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COMPARATIVE STUDIES OF THE PROTEOLYTIC AND THE MILK CLOTTING ACTIVITIES IN THE 'LATEX' OF THREE SELECTED PLANTS

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Abstract

The use of enzymes as catalysts for industrial processes is becoming increasingly widespread, particularly in the food industry. The proteolytic and milk clotting activities of the crude latex of *Calotropis procera, Carica papaya,* and *Musa paradisiaca* were examined. The proteolytic activities of the crude latex were determined using a modified casein digestion method. All the crude latex (16.7 % concentration) yielded significant proteolytic activities of $(5.63 \pm 0.031) \times 10^{-2}$ min^{-1}, $(5.29 \pm 0.015) \times 10^{-2}$ min^{-1} and $(4.97 \pm 0.014) \times 10^{-2}$ min^{-1} for *C. procera, C. papaya* and *M. paradisiaca* repectively. At 16.7 % concentration of the crue latex, *C. procera* and *C. papaya* had the milk clotting activities of 14.21 ± 0.72 min^{-1} and 6.33 ± 0.20 min^{-1} respectively; while *M. paradisiaca* yielded no milk clotting activity. However, preparation of 100% concentrated crude latex of *M. paradisiaca* yielded 4.81 ± 0.11 min^{-1} milk clotting activity. The *t*-tests ($P < 0.05$) of the proteolytic and milk clotting activities of *C. procera, C. papya* and *M. paradisiaca* crude latex show that their activities are significantly different from one another. Nevertheless, the respective relative activities of these three plants are significantly high enough for the m to be used for either proteolytic assay and or milk clotting processes.

1. Introduction

Proteolytic enzymes, also known as proteases, are a group of enzymes used industrially for their ability to transform protein molecules into simpler peptides (Flynn, 1975). They are generally characterized as endopeptidases or exopeptidases (Barette, 1986). For example, trypsin (an intestinal protease) cleaves peptide bonds on the carbonyl side of basic amino acids like lysine and arginine. Prescott *et al.* (2000) and Nelson and Cox (2000) highlighted the role of proteases in humans and other vertebrates, as being basically for protein digestion in the gut, and protective function against some invading microbes (when located in the lysosome). However, they are also often utilized for *in vitro* studies and industrial processes, especially in food industry (Baeza *et al.*, 1990).

Milk is a very clean white secretion from mammary gland of the female warm blooded animals, as a complete diet created by nature for feeding young ones before they can fend for themselves (Babayemi *et al.*, 1991; Oyawole *et al.*, 1997). It can be consumed directly or converted to other products through various preservatives methods, which include pasteurization, fermentation and chemical treatment to increase its shelf life (Oyawole *et al.*, 1997). Milk is a complex mixture of lipids, carbohydrates, proteins and many other organic compounds and inorganic salts dissolved or dispersed in water (Srilakshmi, 2001). Milk protein is of interest in this work.

Milk clotting enzymes are special proteolytic enzymes that affect the milk clotting process by acting on casein (milk protein); affectin its physico-chemical properties. They were first noted in humans and other vertebrates, as seen in the coagulating action of rennin (that is largely found in the stomach of young calves) on the casein of sucked milk (Srilakshmi, 2001).

So far, proteolytic and milk clotting enzymatic activities are notable in animals. Nevertheless, works have been done locally and internationally on some plants to prove the presence of proteolytic and milk clotting enzymes in their crude latex. (Storey, 1986). Such plant is *Calotropis procera. C. procera,* also known as Sodom Apple, is a perennial shrub exuding copious milky sap when cut or broken (Afolabi, 1991). The latex has been reported to be irritant, rubefacient, vesicant, caustic, swellin eyelids and blurring of vision from corneal, and depilatory when applied to skin (Burkill, 1970; Duke-Elder and Macfanl, 1972). These effects might be very connected with its proteolytic activities reported by Atal and Sethi (1962) who isolated calotropain from the latex showed it to be a mixture of at least five proteases; and its milk clotting activites reported by Oseni and Ekperigin (2003).

