

ENRICHMENT OF A FRACTION TOXIC TO GUINEA-PIGS FROM *PACHYSTIGMA PYGMAEUM* (SCHLTR.) ROBYNS

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ABSTRACT

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Pachystigma pygmaeum is one of several species of rubiaceaceous plants which cause delayed heart failure among ruminants after their ingestion at relatively high doses. Using guinea-pigs for toxicity determinations, we were able to separate and enrich a toxic fraction from a fermentation extract of the plant material by countercurrent distribution. It contained virtually no potassium salts, passed through a 500 dalton selective membrane, exhibited lability under acid conditions and was toxic at 1 g/kg *per os*, with a delayed response of 3-4 days.

INTRODUCTION

"Gousiekte" (literally translated as "quick disease") is characterized by sudden death due to heart failure 4-8 weeks after ingestion of a lethal amount of the causative plant (Theiler, Du Toit & Mitchell, 1923). The 5 known causative species are from the *Pachystigma*, *Pavetta* and *Fadogia* genera of the rubiaceaceous family of plants and are indigenous to the Transvaal and bordering areas (Codd & Voorendyk, 1966).

The disease occurs only among domestic ruminants, as monogastric animals are not affected and ruminant game appear to avoid it by selective grazing. *Post-mortem* histopathology of the disease reveals degenerated heart muscle cells and intrusion of connective tissue, especially in the apex (Smit, 1959).

Research on the physiology and biochemistry of "gousiekte" poisoning can be of benefit to both agriculture and medicine, as it might shed light on the complexities of human heart disease. Several attempts at elucidating the mechanism of action of the toxic principle have been made (Pretorius & Terblanche, 1967; Pretorius, Terblanche, Van der Walt & Van Ryssen, 1973; Snyman, Van der Walt & Pretorius, 1982a; Snyman, Van der Walt & Pretorius, 1982b). An impaired contractility of the heart muscle was observed which could be due either to binding of the toxic entity to the contractile proteins or by the inhibition of the normal energy metabolism of the heart cells. The purified toxic principle would greatly assist in resolving these issues. Isolation of the toxic entity has long been hampered by the weak extractability of the toxic entity from the plant material. Recently, we reported a method whereby liberation of an entity toxic to guinea-pigs from *P. pygmaeum* was achieved by fermentation of the plant material with baker's yeast (Verschoor & Potgieter, 1984). A crude extract was thus obtained with sufficient yield of toxicity for use in further isolation work. We are now able to report a significant enrichment of toxicity from the fermentation extract and present data on some physical and chemical properties of the enriched toxic fraction.

MATERIALS AND METHODS

All reagents were of chemically pure grade except those used for analysis, which were of analytical grade.

Plant material

P. pygmaeum was generously supplied by Mr C. J. Sieberhagen of the farm Illmasdale, which is located near Derby in the Western Transvaal. Collections of plant material were made over 8 seasons from 1976-1983. The plants were destemmed, washed with water, distributed in small plastic bags and stored frozen at -10 °C.

Pach F

Frozen leaves were freeze-dried in 1 kg batches in bags of cheese cloth and ground to a fine powder in a Siebtechnik disc mill.* This material was denoted *Pach F*.

Fermentation extracts

Fermentation extracts were made from *Pach F* as described previously (Verschoor & Potgieter, 1984).

Acetone fractionation of fermentation extracts

A 5 % (w/v) solution of lyophilized fermentation extract in water was adjusted to 50 % acetone in the cold (-10 °C). The precipitate was allowed to settle for an hour before it was centrifuged down and discarded. Acetone was removed from the supernatant by flash evaporation at room temperature prior to lyophilization. The dry supernatant was denoted ASF.

Countercurrent distribution (CCD)

The 50 % acetone supernatant fraction (ASF) of fermentation extract was fractionated by 2 subsequent CCD runs as follows:

1. CCD with n-butanol, acetone and water:

A phase system was composed which gave equal volumes of organic (48,0 % n-butanol, 15,0 % acetone and 37,0 % water) and aqueous (12,3 % n-butanol, 10,4 % acetone and 77,3 % water) phases. A 7,5 % solution of ASF in aqueous phase was shaken up with an equal volume of organic phase and loaded on a 500 transfer Craig-Post CCD apparatus** after removal of an interphase. The separation was done at room temperature over 230 transfers with 10 min equilibration times between transfers, while maintaining equal volumes (10 ml) of the 2 phases during the operation. Fractions were collected in pear flasks and concentrated by means of an ebullition tube under nitrogen at room temperature, followed by lyophilization.

