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Full Length Research Paper

Milk-clotting potential of fruit extracts from *Solanum esculentum*, *Solanum macrocarpon* L. and *Solanum melongena*

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Fruit extracts from *Solanum esculentum*, *Solanum macrocarpon* L. and *Solanum melongena* were tested for their milk-clotting potential. The release of substances associated with milk-clotting was highly dependent upon quantity of berries, extraction duration and sodium chloride concentration. The highest milk-clotting activity was obtained after 8 h (for *S. esculentum* and *S. melongena*), and 12 h (for *S. macrocarpon*) of soaking 20 g of fruit in 6% NaCl 4°C. In these conditions, there was a non linear relationship between the milk-clotting time and the amount of fruit extracts from each species. The loss of milk-clotting activity was dramatic after wet-heating of extracts from *S. esculentum* and *S. macrocarpon* at 80°C for 10 min and after dry-heating of fruits at 100°C for 24 h. Heat treatment did not significantly affect the clotting activity of extract from *S. melongena*. Fruit extracts from *S. esculentum* had the greatest milk-clotting activity followed by extracts from *S. macrocarpon* and *S. melongena*. Extracts from *S. esculentum* and *S. macrocarpon* exhibited a proteolytic activity on the casein.

Key words: Milk-clotting activity, *S. esculentum*, *S. macrocarpon, S. melongena*, fruit extracts.

INTRODUCTION

Enzymatic coagulation is very important in the manufacture of cheese. Calf rennet was the first and still is the most widely used in cheese making around the world. It is a milk-clotting enzyme preparation which is extracted from the calf's fourth stomach. This enzymatic preparation contains chymosin, which exhibits specific and limited proteolysis of the Phe_{105}-Met_{106} bond in kappa-casein. High specific milk-clotting activity of calf rennet prevents excessive proteolysis during maturation and ensures the correct ratio between protein and peptides.

The world-wide increase in cheese production, alongside with the reduced supply of calf rennet, have led to an increase in the demand for alternative sources of milk coagulants (Cavalcanti et al., 2004). Microbial rennet produced by genetically engineered bacteria and moulds have proved suitable substitutes for rennin. However, the use of animal rennet may be limited for being against genetically engineered food (e.g. many countries forbid the use of recombinant calf rennet), religious reasons (e.g. Judaism and Islam) and diet (vegetarianism). Recently, the incidence of bovine spongiform encephalopathy (BSE) has reduced both supply and demand for bovine rennet (Roseiro et al., 2003). Consumer constraints on the use of rennets have led to a growing interest in vegetable coagulants.

Natural milk-clotting enzymes extracted from plants have increased the attention for dairy technology (Tavaria et al., 2001). The use of plant coagulants allows the target for cheese production and hence contributes to improve the nutritional intake of people whose use of

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animal rennets is restricted (Gupta and Eskin, 1977).

Cheeses made with vegetable coagulants are normally produced on an artisanal scale, in a farmhouse or small dairy. However, they have an important socio-economical contribution to the dairy sector at local and regional areas of each country. That is, the case of Ngaoundere area in the Adamaoua region of Cameroon. Thus, cheese making contributes to prevent degradation of raw milk in this region.

Several plant preparations have been shown to clot milk. Unfortunately, most of them were proved unsuitable for cheese making owing to their excessively proteolytic character, which lowers cheese yield and produces bitter flavors in the final cheese (Sousa and Malcata, 2002; Roseiro et al., 2003). Moreover, their toxin content constrains their use as food (Silva and Malcata, 2005). Otherwise, the use of certain parts of plant as sources of milk-clotting enzymes, such as: barks, leaves, roots and sap may in the long term lead to an ecological disaster (Brncic et al., 2007).

