IS *PENICILLIUM CITRINUM* IMPLICATED IN SAGO HEMOLYTIC DISEASE?

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**Abstract.** Sago hemolytic disease (SHD) is an acute hemolytic syndrome affecting rural Papua New Guineans who depend on the starch of *Metroxylon sagu* as a staple carbohydrate. It is a suspected mycotoxicosis associated with fungal succession in stored and perhaps poorly fermented sago. Despite a mortality rate of approximately 25%, little is known about the disease. Recent studies have identified *Penicillium citrinum* as a possible candidate in the etiology of SHD. This is based on the frequency of isolation from sago starch and the hemolytic nature of the organism as demonstrated when cultured on sheep and human blood agar. A highly non-polar lipophilic *P. citrinum* fraction from C18 solid phase extraction demonstrated high hemolytic activity in a semi-quantitative assay using both mouse and human erythrocytes. When the red cell membrane proteins were subjected to sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, cleavage of protein band 3 and spectrin was demonstrated. This breach of major structural red cell proteins is consistent with the severe hemolysis found in vivo. Our findings warrant further investigation into the hemolytic activity of *P. citrinum* and its role as the etiological agent of SHD.

**Key words:** *Penicillium citrinum*, sago hemolytic disease, mycotoxicosis

**INTRODUCTION**

Subsistence dwellers in lowland areas of Papua New Guinea (PNG) derive the majority of their dietary carbohydrates from the pith of the sago palm, *Metroxylon sagu*. Familial outbreaks of acute intravascular hemolysis, known as sago hemolytic disease (SHD), have been associated with the consumption of stale sago starch (Taufa, 1974; Donovan *et al*, 1976, 1977). The disease is characterized by acute hemolytic anemia, severe dehydration, fever, headache, vomiting, jaundice, lethargy, grinding of teeth, hematuria and splenomegaly. Clinical signs of SHD develop rapidly and are associated with a mortality rate of approximately 25%, with
death typically occurring within 72 hours of disease onset (Taufa, 1974; Donovan et al, 1976, 1977). The incidence of SHD remains speculative, but is likely to be under diagnosed because of a lack of healthcare facilities where it is endemic (Greenhill, 2006).

On account of the apparent food-borne nature of the illness and the rapid onset of symptoms, it seems likely microbial contamination, and more specifically, preformed microbial toxins, play a role in SHD. Although several studies of the microbiology of sago starch have been conducted (Donovan et al, 1977; Greenhill et al, 2007a, b) the etiology of SHD remains elusive. The absence of common food-borne mycotoxins in sago starch (Greenhill et al, 2008) led to studies that identified a number of organisms that were intensely hemolytic in vitro (Greenhill et al, 2010a). One of the most commonly occurring organisms was Penicillium citrinum, which was isolated from both stale sago and sago considered fit for consumption (Greenhill, 2006). The purpose of this study was to investigate the in vitro hemolytic activity of a fraction of P. citrinum and to determine its action on human erythrocyte proteins. We also compared the activity of the fraction in human and mouse erythrocytes, to gauge the viability of using mice as an animal model for SHD research.

MATERIALS AND METHODS

Fungal extracts

P. citrinum was cultured on moist sterile wheat and extractions conducted in hexane, as previously described (Greenhill et al, 2010a). Extractions were fractionated using methanol:water (80:20) on a C18 solid phase extraction column (Varian Bond Elut LRC-SI, Australia). Activity driven fractionation using the hemolytic assay developed by Greenhill et al (2010a) resulted in a non-polar lipophilic hemolytic fraction that was dried and stored at -20°C. The extract was resuspended in methanol at a concentration of 1 g/ml as a stock solution, from which working concentrations were made.

Human and mouse erythrocytes

Blood samples were taken from a human volunteer (n=1) and male 7-13 week old BALB/c mice (n=15). Mice were euthanized with CO2 and blood was subsequently collected from the aorta. The use of the animals was approved by the Ethics Committee for the Use of Animals at James Cook University.

Hemolytic assay

Screening for hemolytic activity was based on the method developed by Greenhill et al (2010a) with minor modifications to adjust for the fragility of mouse blood. Whole blood was centrifuged at room temperature in a microcentrifuge for 5 minutes at appropriate centrifugation speeds to ensure the physical integrity of erythrocytes was maintained (3,000 g for human blood, 1,000 g for mouse blood). The plasma and buffy coat were removed and erythrocytes were washed in 20 volumes of phosphate buffered saline (PBS) and centrifuged as above. This process was repeated three times.

Slightly different volumes of human and mouse erythrocytes were used so a comparable mass of erythrocytes was used in both assays, taking into account the different sizes of human and mouse erythrocytes. Dilutions were made in PBS until the packed cell volume was comparable for both species.

For quality control, the hemolytic activity of P. citrinum was compared to C. perfringens phospholipase C (Sigma, NY), as its hemolytic activity has previously
been established (Bowman et al., 1971; Sakurai et al., 2004).

Human and mouse erythrocyte suspensions (150 µl) were incubated with 0.5 mg of P. citrinum fraction or phospholipase C. Both the P. citrinum fraction and phospholipase C were at a final concentration of 3.3 µg/µl. Duplicate negative controls (reagent blank) and one positive control (erythrocytes completely hemolyzed by the addition of 50 µl of 10% Triton-X 100) were used. Sampling occurred after incubation periods of 30, 60, 120 and 240 minutes. The samples were centrifuged (1,000 g, room temperature, 5 minutes) and absorbance of the supernatant was read at 540 nm (Labsystems Multiscan EX, Pathtech, Australia). The negative control values obtained in the assay were subtracted from the test values and presented as percentage hemolysis of the positive control.

