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POSTHARVEST INSTITUTE FOR PERISHABLES

PROPOSAL FOR REMOVAL OF HYPOGLYCIN IN ACKEE

by

Dr. Jorg Augustin

GTS Report Number

89

University of Idaho

in cooperation with

United States Agency for International Development

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PROPOSAL FOR

REMOVAL OF HYPOGLYCIN IN ACKEE FRUIT

IN JAMAICA

Introduction

The following proposed project on removal of toxic hypoglycin in Ackee fruit was completed for the USAID Mission in Jamaica on a contract between the Mission and the Postharvest Institute for Perishables. Dr. Jorg Augustin, of the Unviersity of Idaho Bacteriology and Biology Department, consulted for PIP to prepare the scope of work.

The Jamaican AID Mission plans to select an institution to conduct the research as outlined in this proposal. The objective of the research will be to remove the import alert status of Ackee entering the U.S. and allow Jamaican exporters to market their national fruit in this country.

OBJECTIVES

The overall objective of the proposed work is to take the necessary steps to induce the FDA to lift the import alert against ackee fruit imported to the U.S.

Specifically, the procedure necessary to remove the import alert will involve one or more of the following steps:

1. The development of reliable, analytical methods for the quantitative determination of hypoglycin A and B.

2. The establishment of a base line of analytical values for the toxins in the ackee arilli in immature and mature fresh fruits, as well as in the canned fruits, involving existing varieties and various cultural practices.

3. The development of ackee processing methods designed to reduce hypoglycin A and B in the canned fruit.

4. Conducting toxicological studies for the purpose of establishing a no-effect level for hypoglycin.

It is conceivable that the FDA may establish a tolerance level following completion of objectives 1 and 2, and perhaps 3. As each step is completed, the results will be submitted to the FDA for review and approval. Thus it may be possible to eliminate steps 3 and 4. Since toxicological studies are costly and time consuming, they would be undertaken only if deemed necessary.

BACKGROUND INFORMATION

The Ackee fruit (Blighia sapida), the national fruit of Jamaica, is a common

dietary item there. The fruit is about the size of an orange and has a hard shell containing dark seeds, each of which is surrounded by a fleshy arillus, the fruit's only edible part. Ingestion of the unripe arilli has reportedly been the cause of an illness symptomized by severe vomiting. Other related symptoms observed include lethargy, prostration and coma, in some instances leading to death. Accompanying the illness are hypoglycemia, depletion of liver glycogen, accumulation of fat in the liver, and increased plasma fatty acid accumulations (Feng, 1969). Most severely affected by this type of toxicity are undernourished children. It is reported that some 5000 deaths have been associated with the vomiting sickness between 1886 and 1950 (Mitchell, 1974). However, according to Stuart et al (1955), vomiting sickness, including some of the fatal cases associated with it, may be attributable to causes other than the ackee fruit.

Although there may be other causes of the illness, Hassall and Reyle (1954, 1955 a, b) isolated two toxic compounds from immature ackee which, when used in animal studies, resulted in the duplication of the symptoms outlined above. The two compounds, hypoglycin A and B, were demonstrated to possess toxicity associated with vomiting sickness. Hypoglycin A, or hypoglycin, is a non-protein amino acid analog of leucine: α-amino-β-(α-methylene cyclopropyl)propionic acid. It is found primarily in the arilli of the fruit. The less toxic hypoglycin B, or the non-protein amino acid analog, γ-L-glutamyl-α-amino-β-(2-methylene- cyclopropyl)propionic acid dipeptide, or more simply referred to as γ-L-glutamylhypoglycin, is concentrated in the ackee seeds.

Toxicity studies using rats showed LD50 values (lethal dosage for 50% of animals) of approximately 90 mg/kg body weight in adult rats with hypoglycin A (Hassal and Reyle, 1955 b; Feng and Kean, 1955). The LD50 dropped to 20 mg/kg when rats were fed a low protein diet. Hypoglycin B showed only half the toxicity of hypoglycin A. Sherratt (1986) reported on the development of hypoglycemia in fasted animals when fed hypolycin in doses ranging from 10-150 mg/kg body weight in guinea pigs, rabbits, dogs, cats, rats and mice in order of decreasing sensitivity.