The increasing attention has been directed towards the use of fruits or flowers as sources of milk-clotting enzymes (Lopes et al., 1998). Fruits of some plants have been tried to that subject: *Balanites aegyptiaca* (Libouga et al., 2006); *Albizia lebbeck* and *Helianthus annuus* (Egito et al., 2007); *Solanum innacum* (Suleiman et al., 1988); *Solanum dobium* (youssif et al., 1996). Fruits of same genus: *Solanum esculentum*, *Solanum macrocarpon* L. and *Solanum melongena* are abundant in central, northern and western Cameroon. These plants belong to the Solanaceae family. They are a woody herb with a solid erect stem, green in colour and about 30 cm high. The leaves are alternate, long petiole, simple, ovate, acuminate or obtuse at the apex and pale green in colour while the rootlets are brown and the root about 5 mm thick and 15 cm length. The flowers are a hermaphrodite. Unripe fruits are green (*S. macrocarpon*); white (*S. esculentum*) and violet (*S. melongena*), while the ripe fruits are yellow (*S. macrocarpon*), brown (*S. esculentum*) or violet (*S. melongena*). Theirs fruits are edible berries (Dupriez and De Leener, 1987; Bukenya and Bonsu, 2004). The milk-clotting potential of the preparation extracted from fruits of these species have not yet been carried out. Therefore, the aim of this work was to study the potential ability of extracts from *S. esculentum*, *S. macrocarpon* L. and *S. melongena* fruits to clot milk.

MATERIALS AND METHODS

Materials

Ripe berries of *S. esculentum*, *S. macrocarpon* and *S. melongena* were bought during the rainy season 2006/2007 in the Ngaoundere market area (Adamawa region of Cameroon). Calf rennet (1/10000), Bromelain and Casein were from SIGMA (Saint Louis, MO, USA). Low skim milk powder was from NILAC™ (NIZO, Ede, The Netherlands). Otherwise stated, all chemicals used in this study are of reagent grade.

Preparation of crude extract

The berries of each species were sorted out, washed, cut in slices and dried at 40°C for 36 h, then ground with a cereal-grinder. The extracts were prepared by immersing 5, 10, 15 and 20 g of each sample (powder of *S. esculentum*, *S. macrocarpon* and *S. melongena* berries) in 100 mL of 0, 3 and 6% (w/v) sodium chloride solution, respectively. The mixtures were stirred at room temperature (22°C). The extraction procedure was continued for 2, 5, 8, 12 or 24 h at 4°C. Then, extracts were filtered using whatman No.4 filter papers, cooled and maintained at 4°C.

Determination of milk-clotting activity

The milk-clotting activity of fruit extracts from *S. esculentum*, *S. macrocarpon* and *S. melongena* fruits were determined following the procedure described by IDF (1992). 60 g of low-heat skimmed milk powder was reconstituted in 500 mL 0.01 M $CaCl_2$ (pH 6.5); this mixture was stored at 4°C. Extracts were added at a proportion of 0.1 mL per mL of milk. These extracts were added to reconstituted milk at final volumes in the range 0.1 to 2 mL. Sodium azide was added at 0.03% (w/v) to prevent microbial growth (Silva and Malcata, 2005). The coagulation point was determined by periodic manual rotating of the test tube, at very short time intervals. The clotting time was recorded when discrete particles were discernible. One milk-coagulating unit (U) was defined as the amount of protein that coagulates 10 mL of reconstituted low-heat skimmed milk powder at 30°C in 100 s (Berridge, 1952). The milk-clotting activity (MCA) of each extract was measured, assuming that all the soluble proteins are enzymes which coagulate milk at 30°C.

Effect of heat treatment on the milk-clotting activity

Two heating regimes were applied to test the inactivation of substances associated with milk-clotting. Firstly, in the best conditions of extraction, 10 mL of fruit extract from *S. esculentum*, *S. macrocarpon* and *S. melongena*, as well as calf rennet 5% (w/v) diluted 800 times were placed in seven test tubes and heated to 30, 40, 50, 60, 70, 80 and 90°C, respectively, for 10 min using a water bath. Secondly, 20 g of each sample was dry-heated in an oven at 105°C for 24 h. These heated powders from *S. esculentum*, *S. macrocarpon* and *S. melongena* fruits were soaked to extract any substance associated with milk-clotting. Heated extracts and control extracts (unheated) were tested for their milk-clotting activity.