**Extract activity on erythrocyte membrane proteins**

Equal concentrations of hemoglobin-free human and mouse erythrocyte membranes (ghost cells) were prepared as described by Dodge et al. (1963). This was to accurately compare the proteolytic activity of the P. citrinum fraction on the two species. Protein estimation was conducted on the ghost cells using the BCA Protein Assay Kit (Pierce, IL), following the manufacturer’s instructions.

Aliquots of the hemolytic fraction of P. citrinum (12.5 µg/µl) were added to an equal volume of erythrocyte proteins (750 µg/ml) to give a final concentration of 6.25 µg/µl in a 30 µl reaction mixture. Reactions were incubated at 37°C for 1 hour. Two sets of negative controls were employed; ghost cells in lysis buffer, and the P. citrinum fraction in lysis buffer (12.5 µg/µl). The positive control consisted of phospholipase C in lysis buffer (2.5 µg/µl) and 750 µg/ml erythrocytes. All reactions were incubated at 37°C for 1 hour.

Following incubation, 15 µl of each sample was added to 15 µl of sample buffer and heat treated at 100°C for 3 minutes in preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein separation of samples was conducted using 10% polyacrylamide gel (Pierce, Australia). The gel was run in a Mini-PROTEAN 3 SDS-PAGE tank (Bio-Rad, CA) and electrophoresed at 100 V for 60 minutes in Tris-HEPES-SDS running buffer (Pierce, IL). Thereafter, the gel was stained with silver stain. Images were captured by digital photography with Gel Doc 1000 hardware (BioRad, CA) and a hand held digital camera.

**RESULTS**

**Hemolytic activity**

The hemolytic assay demonstrated the non-polar lipophilic fraction from P. citrinum is hemolytic in vitro. There was a notable difference observed in the hemolytic activity using mouse and human erythrocytes. In human erythrocytes the P. citrinum fraction and phospholipase C were both highly active, resulting in 80-100% hemolysis within 30 minutes. In comparison, 50-80% hemolysis was observed in mouse erythrocytes (Fig 1).

**Activity of fraction on erythrocyte membrane proteins**

Qualitative differences were observed by SDS-PAGE between species. Proteolytic activity of the P. citrinum fraction was detected on both human and mouse erythrocyte membrane proteins (Figs 2 and 3). The P. citrinum fraction incubated with the human membrane proteins resulted in changes to α and β spectrin, ankryin and in particular protein band 3, relative to the
Fig 1–Hemolytic activity of Penicillium citrinum fraction (3.3 µg/µl) and phospholipase C toxin (3.3 µg/µl) on human and mouse erythrocytes.

DISCUSSION

We have demonstrated the hemolytic activity of the P. citrinum fraction used in the current study is proteolytic and acts on major structural proteins of the red cell membrane. The fraction was active on human erythrocytes, consistent with a role in human disease; and mouse erythrocytes, suggesting that an animal model may be useful in future studies to further elucidate the pathophysiology of this disease.

Two different forms of cleavage were observed by the P. citrinum fraction on human and mouse erythrocyte membrane protein band 3 (Figs 2 and 3). On silver stained human erythrocyte membrane protein, protein band 3 was less diffuse than the control (Fig 2). No extra bands could be identified with the highly sensitive silver stained gel. However, in mouse erythrocyte membrane proteins treated with the P. citrinum fraction, two additional bands could be observed of approximately 58 and 35 kDa (Fig 3). The estimated molecular weight of these two bands add up to 93 kDa, which closely approximates the molecular weight of the erythrocyte membrane protein band 3. This suggests the proteolytic activity of the P. citrinum extract is predominantly toward protein band 3. Although band A (α and β spectrin) also appeared to be affected by P. citrinum extract, the size of the new protein bands probably does not represent spectrin, a larger molecule with a molecular weight of approximately 240 kDa. The slight differences seen between the hemolytic activity of the P. citrinum fraction on human and mouse erythrocytes in the hemolytic assay and the nature of the proteolytic activity observed in this study may be due to slight structural differences between the mouse and human protein band 3. Glycophorins on the mouse erythrocyte exist in lower concentrations than...
in humans and structural differences also exist between the erythrocytes of the two species (Howard et al, 1979).

Many of the hemolytic compounds isolated from bacteria and fungi are proteins, peptides or lipophilic compounds (Schmitt et al, 1999; Bennett and Klich, 2003). Phospholipase C produced by bacteria such as C. perfringens is a well characterized toxin capable of causing hemolysis both in vitro and in vivo (Matsumoto, 1961). Phospholipase C hydrolyses membrane phospholipids in humans which results in hemolysis secondary to osmotic pressure which bursts the cell (Bowman et al, 1971). In this study, similar in vitro hemolytic activity of the P. citrinum fraction and phospholipase C was observed in mice and human erythrocytes.

The occurrence of SHD negatively impacts food safety and food security in areas of high sago dependency. Although efforts have been made to address the food safety of sago starch (Greenhill et al, 2010b), there will remain a risk of disease until the etiology is determined. This study has made important preliminary findings and paves the way for further investigations to elucidate the role of P. citrinum in the etiology of SHD, which are ongoing.
ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Centre for International Agricultural Research. We thank Lisa Elliot for technical assistance setting up the SDS-PAGE analysis.

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