2. CCD with diethyl ether, ethanol and water:

The toxic fraction 3 obtained from CCD with butanolic phase system (3 g) was suspended in 50 ml aqueous phase (16 % diethyl ether, 26,5 % acetone and 57,5 % water). After sedimentation of the insolubles, the supernatant was loaded in the first 5 tubes of the Craig-Post apparatus and developed over 150 transfers with organic phase (71,5 % diethyl ether, 20,5 % ethanol and 8 % water) in equal phase volumes. Concentration and lyophilization of fractions were done as already described.

Thin layer chromatography (tlc) of fractions

The effectiveness of the attempts at purification was monitored by tlc on pre-manufactured, 5 × 10 cm plates (silica gel plates 60 F254)*** developed in n-butanol: water:acetone (50:30:15). Sample solutions of 0,5 %

* Ferguson Industrial Equipment Co., Johannesburg.

** H.O. Post Scientific Co. Inc., Middle Village, N. Y.

*** Merck Chemicals, Johannesburg

TABLE 1 Countercurrent distribution of ASF in n-butanol-acetone-water

| Fraction No. | Distribution constant (Kd) | Mass yield (%) | Toxicity (%) | Toxicity g/kg | Acid content (m.eq/g) | Potassium content (%) | Nitrogen content (%) |
|-------------------------|----------------------------|----------------|--------------|---------------|-----------------------|-----------------------|----------------------|
| Starting material (ASF) | — | 100 | 100 | 12/acute | 1,4 | 3,6 | 0,74 |
| 1 | 6,58 | 2 | — | — | — | — | 0,48 |
| 2 | 2,55 | 3 | 11 | 3/delayed | 1,4 | <0,1 | 0,34 |
| 3 | 1,51 | 8 | 30 | 3/delayed | 1,2 | <0,1 | 0,36 |
| 4 | 0,86 | 10 | 7 | 16/acute | 3,7 | <0,1 | 0,76 |
| 5 | 0,43 | 62 | 43 | 20/acute | 1,0 | 4,7 | 0,69 |
| 6 | 0,15 | 15 | | | 0,3 | 5,9 | 0,68 |

TABLE 2 Enrichment of toxicity from fermentation extracts of *P. pygmaeum* by countercurrent distribution

| Material | Mass yield (%) | Toxicity ⁽¹⁾ (g/kg) | Toxicity yield (%) | Enrichment |
|----------------------------------|----------------|--------------------------------|--------------------|------------|
| Fermentation extract | 100 | 16/acute | 100 | 1,0 |
| 50 % acetone supernatant (ASF) | 77 | 12/acute | 100 | 1,3 |
| Butanolic CCD fractions 2 and 3 | 8 | 3/delayed | 42 | 5,3 |
| Toxic fraction from ethereal CCD | 1,4 | 1/delayed | 22 | 16 |

⁽¹⁾Owing to seasonal variation of toxicity, repeats of the enrichment procedure showed variations in toxicity of fractions, but this did not significantly affect the toxicity yields

were made in suitable solvents and 10 $\mu\ell$ spotted on the plates, CCD separations were monitored by spotting 10 $\mu\ell$ of the organic phase of each tube. Visualization was done under UV light at 254 and 366 nm.

Preparative tlc

Preparative tlc separation of the toxic fraction obtained by CCD in the ether-ethanol-water phase system was performed on premanufactured plates of 200 \times 200 \times 2 mm (Kieselgel 60 F254)*. The material was toxic at at least 2 g/kg (n=2). Each plate was loaded with 100 mg material in ethereal upper phase by means of an appropriate applicator** and developed with n-butanol:water:acetone (50:30:15) over 15 hours. The bands visualized under UV light were scraped off, extracted with methanol and concentrated and lyophilized as described for CCD fractions.

Toxicity determinations

Toxicity determinations were done by *per os* dosage to Albino Wistar guinea-pigs of both sexes. The animals were deprived of food for 6 hours prior to dosing. Aqueous suspensions were made and administered by means of a stomach tube (a shortened, nasal paediatric feeding tube). Acute toxicity is defined as death occurring within 24 hours after dosing, while delayed toxicity applies to death occurring between 2 and 4 days after dosing.

Heat and acid lability

Heat lability tests were previously done by heating an aqueous suspension of a lethal dose of a toxic fraction in a boiling water bath for 20 minutes, followed by cooling and dosing (Verschoor & Potgieter, 1984). To correlate heat lability with possible decomposition of toxicity under acidic conditions, the procedure was applied to toxic fractions, but after neutralization to pH 7,0 with alkali. After the heat treatment, the suspension was acidified back to its original pH (2,7) with dilute sulphuric

acid and dosed. As control a non-neutralized sample was heated, an equivalent amount of sodium sulphate added, and dosed.