Determination of proteolytic activity

Proteolytic activity was estimated according to the method of Bergkvist (1963) with slight modifications. Casein solution (1% (w/v)) was subjected to hydrolysis at 30°C in 0.01 M phosphate buffer (pH 6.7). Sodium azide was added at 0.03% (w/v) to prevent microbial growth (Silva and Malcata, 2005). The hydrolysis was initiated by addition of 1 mL of each fruit extract from *S. esculentum*, *S. macrocarpon* and *S. melongena*, as well as rennin 5% (w/v) diluted 800 times and bromelain 5% (w/v) diluted 300 times to 10 mL of the casein solution (1% (w/v)). At selected durations (30 min, 1, 2, 4 and 6 h), 1 mL of mixture was taken and the reaction was quenched by heating at 100°C for 10 min. The mixtures of each sample were treated with 12% (w/v) trichloroacetic acid (TCA) at a volumetric ratio of 1:2. The mixture was allowed to settle for 10 min and then centrifuged at 12,000 g for 10 min. The proteolytic activity was quantified by evaluating the tyrosine concentration in the supernatant. One unit (U) of enzyme activity was taken as the amount of enzyme which liberates 1 µ mole of tyrosine/ml/min.

Table 1. Variation of milk-clotting time with the type of extracts (*S. esculentum, S. macrocarpon* and *S. melongena*) and the extraction factors.

Type of coagulants	Milk-clotting time (min)		
	S. macrocarpon	***S. esculentum***	***S. melongena***
% Berries (w/v)			
5	$177 \pm 13^{d.\ 2}$	$146 \pm 12^{d.\ 1}$	$266 \pm 17^{d.\ 3}$
10	$101 \pm 16^{c.\ 2}$	$73 \pm 9^{c.\ 1}$	$152 \pm 13^{c.\ 3}$
15	$60 \pm 9^{b.\ 1}$	$59 \pm 13^{b.\ 1}$	$126 \pm 18^{b.\ 3}$
20	$44 \pm 2.1^{a.\ 2}$	$38 \pm 7^{a.\ 1}$	$93 \pm 12^{a.\ 3}$
Extraction duration (h.)			
2	$136 \pm 13^{h.\ 2}$	$90 \pm 16^{g.\ 1}$	$197 \pm 13^{i.\ 3}$
5	$110 \pm 12^{g.\ 2}$	$69 \pm 15^{f.\ 1}$	$152 \pm 9^{g.\ 3}$
8	$78 \pm 11^{f.\ 1}$	$59 \pm 17^{e.\ 1}$	$134 \pm 14^{e.\ 2}$
12	$68 \pm 7^{e.\ 1}$	$61 \pm 13^{e.\ 1}$	$146 \pm 19^{f.\ 2}$
24	$86 \pm 10^{f.\ 1}$	$151 \pm 18^{h.\ 2}$	$167 \pm 10^{h.\ 3}$
% NaCl (w/v)			
0	$101 \pm 7^{k.\ 1}$	$91 \pm 4^{j.\ 1}$	$164 \pm 12^{k.\ 2}$
3	$95 \pm 13^{j.\ 1}$	$85 \pm 10^{i.\ 1}$	$157 \pm 9^{j.\ 2}$
6	$90 \pm 5^{i.\ 1}$	$83 \pm 11^{i.\ 1}$	$156 \pm 8^{j.\ 2}$

Values are means with standard deviations; values with different superscript letters are significantly different ($p < 0.05$) in the same column for each factor. Values with different superscript number are significantly different ($p < 0.05$) in the same line.

Statistical analysis

The experiments were conducted in triplicate and the results are the averages with standard deviation of these three independent trials. For the extraction procedure a randomized split-plot design was used, with fruit from *S. esculentum*, *S. macrocarpon* and *S. melongena* berries as the main factor; extraction duration and sodium chloride concentration were used as the secondary factors. Statistical analysis was carried out using Statgraphics Centurion (2007, StatPoint, Inc., IL USA), Excel 2007 (Microsoft Office 2007 Professional) and SigmaPlot (SPW11).

RESULTS AND DISCUSSION

Extraction

Extraction of substances associated with milk-clotting differed according to the type of extract, amount of berries, duration of extraction and sodium chloride concentration. Therefore, there were significant differences ($P < 0.001$) in the milk-clotting time of the extracts depending on which species of *Solanum* was used as source (Table 1). The amount of soaked fruit affected the clotting point ($P < 0.001$). The milk-clotting time depended on the time of soaking fruit ($P < 0.01$). Moreover, extractant solution (distilled water, 3 or 6% sodium chloride) affected the milk-clotting time ($P < 0.05$). The interaction between these extraction factors was highly significant ($P < 0.0004$). The compound associated with milk-clotting were mostly extracted after 8 h (for *S. esculentum* and *S. melongena*) and 12 h (for *S. macrocarpon*) of soaking 20 g of fruit in 6% NaCl.