Aside from acute toxicity, it has been shown that hypoglycin A also may have some chronic toxic effects as a teratogenic agent (a compound causing birth defects). Persaud (1967, 1972, 1975) and Persaud and Kaplan (1970) reported such effects to vary depending on the species of test animal used. Peritoneal administration of 30 mg/kg body weight of hypoglycin A in pregnant rats resulted in congenital anomalies and fetal resorptions whereas pregnant mice given 120 mg/kg body weight were unaffected. Rabbits intravenously injected with 0.5 mg/kg body weight showed increased fetal resorption and stunting but no congenital malformation. Hypoglycin A administered to chick embryos in amounts ranging from 20 to 200 mg per egg caused no increase in abnormalities in comparison to the controls. In all of these experiments no animals suffered from vomiting, convulsions, or death. Studies with rats using 1.25 mg/kg hypoglycin A had no adverse effects on the animals.

Concentrations of hypoglycin A and B in ackee are poorly documented, due to a lack of proper analytical methods, and the unavailability of a commercial supply of hypoglycin standards. Ellington (1975), using ion-exchange chromatography, reported hypoglycin A values of 111 mg/100g and 8 mg/100g in

unripe and ripe ackee, respectively. Earlier, Hassall and Reyle (1955 a) obtained identical values with paper chromatography. Fincham (1975), using an automated ion-exchange chromatography amino acid analyzer, determined the hypoglycin A content in immature ackee arilli to be 270 mg/100g. The comparatively high value was explained on the basis of a more efficient extraction procedure. Abrahams and Kean (1969), using dansyl derivatization and paper chromatography separation, found 90 mg/100g of hypoglycin A in unripe ackee arilli. Kean (1978), by fluorometrically measuring formaldehyde generated by permanganate oxidation, found 105 mg/100g of hypoglycin A in immature ackee arilli. Scott et. al. (1974), using a Beckmann Amino Acid Analyzer, reported 10.8 to 26.0 and 12.2 to 28.7 mg/100g in canned ackee fruit and its corresponding brine, respectively, with considerable leaching of hypoglycin A from the fruit into the brine during canning.

Because of the toxicity associated with ackee, the U.S. Food and Drug Administration (FDA) in 1972 declared an import alert on this product. This is essentially the same as a ban on the import of the fruit into the United States. Import alerts have not been initiated in other ackee importing countries. England and Canada, the two largest importers of ackees, have not reported either illness or death associated with the canned fruit. The current export volume for canned ackee is 73,396 and 54,758 cases in 1986 and 1985 respectively, with a total U.S. dollar value of up to 3.7 million (Jamaican Bureau of Standards, 1987).

This proposal is based on an on-site visit in Jamaica, study of past FDA correspondence regarding the matter, and subsequent consultation with FDA representatives. FDA personnel have reviewed the experimental outline of this

proposal and submitted comments, essentially approving the proposal by oral communication. The FDA has also advised us to delay toxicity studies until accurate data on the hypoglycin content in canned ackee fruits is available (Thomas, 1987). Based on the above, this proposal appears to be the only option that will induce the FDA to lift the current import alert on ackee fruits.

EXPERIMENTAL APPROACH - CONDUCT OF THE STUDY

The outline of the proposed research program is based on the objectives stated above.

A. Development of an analytical method. The development of a sound analytical method is crucial to the success of the project as a whole.

Following initial procurement of hypoglycin A and B standard compounds, attempts will center on the development of a method utilizing high performance liquid chromatography (HPLC) and utilizing the method used by Heinrikson and Meredith (1984) and Bidlingmeyer et. al. (1984). This method entails the derivation of amino acids, including hypoglycin A and B, with phenylisothiocyanate followed by their separation on a reverse-phase column using UV (ultraviolet) detection. Other HPLC methods may be developed for the successful determination of the toxins. Once the method is developed and tested, efforts will focus on the development of a more simplified method for field work, e.g., the fluorometric method developed by Kean (1978).

One of the problems associated with the development of analytical methods for hypoglycin has been the lack of a source of pure hypoglycin. This compound is unavailable through commercial sources and, thus, will have to be isolated in the laboratory. Initially, and if unavailable from any other source, the method by Kean (1974) will be used to isolate hypoglycin.

The above outline constitutes the initial approach for the methods development phase. Another HPLC method may be more feasible as a practical solution to the problem, but this remains to be seen. The FDA will be kept informed on any progress or achievements and will be consulted with to insure their approval of new analytical methods.

B. Baseline establishment. Following the successful development of an analytical procedure, a survey will be conducted on the hypoglycin content in ackee arilli involving immature, mature and canned samples. Individual ackee processing plants will be sampled for a period of one year or at least during the major processing season. An integral part of this study will be the establishment of a correlation between hypoglycin content and fruit maturity development. Also included in the study will be an assessment of the hypoglycin in different ackee varieties and the effect of cultural practices on toxicity. It is hoped that from the information gathered in this phase, processors can be advised of the optimum selection time of the mature fruit for canning.