Membrane ultrafiltration

A solution of fermentation extract in water (5 %) was filtered through and exhaustively washed on an UMO5 membrane in a Diaflo ultrafiltration apparatus* with nitrogen pressure. After freeze-drying, both the retentate and filtrate were dissolved to 2,5 % in the aqueous phase of the butanolic system used in CCD (see above) and shaken up with an equal volume of upper phase. After separation, the phases were separately concentrated and lyophilized, as described for CCD fractions.

Potassium, acid and nitrogen determinations

Potassium and acid contents of fractions were determined as previously described (Verschoor & Potgieter, 1984). Nitrogen was determined by a modified Kjeldahl procedure (Bradstreet, 1954). Values for nitrogen and potassium content are expressed as % of oven-dried (110 °C, 16 h) material and acid content as milli-equivalents per gram of lyophilized sample.

RESULTS AND DISCUSSION

Acetone fractionation of fermentation extracts

A macromolecular fraction toxic to guinea-pigs was previously enriched from pressed out juice of *P. pygmaeum*, but at such low yields that alternative methods for the preparation of a toxic crude extract with sufficient yield of toxicity were sought (Potgieter, Jordaan, Cronje & Meij, 1975). Fermentation of aqueous suspensions of *Pach F* with baker's yeast resulted in an extract which apparently gave quantitative recovery of toxicity as measured in guinea-pigs and extrapolated to the LD 100 (200 g/kg) of the fresh plant material in sheep (Cronje, 1976). Fractionation of fermentation extract with 50 % acetone gave quantitative recovery of toxicity in the supernatant, which indicated that the entity toxic to guinea-pigs had lost its macromolecular properties during fermentation.

* Merck

** Burkard SA 40, Burkard Manufacturing Co., Rickmansworth, England

* Amicon Corporation, Cambridge, Massachusetts

TABLE 3 Preparative tlc of the toxic fraction from the ethereal CCD

| Band No. | R _f | Visual appearance at 366 nm | Mass yield (%) | Delayed toxicity ⁽¹⁾ (g/kg) |
|----------|----------------|-----------------------------|----------------|--|
| 1 | 0,82 | White | 16 | 1,2 |
| 2 | 0,70 | Brown | 23 | 1,7/NT |
| 3 | 0,62 | White | 5 | 1,4/NT |
| 4 | 0,56 | White | 13 | |
| 5 | 0,48 | Brown | 25 | 1,9/NT |
| 6 | 0,37 | Blue | 11 | 0,9/NT |
| 7 | 0,29 | Yellow | 7 | 0,6/NT |

⁽¹⁾Determined by dosing the total yield recovered of each band from 11 preparative plates. NT = non-toxic

TABLE 4 Heat and acid lability of a lethal dose of the enriched toxic fraction obtained after butanolic CCD with a pH of 2,7 in aqueous suspension

| Experiment | Action taken | | | Toxicity at 5 g/kg |
|-------------------------------------|-----------------------|----------------------------|------------------------|--------------------|
| | Neutralized to pH 7,0 | Heat treated 90 °C, 20 min | Re-acidified to pH 2,7 | |
| Control | — | — | — | Lethal |
| Heat treatment | — | — | — | Non-toxic |
| Neutralization | — | — | — | Non-toxic |
| Neutralization and re-acidification | — | — | — | Lethal |
| Heat treatment at neutral pH | — | — | — | Lethal |

Countercurrent distribution of ASF

Enrichment of toxicity from ASF was obtained by countercurrent distribution with a phase system composed of n-butanol, acetone and water. Results of one such an experiment appear in Table 1. The major achievement of this attempt was the separation of components which caused acute death at relatively high doses (16 to 20 g/kg) in fractions 4–6 from components which caused a delayed response at relatively low doses (3 g/kg) in fractions 2 and 3. Acid and potassium determinations revealed that 71 % of the organic acids and virtually all the potassium salts of the ASF resided in fractions 4–6 after the distribution. Thus, the acute toxicity caused by fractions 4–6 in guinea-pigs was probably due to metabolic acidosis and potassium toxicity, while the delayed toxicity of fractions 2 and 3 was due to some unique toxin or toxins from *P. pygmaeum*. In repeats of the experiment, the toxic fractions 2 and 3 could be obtained at $1,65 \pm 0,04$ g (n=14), or 11 % yield of the fermentation extract and toxicity of the combined fractions was ascertained by a single successful dosing at 4 g/kg.