Another plant is *Carica papaya*, which is also known as pawpaw. It is an erect, fast-growing, usually unbranched tree or shrub, with copious latex (Duke, 1983). Externally, the latex is irritant and

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dermatogenic. Internally, it causes severe gastritis. This toxicity may also be very connected with the proteolytic activities (Arnon, 1970; Baines and Brocklehurst, 1979; Brocklehurst *et al.*, 1984; Polgar, 1984) and milk clotting activities recorded by Duke (1983).

Musa paradisiaca, commonly called cooking Banana, is a tall perennial monocotyledon herb cultivated in the tropics and subtropics of the world (Simmonds, 1966; Uphof, 1968). There has been no report of the proteolytic and or milk clotting activities in its 'latex'. However, Frison and Sharrock (1999) recorded that the sap is used as dye and the pseudostem used as soap. More so, its fruit aid digestion by relieving condition like constipation. These reports indicate that the plant extracts may have some biological properties like proteolytic and milk clotting activities. In addition, when various parts of *M. paradisiaca* are used, especially for non-nutritional purposes, like the making of crafts, other product like sap will most likely be enormously wasted. If this sap can be used simultaneously, especially from extensive plantain cultivation, it will be an added economic advantage to the plant.

Thus, *Calotropis procera, Carica papaya* and *Musa paradisiaca* have been selected for study in this work. The review so far shows that there is need to clarify uncertainties about these plants (*C. papaya* and *M. paradisiaca*), as regards the presence of proteolytic and or milk clotting enzymes in their 'latex'. More so, the relative activities of these enzymes in the three plants are necessary to be studied; as well as the relationships between the enzymes.

2. Experimental Design

The plants (*Calotropis procera, Carica papaya,* and *Musa paradisiaca*) were obtained from Illisan Remo town, Ogun State Nigeria. The latex of *C. procera* was collected in a sterile plastic container by breaking off the leaves, which allowed the out flow of the white viscous liquid from the base of the petiole and just below the node point.

The latex of *C. papaya* was collected by making several certical incisions into the surface of the unripe fruit with a sharp penknife. The cut was not too deep (0.5-1.0 mm), to avoid contamination of the preparation. The latex was also collected in a sterile plastic container (Burdick, 1984).

To collect the 'latex' of *M. paradisiaca*, a very smooth stem portion was first located, cleaned up, and a horizontal incision (1.5-2.0 mm deep) was made on the surface, 130 cm above the ground level. The upper part 1 cm from the incision was gently depressed and the very light grown 'latex' was simultaneously collected with a sterile disposable syringe (without the needle affixed); as it flows down the stem. This was immediately transferred into a sterile plastic container as well.

The crude latex samples obtained from the three plants were prepared almost immediately after collection. 4 ml of each crude latex was mixed with 20 ml of 0.1 M sodium acetate buffer of pH. 5.5 and filtrated. The filtrate was centrifuge at 3000 r.p.m for 15 minutes. The supernatant was then obtained and stored in the refrigerator for analysis. The corresponding pH of the various crude latex was measured with a pH meter, before and after the preparation.

Casein Digestion: Protease activity was measured using a modified version of the Casein Digestion Method (Oseni and Ekperigin, 2003). 1 % casein solution was prepared in 0.1 M citrate phosphate buffer pH 7.5 and heat denatured at 100 °C for 15 minutes in water bath, cooled and used as substrate. The reaction mixture consist of 1 ml of the extract, thoroughly mixed together. This was incubated for 1 hour at 37 °C, the reaction was terminated by adding 3 ml of cold (2 °C) 10 % trichloroacetic acid (TCA). The tube was allowed to stand for a minimum of 1 hour at 20 °C in a refrigerator to allow the undigested protein precipitate. It was then centrifuged at room temperature for 30 minutes and the absorbance of the clear supernatant was measured at 280 nm.