C. Process development. Efforts will focus on modifying current canning processes for the purpose of further reducing hypoglycin values in the finished, canned product. In current canning practices, the fresh, cleaned arilli are not precooked in water before canning. The fact that Scott et. al. (1974) reported hypoglycin to occur both in the arilli and the brine of the canned product indicates leaching of the toxin from the arilli into the brine during the sterilizing process. Based on this information, it is reasonable to assume that precooking (blanching) the fruit prior to canning will result in additional leaching of hypoglycin and thus result in further reductions of the toxin in the finished product.

D. Toxicological studies. Following, or even during phases B or C, FDA representatives will be contacted to establish the need for toxicological studies. The FDA has advised not to conduct toxicity studies until completion of phases A-C (Thomas, 1987) as such studies

may not be necessary . This indicates that the FDA may decide to lift the existing import alert on the basis of data generated in phases A, B, and possibly C. Such studies will be undertaken unless zero hypoglycin levels are achievable, or the FDA decides to set a maximum tolerance limit on the basis of the analytical values obtained during phases A and B. Should such studies be required, they will involve both long and short range investigations designed to establish no-effect levels for hypoglycin.

If a toxicological investigation is required, the following studies will be included:

- a lifetime feeding study (approx. 2 years) using rats with *in-utero* exposure for carcinogenesis and chronic toxicity,

- a lifetime feeding study (approx. 2 years), using rats to evaluate carcinogenesis,

- a short-term feeding study (approx. 6 to 12 months) using non-rodent animals,

- a multigeneration reproduction feeding study (3 generations, 2 litters/generation with teratology phase) using rats, and

- a teratological study using non-rodents.

Pending approval by the FDA, these toxicological studies could be streamlined in the following manner: chronic exposure studies would be combined with carcinogenicity and reproduction studies in rats. Male and female rats would be treated for three months with the toxin (3 doses) and then bred. Effects on reproduction would be assessed on these animals and their litters. Offspring from these litters (50 males and 50 females/treatment group) would be maintained and utilized to assess chronic exposure effects and

carcinogenicity (18 months w/samplings at 6 and 12 months). The parents could be utilized to assess subchronic exposure effects. Subchronic exposure would also be assessed in a non-rodent species (rabbits) treated for three months (three doses).

Potential teratogenic effects would be assessed using two strains of mice treated with the toxin during the organogenesis stage of gestation.

LABORATORY FACILITIES

The Food Research Center at the University of Idaho is equipped with adequate laboratory space and equipment to carry out the proposed study. In addition to general laboratory equipment capable of performing physio-chemical analytical work, the following laboratory facilities, equipment and instrumentation are

located at the center:

- Walk-in freezer and refrigerator, appx. 200 sq. ft. each
- Blending apparatus (Polytron, Virtis, Waring, Omni)
- Spectrophotometers
- Analytical scales
- Distillation apparatus
- Millipore water purification system
- HPLC equipment including fixed and scanning UV detectors, flourescence detectors, and a refractive index detector
- PICO-TAG amino acid derivatizer
- Freeze dryer (3.5 l condenser capacity)

Under existing programs, the HPLC unit, with the exception of the detectors, is running at full capacity. An additional pumping system will therefore be

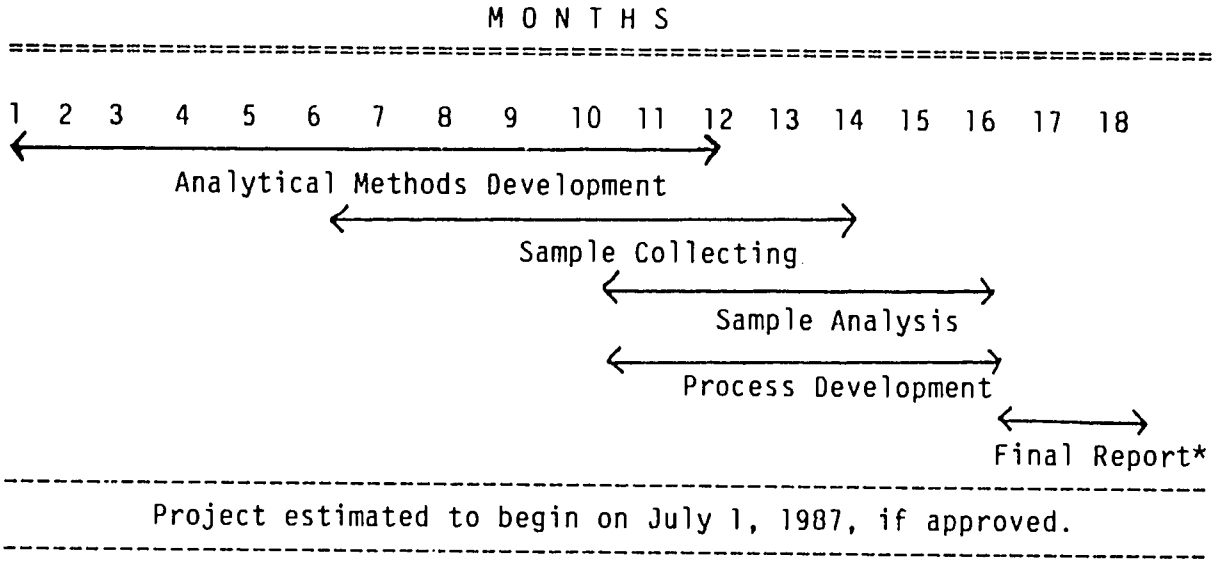
required to handle the extra load associated with the project. Except for the lack of a vacumm pump, the existing PICO-TAG derivatizer capacity can handle the hypoglycin work.

