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USES, PREPARATION, AND PROPERTIES OF PINGUINAIN, THE PROTEIN-SPLITTING ENZYME OF THE *MAYA* FRUIT

By C. F. ASENJO and M. del C. C. de FERNÁNDEZ

From the Department of Chemistry of the School of Tropical Medicine and the Agricultural Experiment Station of the University of Puerto Rico

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The existence of a protein-splitting enzyme in *maya* (*Bromelia pinguin* L.) was first established by Asenjo and Fernández in 1941 (1) and the name "pinguinain" suggested for this new enzyme. Pinguinain belongs to that group of enzymes known as papainases, that is, enzymes similar to papain which clots milk and digests proteins and live tissue. The milk-clotting activity of pinguinain is similar in many respects to that of the renin enzyme. Preliminary experiments carried out on it suggest its use as a substitute for this animal enzyme in the manufacture of cheese.

The above-mentioned properties of the papainases have already been put to practical usage both in industry and in medicine. Every year, beer manufacturers utilize a large amount of papainases in the preparation of "chill-proof" beers. When chilled, some beers become turbid because of small particles of protein and other associated substances that precipitate during the chilling process; when beers are pretreated with a proteainase (Wallerization), such as papain or pinguinain, such particles are digested and a perfectly clear beer is produced at low temperatures. Pinguinain

has already been tried by several consulting laboratories who report that it has proved very satisfactory for the above purpose.

The papainases are also abundantly used in the meat-packing industry, therefore another practical usage of pinguinain would be as a meat-tendering agent. Pinguinain has been found to soften meats readily and could compete favorably with papain and bromelin—the two enzymes now currently used as tenderizers—if produced on a commercial scale.

Many pounds of protein-splitting enzymes are used yearly in the tanning industry, since raw hides have to be treated with a proteinase to make them softer and more porous before being submitted to the action of the tanning reagent.

Lastly, the authors wish to call attention to the worm-digesting activity of pinguinain. Experiments have shown that *maya* juice, or the crude enzyme obtained from it, readily digests live intestinal parasites *in vitro* and *in vivo*, in experimental animals. For a long time now the juice of the *maya* fruit has been utilized as an anthelmintic in the folk medicine of the West Indies.

These potential applications of pinguinain in the practical field suggest the desirability of developing its production; so towards this end the Department of Agronomy of the Agricultural Experiment Station at Río Piedras, Puerto Rico, is studying the methods of planting and harvesting the *maya* fruit on a commercial scale.

In the present communication the authors report several observations dealing with the preparation and properties of pinguinain.

EXPERIMENTAL

The maya plant. This plant (Fig. 1) belongs to the pineapple family and has been described by Britton (2) as follows:

"*Bromelia pinguin* L.: Leaves many, tufted stiff, linear long-attenuate, 1–2 m. long, 2–4 cm. wide, light green, the margins armed with stout, rather distant hooked prickles 5–10 mm. long. Inflorescence paniculate, shorter than the leaves, stout, densely white-floccose; bractlets narrow, 5–25 mm. long; sepals narrow, erect, triangular-subulate; petals white or pinkish, about 3 cm. long, linear-elliptic, united below, the apex white-tomentose; stamens about 2 cm. long; anthers yellow, linear, 1–1.4 cm. long; ovary white-farinose, subterete. Berry ovoid, yellow, beaked, verruculose, 3–4 cm. long.

Thickets, hedges, and waste grounds, Porto Rico; St. Thomas, St. John, St. Croix: —West Indies and continental tropical America. Piñuela, Maya, Pinguin."

The plant is extensively utilized in the rural districts of Puerto Rico as a natural barrier or boundary. Attempts were made in the past to use its leaf fibers industrially.

The Maya fruit. The *maya* fruit grows in the center of the plant in the form of a bunch, as shown in Fig. 2, consisting generally of about 63 per cent fruit and the rest stalk. The fruit is available in appreciable amounts between May and November, but is difficult to obtain during the remainder of the year.

