0.04×10² (cfu/mL) of moulds and yeasts, 2.9±0.1×10² (cfu/mL) of *Lactobacilli*; while *Salmonella* spp, *E. coli* and *Clostridium* spp were absent. At ambient temperature, the SAS extract strength was lost after three days of storage. For this reason, the effect of storage on the changes in microbial populations and activity of SAS extract was conducted at low temperature (4°C). Results concerning total viable count showed that the percentage of growth decreased when acetic and citric acids were used as additives, while it increased with sorbic acid and control. For moulds and yeasts, the rate of growth increased regardless of the preservative used, with a maximum of 68.7±2.4% (sorbic acid). The percentage of *E. coli* growth increased from the second month when sorbic acid was used. Regarding *Lactobacilli*, the rate of growth decreased with theses additives except control. *E. coli* appeared at the third, second and third months of storage respectively in control and sorbic acid; this can seriously limit the use of stored SAS extract. The SAS extract retained 58.1±2.7% of activity in the control, followed by citric acid (54.8± 5.0%), sorbic acid (44.1±1.5 %) and acetic acid (24.2±1.3%) after three months of storage. These results suggested that SAS extract can be stored without preservatives for two months, while citric acid was the most suitable preservative for long term storage.

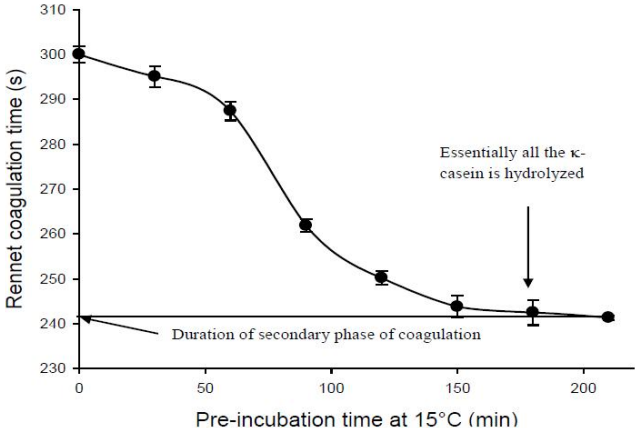

Figure 6 : Effect of pre-incubation at low temperature on rennet coagulation time. Reconstituted milk was cooled to 15°C before renneted with 10% with SAS extract. At regular intervals of 30 min, RCT was determined at 35°C. Values were presented as mean of five replicates, and error bars represent the standard deviation.

Effect of SAS extract on curd yield

The Zebu milk used for this study was composed of 7.8 ± 0.4 g/kg ash, 45.3 ± 4.2 g/kg fat, 49.1± 8.0 g/kg lactose monohydrate, 37.2 ± 4.0 g/kg proteins and 139.4 ± 16.0 g/kg dry matter; with a pH of 6.69. Cheese yield, defined as the weight of cheese obtained from a given weight of milk sample, is considered a major factor affecting efficiency and profitability of

FULL PAPER

TABLE 2 : Effect of storage period at 4°C and additives on the microbial changes in SAS extract and remaining activity

Additives	Storage (months)	Microbial populations in SAS extract (10^2 cfu/mL)						Residual activity (%)
		Total viable count	Moulds and yeasts	Salmonella spp	*Lactobacilli*	*E. coli*	*Clostridium spp*	
Control	0	7.2 ± 0.1	0.31 ± 0.04	/	2.9 ± 0.1	/	/	100
		Percentage of microbial growth from 0 storage month (%)						
	1	+1.4 ± 0.3	+12.6 ± 2.1	/	+6.9 ± 0.2	/	/	84.3± 4.3
	2	+13.9 ± 1.5	+25.2 ± 1.4	/	+10.4 ± 0.1	/	/	72.8 ± 4.1
	3	+66.7 ± 3.1	+29.7 ± 1.5	/	+10.4 ± 0.3	24 ± 2.2	/	58.1± 2.7
Acetic acid	1	- 1.4 ± 0.3	+1.2 ± 0.2	/	- 6.9 ± 0.2	/	/	67.3± 2.7
	2	- 4.2 ± 0.7	+9.6 ± 0.7	/	- 10.2 ± 0.1	/	/	43.5± 5.1
	3	- 9.7 ± 0.6	+17.4 ± 1.4	/	- 34.5 ± 0.3	/	/	24.2± 1.3
Sorbic acid	1	+1.4 ± 0.3	+11.7 ± 0.2	/	- 24.2 ± 1.0	/	/	77.2± 2.4
	2	+9.8 ± 0.5	+26.1 ± 1.3	/	- 37.9 ± 2.5	25.0±2.0	/	53.0± 2.5
	3	+18.0 ± 0.6	+68.7 ± 2.4	/	- 48.3 ± 2.0	39.0±1.5	/	44.1± 1.5
Citric acid	1	- 1.4 ± 0.3	+2.9 ± 1.2	/	- 6.9 ± 0.0	/	/	81.6± 2.2
	2	- 4.1 ± 0.6	+5.4 ± 0.1	/	- 20.6 ± 0.5	/	/	64.1± 3.0
	3	- 9.5 ± 0.7	+16.3 ± 1.5	/	- 27.4 ± 0.9	/	/	54.8± 5.0