0.25 g of powdered milk was weighed into a clean test tube, followed by 0.75 ml of 0.1 M sodium acetate buffers of pH 5.5. The test tube was shaken vigorously until the milk dissolved and was placed in water bath for 10 minutes at 35 °C. After this, 1 ml of the prepared crude latex was added and the time taken for the milk solution to clot was recorded. It was further observed for 1 hour (Oseni and Ekperingin, 2003).

3. Results and Discussion

The data in Table 1 shows the pH values of the crude latex used. The pH 5.6 recorded for *Calotropis procera* latex used is slightly higher than the 5.30 pH, reported by Oseni and Ekperigin (2003). The variation may be due to varied environmental factors in locations (Ilisan and Akure) of obtaining the plants. The variation that exists in the pH of the dilute latex is a reflection of their initial pH values. So the latex have similar decreasing acidity trend before (4.62, 5.91 and 6.24 for *C. procera, M. paradisiaca* and *C. papaya* respectively) and after (5.36, 5.49 and 5.62 respectively) the dilution process. However, the relatively acidic pH of the dilute crude latex aid their activity towards the casein as substrates in a citrate phosphate buffer at pH 7.5, which is relatively basic.

Table 2 shows the proteolytic activities of the three selected plants. *C. procera* has the highest proteolytic activity while *M. paradisiaca* has the least. This

Table 1: The pH of Crude Latex of the Selected Plants

Plant Source	pH of Crude Latex before Dilution	pH of Crude Latex after Dilution
Calotropis procera	4.62	5.36
Carica papaya	6.24	5.62
Musa paradisiaca	5.91	5.49

Table 2: The Proteolytic Activities of Dilute Crude Latex of the Selected Plants

Plant Source	Amt. of dil. latex used (ml)	Absorbance at 280 nm	Proteolytic Activity (min^{-1}) $x10^{-2}$	Mean Proteolytic Activity (min^{-1}) $x10^{-2}$	Relative Activity (%)
**Calotropis procera*	1	3.325	5.54	5.63 ± 0.031	100.00 ± 0.55
	1	3.378	5.63		
	1	3.385	5.64		
	1	3.413	5.69		
**Carica papya*	1	3.195	5.33	5.49 ± 0.015	93.96 ± 0.27
	1	3.169	5.28		
	1	3.174	5.29		
	1	3.156	5.26		
**Musa paradisiaca*	1	2.955	4.93	4.97 ± 0.014	88.28 ± 0.25
	1	2.992	4.99		
	1	2.986	4.98		
	1	2.989	4.98		

* The Crude Latex used was of 16.7 % Concentration.

agrees with the report of Atal and Sethi (1962) that the proteolytic activity of *calotropain* (a mixture of at least five proteases in *C. procera* latex) is greater than that of *papain, ficin and bromelain.* The proteolytic activity (5.63±0.031) x 10^{-2} min^{-1} of *C. procera* in the table is also higher than the record (5.2 x 10^{-2} min^{-1}) of Oseni and Ekperigin (2003). The reason may still be as previously discussed above - varied locations of the plant.

The statistical analysis (*t*-tests) of the data in Table 2 show that the difference in their activities is statistically significant. But the relative proteolytic activities (93.96±0.27 % and 88.28±0.25 %) shown respectively by the latex of *C. papaya* and *M. paradisiaca* prove beyond doubt that the two plants have very significant proteases.

It is not very surprising that high protease level in *C. papaya* latex was detected, since this is in agreement with the various report of the presence and activity of *papain* in *C. papaya* latex. Nevertheless, the presence of proteolytic enzymes in the latex of *M. paradisiaca* has not been previously reported and is certainly a discovery. Moreso, this activity is relatively high.