Two varieties of *maya* have been observed: a long, thin one that gives a poor yield of juice and a round, thick one, shown in Fig. 2, which yields a goodly amount. The first variety is found in sandy soils near the seashore,

FIG. 1. *Maya* plant with fruit stalk

while the latter grows in the interior of the Island at higher elevations. The authors have primarily utilized the last variety in their work.

Expression of the juice. The juice is prepared by squeezing the pulp in a cheesecloth as follows: the fruit is cut in four pieces; the pulp removed and pressed until dry of juice. The expressed juice is somewhat viscose but, left overnight in the icebox, the sludge settles to the bottom of the vessel and the supernatant fluid may be syphoned and used in the preparation of the enzyme. Attempts to centrifuge out the viscose sludge have proved unsuccessful; it may be removed only as described above, that is, by slow settling in the icebox. It is very important to remove this substance, which is primarily present in the rind of the fruit, before precipitating the enzyme; otherwise the latter is rendered sticky and difficult to dry. The author's

attempt to press the whole fruit produced an exceedingly viscose juice which could not be used in the preparation of the enzyme.

The pH of 10 different samples of *maya* juice fluctuated between 3.70 and 4.30 with an average of 3.75.

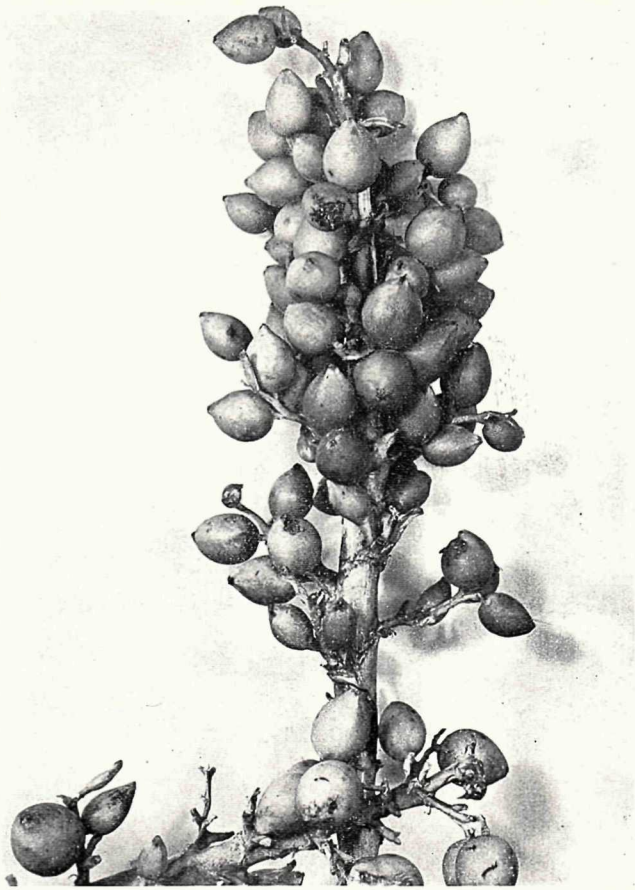

FIG. 2. Close view of fruit raceme

The activity of this juice varies somewhat. Such variation is recorded in table I for three different batches of juice in terms of milk-clotting units[1] and formol titrations (ml. 0.01N NaOH per ml. of juice).[2]

[1] The milk-clotting method of Ball and Hoover (3) was used throughout this work. Only 5 ml. of milk solution per tube were used hence, the milk-clotting units obtained were 0.546 times smaller than those resulting from the use of 10 ml. of milk.

[2] Throughout these studies, the technique utilized in formol titration was as follows: 2 per cent gelatin solution containing 1 per cent toluene, as preservative, was

Preservation of the juice. During the time that the authors have been working with *maya* juice, they observed that its activity diminished rapidly at room temperature, produced primarily by fermentation that set in from four to five hours after the juice was expressed. However, icebox temperatures of 4 to 8°C. preserved the juice for 24 to 48 hours without appreciable loss of activity, after which the activity again began to diminish rapidly.