(+) Increase, (-) Decrease, (/) Absent

TABLE 3 : Mass, dry matter content, and solids in vat milk, curd and whey of five laboratory scale trials

Parameters	Calf Rennet	SAS extract
Milk		
Mass in vat (g)	20.0 ± 1.2	20.0 ± 1.2
Dry matter content (g/kg)	139.4 ± 16	139.4 ± 16
Milk solids in vat (g)	2.8 ± 0.2	2.8 ± 0.2
Curd		
Curd mass from vat (g)	5.6 ± 0.7	5.5 ± 0.7
Milk solids in curd (g)	2.0 ± 0.1	1.9 ± 0.2
Dry matter content (g/kg)	357.1 ± 4.1	349.1 ± 5.5
Whey		
Mass from vat (g)	14.4 ± 0.68	14.5 ± 0.7
Milk solids in whey(g)	0.8 ± 0.2	0.9 ± 0.20
Dry matter content (g/kg)	92.1 [a] ± 15.4	100.7 ± 14.9
Yield		
[a]Estimated yield$_1$ (%)	28.0 ± 0.6	27.8 ± 1.5
[b]Estimated yield$_2$ (%)	17.1 ± 0.5	15.6 ± 1.2

Dry matter, milk mass and curd mass were measured. All other data were calculated; No statistically significant difference was observed between calf rennet and SAS extract; [a] yield calculated with fresh curd; [b] yield calculated with curd dry matter

cheese-making[31]. Indices of cheesemaking efficiency include cheese yield, and/or the recovery of milk constituents in curd or their loss in the whey, in particular the casein and fat[32]. However, judgement on the appropriateness or suitability of such substitutes has always been limited to coagulating and proteolytic activities: with little or no information relative to specific transfer of milk solids from milk to curd. As shown in TABLE 3, no statistically significant difference was observed between calf rennet and SAS extract ($P > 0.05$). However, curd dry matter (357.1 ± 4.1 g/ kg; 349.1 ± 5.5 g/kg), milk solids in curd (2± 0.1 g; 1.9 ± 0.2 g), mass curd (5.6 ± 0.7g; 5.5 ± 0.7 g), estimated fresh curd yield (28.0 ± 0.6%; 27.8 ± 1.5 %) and estimated curd dry matter yield (17.1 ± 0.5 %;15.6 ± 1.2 %) were higher when calf rennet was used. This may be either because nonspecific proteolysis of SAS extract or as the loss of milk solids was more important with SAS extract than calf rennet during drainage[22]. Despite this slight difference in yield, SAS extract could be used as a plant coagulant alternative to calf rennet. Further studies would be necessary on the microbiological, physicochemical, rheological and sensory characteristics of cheese made with SAS coagulant.

CONCLUSIONS

The present study was carried out to investigate the possibility to use SAS fruits in cheese-making. Milk-clotting enzymes were uniformly distributed in coats and

seeds parts of SAS fruit. Fruits harvested from six to eight weeks maturity were the best source of milk-clotting enzymes, irrespective of Cameroon's agro-ecological zones. Remarkable strength of SAS coagulant can be observed when 4% NaCl solution used as extractant medium. As with other milk-clotting enzymes, SAS extract was pH, temperature, calcium and enzyme concentration dependent. It was more thermosensitive than other proteases from plant. This property enhances the use of resulting whey in other food processes. Moreover, SAS extract was associated with strong coagulating activity and least proteolytic action. SAS extract can be stored without preservatives for two months; and citric acid can be used as additive for long term storage. In addition, there was no significant difference between yield of curds obtained with SAS extract and calf rennet. The results of this study indicate that SAS extract could be used as a plant coagulant alternative, to ameliorating the nutritional input of Sub Saharan Africa people whose use of animal and microbial coagulant is constraint. However, further studies about biochemical characteristics of both the SAS extract and the resulting milk curd are needed to confirm its convenience in cheese-making.

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FULL PAPER

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