The toxicity in fraction 3 of the butanolic CCD separation was enriched further by CCD with a biphasic solvent system composed of diethyl ether, ethanol and water. In this experiment toxicity was found in the non-polar half of the distribution pattern. Other fractions were found to be non-toxic at the level of the starting material (3 g/kg). In total, a sixteenfold enrichment of toxicity of the delayed type was obtained with a potential toxicity yield of 22 % calculated from the fermentation extract (Table 2). Repeats of the experiment were done on the combined butanolic fractions 2 and 3. Toxicity (better than 2 g/kg, n=2) was found in the Kd range of 1,0 and was represented in a fraction of $1,04 \pm 0,13$ g yield, or 10,4 % of the material loaded. Enrichment of toxicity was found to be inversely proportional to the nitrogen content of the fractions.

Preparative tlc

In spite of the enrichment, the toxic fraction from the ethereal CCD was still complex and exhibited at least 8

components with tlc with R_f ranging from 0,2–0,9. Preparative tlc of this fraction indicated that the toxicity was concentrated in the least polar part of the chromatogram visualized as a white fluorescent band of R_f 0,82 under UV-radiation at 366 nm (Table 3). It consisted of only 16 % of the material chromatographed. The dosed guinea-pig appeared perfectly healthy for 2 days after administration of the fraction, but died during Day 4.

Heat and acid lability

The results of experiments done to illustrate heat and acid lability of the combined fractions 2 and 3 of the butanolic CCD are summarized in Table 4. The pH of an aqueous suspension of this material was 2,7.

A loss of toxicity was observed when this fraction was heat treated. Adjustment of the pH to 7,0 resulted in a loss of toxicity which could be reversed by acidification back to pH 2,7. No loss of toxicity was observed when heat treatment was done on material neutralized to pH 7,0 and re-acidified before dosing. These results suggest that toxicity is heat labile only under acidic conditions.

Membrane ultrafiltration

In an attempt to estimate the molecular mass of the entity toxic to guinea-pigs from fermentation extracts, aqueous solutions of the material were passed through selective membranes. Even with the smallest membrane (a 500 molecular mass cut-off UM 05) we observed only a partial retention. Toxicity due to acids and potassium salts causing metabolic acidosis was distinguished from heat labile toxicity by fractionating the retentate and filtrate by phase separation in the butanolic CCD phase system. Accordingly, delayed responses with low dose levels were obtained with the organic phases, while acute (acidotic) responses were obtained at high dose levels with the aqueous phases (Table 5). The toxicity of the organic phase could only partially be retained on the membrane, suggesting a molecular mass in the range of 500.

TABLE 5 Toxicities of fractions of a fermentation extract of *Pach F* after UM 05-membrane filtration and phase separation

| | Mass yield (%) | Acute toxicity (g/kg) | Delayed toxicity (g/kg) |
|--|----------------|-----------------------|-------------------------|
| Starting material fermentation extract | 100 | 16 | — |
| (1) <i>Retentate</i> | 77 | Not determined | Not determined |
| (a) Organic phase ⁽¹⁾ | 12 | — | 10 |
| (b) Aqueous phase | 65 | 28 | — |
| (2) <i>Filtrate</i> | 23 | Not determined | Not determined |
| (a) Organic phase ⁽¹⁾ | 3 | — | 4 |
| (b) Aqueous phase | 20 | 10 | — |

⁽¹⁾ The phase system was the same as that used in the butanolic CCD

CONCLUSION

A fraction toxic to guinea-pigs and eliciting a delayed response, was enriched sixteenfold from fermentation extracts of *P. pygmaeum* by 2 subsequent countercurrent distributions, with phase systems composed of n-butanol-acetone-water and diethyl ether-ethanol-water respectively.

The heat labile part of the toxicity of the fermentation extract previously observed (Verschoor & Potgieter, 1984), could be separated from heat stable toxicity by CCD with the butanolic phase system. The high acid and potassium contents of the heat stable toxic fractions, as well as the low level of toxicity and the acute response they induced, suggest that their lethality might be the result of metabolic acidosis and potassium poisoning only. The heat lability of the enriched toxic fraction was shown to be due to an accelerated decomposition under acidic conditions.

It was found that the buffering capacity of the enriched dosed material affected the normal pH of the stomach for at least 1 hour after dosing even at 3 g/kg (data not shown). The reversible loss of toxicity upon increasing the pH of the enriched toxic fraction before dosing may suggest that absorption of toxicity from the stomach or intestines requires a low pH. This property should be borne in mind when using small laboratory animals in comparisons of the relative toxicities of these fractions.

The results obtained by membrane filtration and phase distribution of fermentation extracts suggest that the heat labile entity toxic to guinea-pigs has a molecular mass of approximately 500, or that it may exist in different molecular sizes, the smallest of which is smaller than 500.

This work does not prove that the toxic entity that was enriched is indeed the causative agent of "gousiekte". However, its heat lability and the delayed response which it induced in guinea-pigs are to some extent in agreement with observations with toxic plant preparations in ruminants.

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