TABLE I

Proteolytic activity of maya juice

Batch	Milk-clotting units per ml. of *maya* juice	Formol titration ml. 0.01N NaOH per ml. of *maya* juice
1	50.0	3.80
2	45.0	3.70
3	47.6	4.03
Average.....	47.5	3.84

TABLE II

Preservative action of different substances on the milk-clotting activity of maya juice at room temperature (25–28°C.)

No. of days standing at room temperature	Percentage of the original activity lost					
	Thymol 90 mg./100 ml.	Sodium bisulphite 30 mg./100 ml.	Toluene 90 mg./100 ml.	50–50 toluene chloroform mixture 90 mg./100 ml.	Sodium benzoate 90 mg./100 ml.	Merthiolate 30 mg./100 ml.
1	25	38	46	33	52	4
2	50	52	55	54	65	4
6						4
12						4
29						46

Several reagents were tried as preservatives and their effect on the proteolytic activity of the juice was measured by means of the milk-clotting assay at different time intervals (Table II). Merthiolate (Sodium Ethyl Mercuri Thiosalicylate) was the only reagent that gave satisfactory results. Thirty milligrams kept 100 ml. of juice at room temperature for 12 days

used as a substrate. One ml. of the juice to be tested was then added to 10 ml. of this gelatin solution and one ml. samples taken for titration. The indicator was a solution containing 0.2 per cent phenolthalein in 50 per cent alcohol. One ml. of 40 per cent formaldehyde was then added to each volume of mixture to be titrated; the strength of the sodium hydroxide solution was 0.01N. These tests were run for a period of 24 hours at an incubation temperature of 40°C.; no buffer was used. The natural pH of the mixture was found to be in the neighborhood of 4.5 to 5.

with only a slight loss of activity. However, after 29 days, the percentage loss of the original activity was 46 per cent. The authors used merthiolate at the above concentration all through the work to preserve juices and enzyme solutions which were kept for a few days with very good results.

TABLE III

Percentage yield of pulp, juice and enzyme preparation by weight of fresh fruit

Fruit batch no.	Percentage of pulp by weight of fresh fruit	Percentage of juice by weight of fresh fruit	Percentage enzyme yield by weight of fresh fruit
1	57.0	29.5	0.5
2	59.9	30.5	1.0
3	59.5	21.6	0.4
4	65.0	36.4	2.3
5	66.6	32.3	1.7
Average.....	61.6	30.1	1.1

TABLE IV

Proximate composition of crude pinguinain

Total moisture per cent.....	6.0
Reducing sugars per cent.....	11.0
Nitrogen per cent.....	2.7
Substances soluble in ether per cent.....	1.1
Ash per cent.....	10.2

TABLE V

Milk-clotting activity of pinguinain

Batch No.	Milk-Clotting Units Per Gram
1	434.7
2	500.0
3	526.8
4	416.7
Average.....	469.5

Preparation of the enzyme. The crude enzyme was prepared by adding three volumes of acetone to one of juice, the precipitate obtained thereby separated by centrifugation, washed with fresh acetone and diethyl ether, and dried in a vacuum desiccator over $CaCl_2$.[3] The yield of enzyme for

[3] In the original communication (1) the authors recommended dissolving this precipitate in 0.02 M NaCN and reprecipitating it with acetone. Later on they found that this step could be eliminated without affecting the quality of the final product.

several batches of fruit is recorded in Table II and averaged 1.1 gm. per 100 gm. of fruit, or 3.7 gm. per 100 gm. of juice.