However, above these extraction durations, the clotting time increased significantly ($P < 0.005$). It could be that, there was a release of inhibitor substances into the medium of clotting compound. This observation seems consistent with the observations of Yousif et al. (1996), who extracted a milk-clotting enzyme from a similar plant species, *Solanum dobium*. More than one substance might have been extracted. Further work is needed to elucidate the nature of clotting compound and their inhibitors in the crude extracts.

With regard to the extractant solution, the milk-clotting compounds are mostly released in 6% sodium chloride (NaCl) solution followed by 3% NaCl solution and then distilled water. This is because NaCl solution increases the ionic force of the extraction medium and releases the maximum of milk-clotting substances (Verissimo et al., 1995; Yousif et al., 1996; Lopez et al., 1998). Sousa and Malcata (1996) found that the effect of NaCl concentration is insignificant within the procedure of milk-clotting enzymes extraction.

Coagulating activity

The variation of the clotting time with the amount of extracts from *S. macrocarpon S. esculentum* and *S. melongena* is shown in Figure 1. Coagulating time decreases significantly with amount of extract and tends

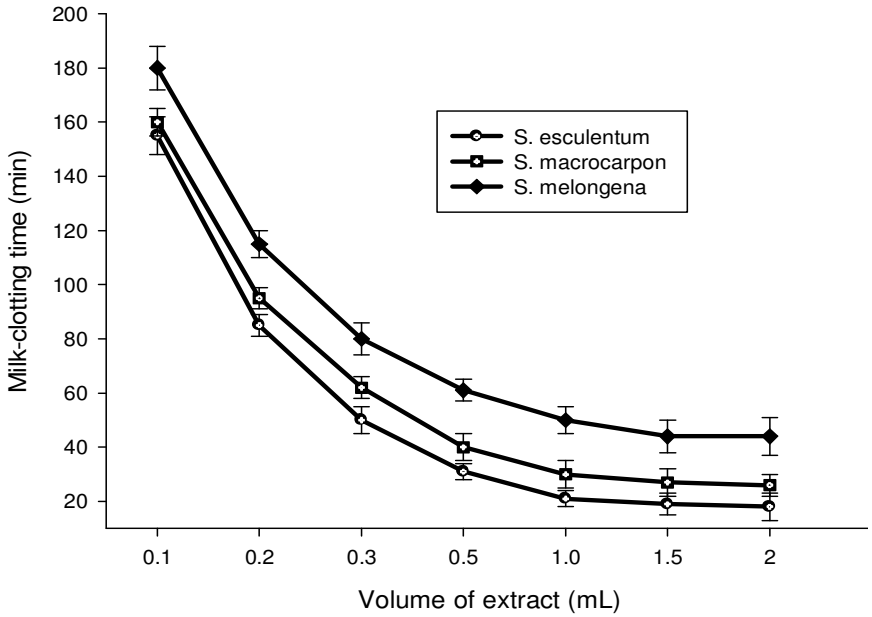

Figure 1. Variation of milk-clotting time with amount of fruit extracts from *Solanum*.

toward an asymptotic value at 1.5 mL of extract.

As with other coagulants (McMahon and Brown, 1985), such as animal rennets, fungal rennets (Fernandez-Lahore et al., 1999) from *Mucor miehei* and *Mucor pusillus* and plant rennets (Silva and Malcata, 2005), the amount of extracts (from *S. macrocarpon S. esculentum* and *S. melongena*) significantly affected the milk-clotting time ($P < 0.001$). There was a high and significant correlation between the milk coagulating time and the amount of extracts ($R^2 = - 0.99$; $P < 0.001$). These results are consistent with those of Silva and Malcata (2005) working on the flowers of *Cynara cardunculus*. There was a non linear regression of inverse exponential type. According to this observation, the extracts from *S. esculentum* had a higher milk-clotting activity followed by extracts from *S. macrocarpon* and then *S. melongena.*

The regression curves did not pass through the origin. This may be either because of a lack of specificity of the extracts (from *S. esculentum*, *S. macrocarpon* and *S. melongena* berries) for the Phe_{105}-Met_{106} bond on O-casein or that other proteases were also present in the extract; as those from *S. dobium* (Yousif et al., 1996).