Table 3 shows the milk clotting activities of the selected plants, with *C. procera* having the highest activity of 14.21±0.72 min^{-1}. When 16.7 % concentrated crude latex was applied, *M. paradisiaca* yielded no milk clotting activity. Nevertheless a preparation of 100 % of its latex helps to clearly show the milk clotting activity of this plant; though of a lower frequency than as found in the latex of *C. procera and C. papaya.*

The statistical analysis (*t*-tests) of the data in Table 3 show that the milk clotting activities of the plants (14.21±0.72 min^{-1}, 6.33±0.20 min^{-1} and 4.81±0.11 min^{-1} for *C. procera, C. papaya and M. paradisiacal* respectively) are significantly different from one another. This is also reflected in their relative activities (100.00 ± 5.07 %, 44.55 ± 1.41 % and 33.85± 0.77 % respectively). Moreso, the analysis of *C. procera and M. paradisiaca* latex shows the largest marginal difference ($t_{4,6}$=12.90), whereas that of *C. papaya and M. paradisiaca* shows the least marginal difference ($t_{5,6}$=6.72).

The milk clotting activity of *C. procera* crude latex in Table 3 doubles the amount documented by Oseni and Ekperigin (2003). The variation may be due to varied locations of obtaining the plant. This also suggests the existence of the varieties of the plant; or the effect of some biotic and abiotic factors on its chemical composition. These latter ideas can only be substantiated by further study.

The leaves of *C. procera,* which has relative activity of 2.5 % to dilute crude latex (Oseni and Ekperigin, 2003), was reported to be used as clotting agent in local production of 'warankasi' (soft cheese) of milk (Ihekoronye and Ngoddy, 1985). So, the dilute crude latex of *C. papaya and M. paradisiaca,* which have relative activities of 44.55 ± 1.41 % and 33.85± 0.77

Table 3: The Milk Clotting Activities of Crude Latex of Selected Plants

Plant Source	Amt. of dil. latex used (ml)	Absorbance at 280 nm	Proteolytic Activity (min^{-1}) $x10^{-2}$	Mean Proteolytic Activity (min^{-1}) $x10^{-2}$	Relative Activity (%)
**Calotropis procera*	1	0.067	14.93	14.21 ± 0.72	100.00 ± 5.07
	1	0.067	14.93		
	1	0.083	12.05		
	1	0.067	14.93		
**Carica papya*	1	0.167	5.99	6.33 ± 0.20	44.55 ± 1.41
	1	0.150	6.67		
	1	0.150	6.67		
	1	0.167	5.99		
**Musa paradisiaca*	1	-	No activity	No activity	-
	1	-	No activity		
	1	-	No activity		
	1	-	No activity		
***Musa paradisiaca*	1	0.200	5.00	4.81 ± 0.11	33.85 ± 0.77
	1	0.217	4.61		
	1	0.200	5.00		
	1	0.217	4.61		

* The Crude Latex used was of 16.7 % concentration.
* * The Crude Latex used was of 100 % concentration.

% respectively, are significantly high to be used in milk clotting process.

The presence of milk clotting enzymes in the crude latex of *C. papaya* agrees with the report of Athal and Sethi (1962) and Oseni and Ekperigin (2003). The milk clotting activity of *papain* (*C. papaya* protease) has also been previously documented (Duke, 1983). However, no report has been noted concerning the presence of milk clotting enzymes in *M. paradisiaca* latex - so, this is also a discovery. Moreso, this detected activity is relatively high as noted in the previous discussion.

4. Conclusion

The dilute crude latex of the three selected plants possesses proteolytic enzymes substantially, with the highest activity in *C. procera.* So, they can all be applied in proteolytic assays.

The crude latex of *C. procera, C. papaya and M. paradisiaca* contain milk clotting enzymes, of which *C. procera* has the highest activity. Though the milk clotting activities of the plants' latex are significantly different; *C. papaya and M. paradisiaca* latex are quite useful as source of milk clotting enzymes while the latex of *C. procera* is a perfect source. The distribution of milk clotting enzymes in these plants makes the effective of one for the other, an inappropriate practice; though complementation could be very appropriate.

Moreso, the presence of proteolytic and milk clotting enzymes in the latex of *M. paradisiaca* is an additional economic value to the plant, especially to the people that largely cultivate it for its non-nutritional products.

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