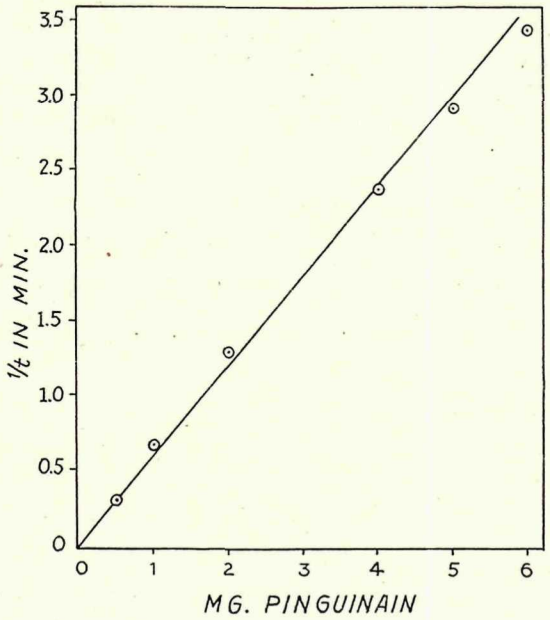

FIG. 3. Milk-clotting activity of pinguinain dissolved in 0.05 M NaCN

TABLE VI

Milk-clotting by pinguinain in 0.05M NaCN

Amount of Enzyme Per 6 ml. (E)	Time of Coagulation (t)	1/t	E.t = K
mg.	*min.*		
6.0	0.29	3.45	1.7
5.0	0.34	2.94	1.7
4.0	0.42	2.38	1.7
2.0	0.77	1.32	1.5
1.0	1.50	0.67	1.5
0.5	3.42	0.30	1.7
Average			1.6

General characteristics of the crude pinguinain. Generally, pinguinain has a very light greenish yellow color. It is an amorphous powder that dissolves somewhat slowly and produces an opalescent solution. This enzyme readily reduced Fehling's solution and gave a positive Millon's reaction for proteins. Its proximate composition is shown in Table IV.

Activity of pinguinain. The milk-clotting activity of four different batches of pinguinain are recorded in Table V, with an average activity of 469.5 milk-clotting units per gram.

The milk-clotting activity of various dilutions of pinguinain were determined by using 0.05M NaCN as diluent and the results obtained plotted in Fig. 3. The reciprocal of the milk-clotting time varied linearly between 0 and 6 mg. of enzyme. As in the case of crystalline chymotrypsin (3), apparently none of the enzyme was inactivated, as the curve passed through

TABLE VII

Digestion of casein in water by different amounts of pinguinain

Enzyme in 6 ml. digestion mixture	N. P. N. in 6 ml. digestion mixture 20 min. digestion
mg.	*m-eq.*
10.0	0.330
5.0	0.249
1.0	0.044
0.5	0.032
0.1	0.014

TABLE VIII

Digestion of hemoglobin in urea at 40°C. by pinguinain dissolved in 0.05M NaCN

Enzyme in 6 ml. of digestion mixture	Tyrosine in 6 ml. digestion mixture after 10 minutes
mg.	*m.-eq.* $\times 10^3$
10.0	6.37
5.0	4.90
3.0	3.6
1.0	1.82
0.5	1.21
0.1	0.19

the origin; therefore, the simple equation $E \cdot t = K$ held for this range of dilutions.

This particular batch of pinguinain, when dissolved in 0.05M NaCN, had an activity of 606 milk-clotting units per gram, as computed from table VI.

The proteolytic activity of pinguinain on casein and hemoglobin was measured by the Northrop (5) and the Anson (6) methods, respectively; the results of these determinations are given in Tables VII and VIII.

The quantity of casein digested, in m-eq., was plotted against the quantity of enzyme (Fig. 4); the variation was approximately linear up to 5 mg. of enzyme. From a straight line drawn tangent to the initial part of this