Effect of heating

The effect of heating on the clotting activity of fruit extracts from *S. esculentum*, *S. macrocarpon* and *S. melongena* is shown in Figure 2. The milk-clotting activity of the extracts remained stable at 40°C of heating for 10 min for the extract from *S. esculentum*, *S. macrocarpon* and *S. melongena*. After heating at 40°C, loss of clotting activity was increased linearly with temperature up to 70°C for Calf rennet, extract from *S. esculentum* and *S. macrocarpon*. In contrast, heat treatment no longer affected the clotting activity of extract from *S. melongena* significantly, whatever the temperature of heating used during this study.

The loss of clotting activity became dramatic at 70°C for calf rennet and 80°C for extract from *S. esculentum*, and *S. macrocarpon*. Calf rennet was the most affected by wet-heating followed by extract from *S. esculentum* and *S. macrocarpon*. Heating to 60 or 70°C, respectively, reduced the clotting activity of extract from *S. macrocarpon* to 45 and 84%; that of *S. esculentum* to 56 and 87%. These results are inconsistent with those of Yousif et al. (1996) who found out 28 and 86% loss of the clotting activity on a similar species (*S. dobium).* There was no clotting activity when berries were heated in an oven to 105°C for 24 h before extraction; except those of *S. melongena*.

Proteolytic activity

One of the fastest methods to evaluate proteolysis is the quantification of soluble peptides in trichloroacetic acid (TCA) at levels between 2 and 12% (w/v); the higher the TCA concentration, the shorter the sizes of soluble peptides (Yvon et al., 1989).

The proteolytic activity indicated the ability of extracts to break peptide bonds, thus provoking an enzymatic milk-clotting. The tyrosine concentration in the presence of 12% TCA increased with the incubation time up to 1 h for calf rennet, 2 h for extract from *S. esculentum* and 4 h for bromelain and *S. macrocarpon* (Figure 3). In contrast, extract from *S. melongena* berries did not hydrolyze

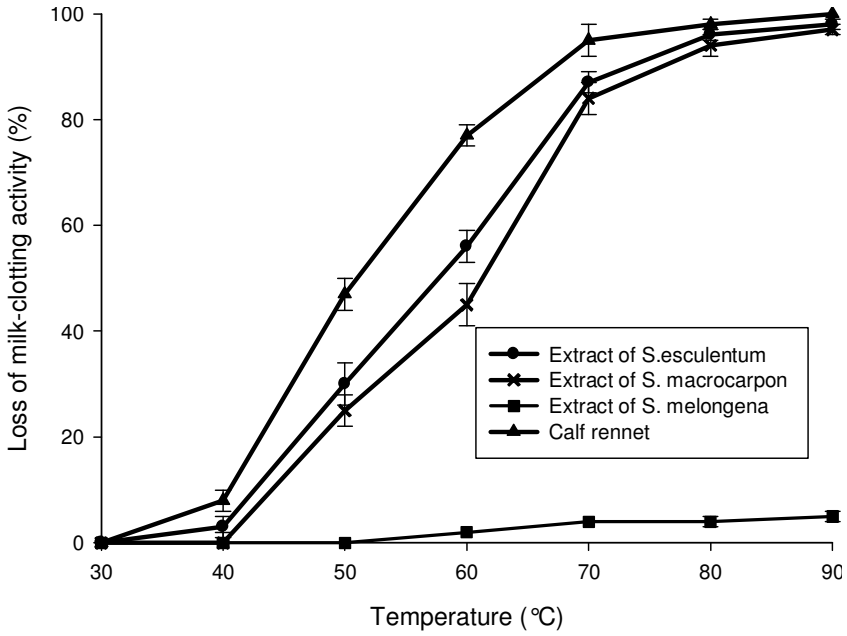

Figure 2. Effect of heating *Solanum* extracts for 10 min on the milk-clotting activity.