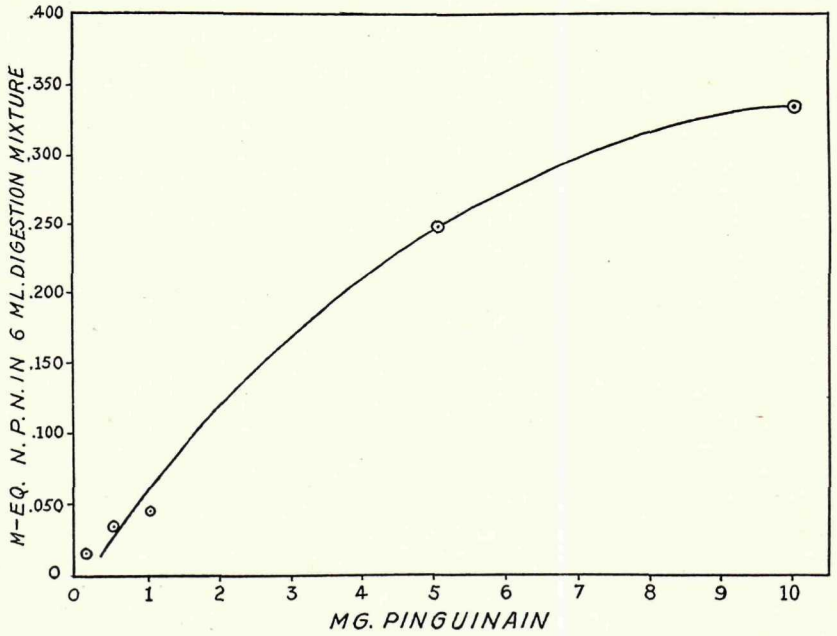

FIG. 4. Digestion of casein by pinguinain

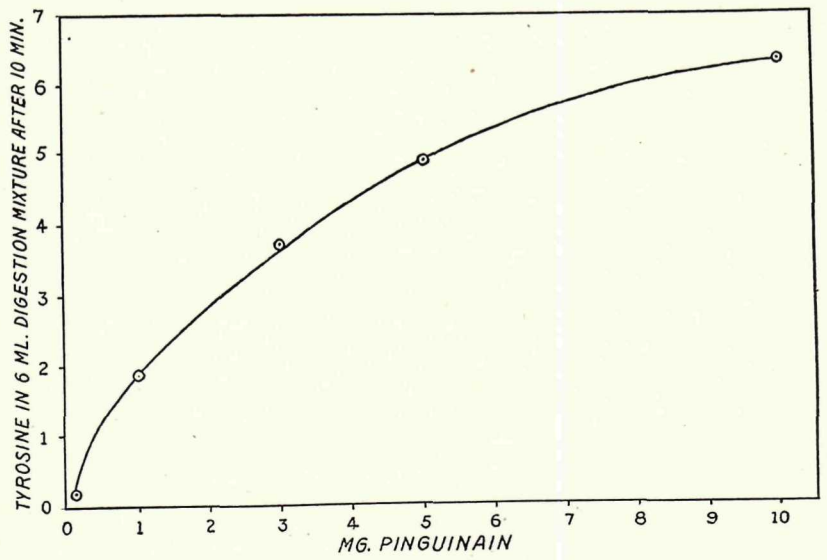

FIG. 5. Digestion of hemoglobin in urea by pinguinain dissolved in 0.05 M NaCN. Tyrosine is in m.-eq. $\times 10^3$

curve, the quantity of N. P. N. (non-protein nitrogen) in 6 ml. digestion mixture, yielded by 1 mg. of pinguinain, was found to be 0.055 m-eq. per mg. of the enzyme.

The proteolytic activity of pinguinain on hemoglobin was evaluated from a plot of tyrosine color against weight of enzyme. The pinguinain was dissolved in 0.05M NaCN to prevent its inactivation by the oxidative action of hemoglobin.

By drawing a line tangent to the first part of the curve, (Fig. 5) the Anson protease unit[4] was calculated. It was found that 0.4 mg. of pinguinain gave a color value of 0.001 m.-eq. of tyrosine in 6 ml. digestion mixture after ten minutes. One Anson unit was, therefore, contained in 0.4 × 1000 × 10 = 4000 mg., or one gram of enzyme contained 0.25 Anson hemoglobin units.

TABLE IX

Activation and inhibition of pinguinain

Reagents added to enzyme solution		Reciprocal of the milk-clotting time in min. 4 mg. enzyme	Ratio of activity to that of untreated enzyme
Inhibitor	Activator		
None*	None	1.560	1.00
0.00014M I_2	None	0.189	0.12
0.00014M I_2	0.2M NaCN	1.190	0.76
0.00028M I_2	None	0.086	0.06
0.00028M I_2	0.2M NaCN	1.062	0.68
0.00014M I_2	None	0.183	0.12
0.00014M I_2	0.3M cysteine HCl	1.390	0.89
0.00014M I_2 and boiled	0.3M cysteine HCl	0.000	0.00
None	H_2S bubbled for 3 min.	2.130	1.36

* The untreated enzyme solution contained 4 mg. enzyme per ml. The pH of the untreated solution was 5.9.