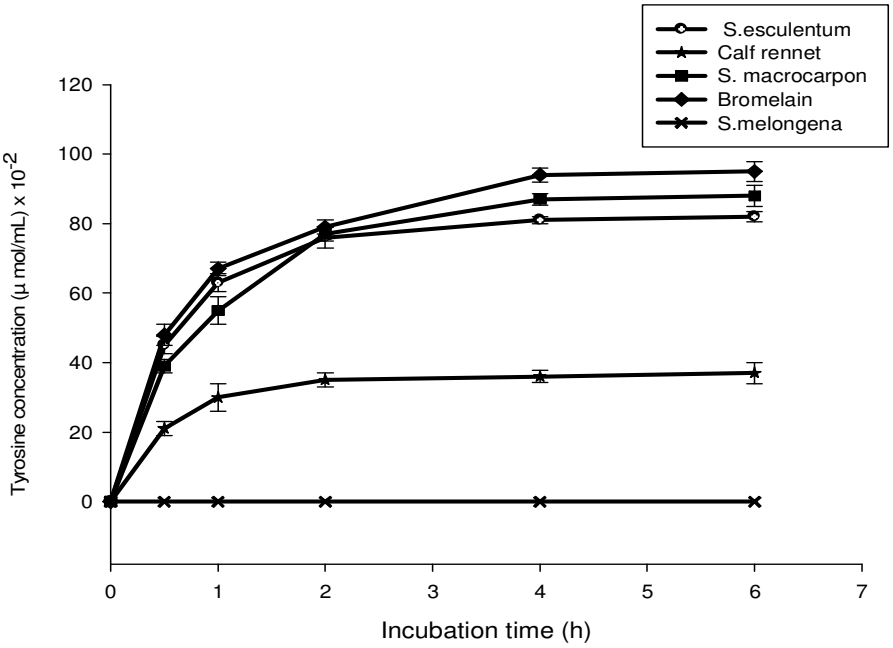

Figure 3. Variation of tyrosine concentration with incubation time.

casein (Figure 3). This may be either because of the fact that the extracts from *S. melongena* berries did not possess any protein having a proteolytic activity on casein or because its proteases were inhibited by other compounds present in the crude extract. It could be that the milk- clotting activity observed previously was caused by the acidity of this extract (pH 4.33 ± 0.07).

Between 30 min and 2 h of incubation, the Duncan's classification showed that there was no significant difference between bromelain and *S. esculentum* ($P > 0.05$); this became significant after 4 and 6 h of incubation ($P < 0.05$). At 1 h of incubation, there was a difference between bromelain and *S. macrocarpon* ($P < 0.05$). There was a high significant difference between

calf rennet and other extracts from *S. esculentum* and *S. macrocarpon*, as same as bromelain ($P < 0.0001$). Between 4 and 6 h of incubation, the variation of proteolytic activity was not significant for these extracts. The variation of proteolytic activity was significant with calf rennet between 0 to 1 h of incubation. This may be because of the fact that β-casein which is very concentrated in tyrosine is sensitive to chymosin (Reid et al., 1997).

Calf rennet exhibited the lowest proteolytic activity, followed by extract from *S. esculentum* and *S. macrocarpon*. Bromelain showed the greatest proteolytic activity. Extracts from *S. esculentum* and *S. macrocarpon* exhibited a proteolytic activity on the casein and might be a potential source for proteases which is useful in dairy technology. Further studies should be conducted to identify the proteases in these extracts.

Conclusion

The milk-clotting time is shorter with extracts from *S. esculentum* followed by those from *S. macrocarpon* and *S. melongena*. The substances associated with milk-clotting are better released in 6% sodium chloride solution than they are liberated in distilled water. The milk-clotting activity was totally lost after wet-heating extracts at 80°C for 10 min (*S. esculentum* and *S. macrocarpon*); as well as the dry-heating of the powders of these berries for 24 h. There is a high relationship between the clotting time and the amount of extracts. The caseinolytic activity was only observed for the extracts from *S. macrocarpon* and *S. esculentum*. The extracts from *S. esculentum* berries had the best milk-clotting potential followed by the extracts from *S. macrocarpon* and then *S. melongena*. These results suggested that crude extracts obtained from *S. esculentum* and *S. macrocarpon* L. fruit might be a potential source for enzymes useful in dairy technology.

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