Activation and inhibition of pinguinain. Pinguinain, like other papainases, responds readily to the activating action of some reducing agents and also to the inhibiting action of some oxidizing ones. Table IX records the action of some of these agents on the milk-clotting action of pinguinain.

Action of pinguinain on intestinal parasites. *Maya* juice, as well as the crude enzyme, readily digests intestinal parasites *in vitro* and *in vivo*. Dog *Ascaris* and *Macracanthorhynchus hirudinaceus* from hog intestines were incubated at 40°C. with *maya* juice and with a one per cent solution of pinguinain. These parasites showed definite signs of digestion in two

[4] Anson defines one protease hemoglobin unit as the amount that will digest hemoglobin under standard conditions at an initial rate so that there is liberated per minute an amount of split products not precipitated by trichloroacetic acid which will give the same color with phenol reagent as one milliequivalent of tyrosine.

hours and were totally disintegrated in ten. Controls, in solution, inactivated by previous heating to 100°C., were not digested.

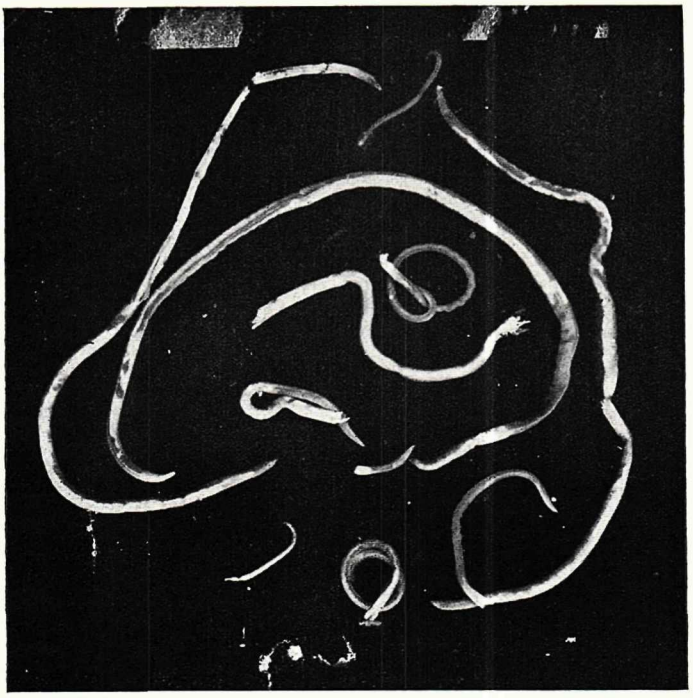

FIG. 6. *Ascarids* digested *in vivo* by pinguinain

TABLE X

Action of maya juice and pinguinain on dog ascaris in vivo

Dog No.	Dog Weight in kg.	Dose Administered	Egg Count		Number of partly digested ascarids in stools	Autopsy Findings
			Before Treatment	After Treatment		
1	2.3	10 gms. Pinguinain	19,972	3,233	5	
1	2.3	200 ml. *maya* juice	3,233	negative	1	No ascarids found in the intestine
2	3.5	200 ml. *maya* juice	5,167	negative	4	No ascarids found in the intestine

Animal experiments were carried out on dogs infected with *Ascaris*. On administering both the juice and the enzyme, by the use of stomach tube, the ascarids were expelled in the feces in a partially digested condition (Fig. 6). The details of this experiment are reported in Table X.

Both the fresh *maya* juice and the pinguinain in the dosages given above readily digest dog *Ascaris in vivo*.

SUMMARY

Some of the uses, methods of preparation, and properties of pinguinain, the protein-splitting enzyme of the *maya* fruit, have been considered and studied herein.

RESUMEN

La pinguinaina es un enzimo proteolítico del grupo de las papainasas, la cual se haya en el zumo de la fruta de la maya (*Bromelia pinguin* L.). En el presente artículo se describen sus posibles usos medicamentosos e industriales y se estudia su preparación, conservación y acción proteolítica sobre varios substratos.

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