SCHEDULING

Phase A is expected t take 12 months t complete. Phases B and C are estimated to last 6 months each, overlapping with the latter part of phase A. Table 1 outlines the proposed sequence of events and time frame. The total duration of Phases A, B, and C is estimated to be 18 months. This is an optimistic estimate, yet every effort will be made to shorten the proposed time periods.

TABLE 1

Critical Pathways of Project Development



* Interaction with FDA to remove import alert.

The duration of phase D, if needed, is estimated to be 24 months. Depending on the decision for the need of toxicological studies, the total time for the project, including phase D, will be between 18 and 48 months. Following the establishment of a maximum tolerance limit by the FDA, a safety assurance program will be developed in cooperation with the Jamaica Bureau of Standards (JBS) and presented to the FDA for approval.

PERSONNEL AND RESPONSIBILITIES

Dr. Jorg Augustin of the Food Research Center at the University of Idaho (UI) is the principal investigator (PI) of the project. Phase A will be conducted at the UI under the direction of the PI, with Dr. Sherry O. Farwell of the UI Chemistry Department as a consulting advisor.

The JBS has informed PIP that it will provide sufficient ackee samples. Mr. James Kerr, JBS, will isolate and purify hypoglycin A and B in sufficient quantities to be used as standards for the project.

Ms. Andrea Robins, Coordinator of the Ackee Project at the JBS and Mr. James Kerr, Head, Chemistry Department, JBS, in cooperation with the PI will be in charge of phase B. It will be the PI's responsibility to train personnel at the JBS in carrying out the analytical work.

Mr. Alwyn Lynch, Head, Food Technology Institute, Scientific Research Council (SRC), will conduct phase C. His group will conduct the process development phase, and Mr. Kerr's group will analyze the samples for their hypoglycin contents.

AID/Jamaica will be asked to monitor this part of the study in Jamaica to assure prompt completion of the tasks. If PIP is required to do this, additional trips and expenses will be required.

Phase D, if necessary, will be coordinated by Dr. Jerry H. Exon at the UI Veterinary Science Department.

Resumes of PIP consultants are attached.

BUDGET

The first increment will cover the research and expenses as outlined in this proposal.

The second increment will cover additional expenses should the planned HPLC method not be entirely successful in generating satisfactory results. The requirment of a second HPLC method would entail the purchase of a fluorometer ($7,500) and a post-column derivatizer ($5,000). Should Increment 2 be required, the total cost would increase to $111,288.00 from $98,788.00.

The toxicology studies, if required by the FDA, are estimated to cost between $150-200,000, and require a 24 month period to complete. This is the third increment.

1st Increment

(Phases A,B,C)

A. Salaries

Principal Investigator (PI) 523 hrs @ $23.23/hr =		$ 12,150.00
Consulting Advisor 50 hrs @ 16.96/hr =		$ 848.00
Technician 2088 hrs @ $7.40/hr =		15,451.00
Irregular Laboratory Help 522 hrs @ $4.50/hr =		2,349.00
	Salary Total:	$ 30,798.00

B. Fringe

12,150 + 15,451 + 848 X 23.5% =		$ 6,686.00
2,349 X 11% =		258.00
	Fringe Total:	$ 6,944.00

C. Travel

2 Trips Idaho/Jamaica/Idaho		$ 2,400.00
1 Trip Washington DC/FDA		800.00
	Travel Total:	$ 3,200.00

D. Per Diem

21 days X 125/day =		$ 2,625.00
7 days X 100/day =		700.00
	Per Diem Total:	$ 3,325.00

E. Equipment

HPLC Pump and Programmer		$ 13,400.00
Vacuum Pump		1,300.00
	Equipment Total:	$ 14,700.00

F.	Laboratory Supplies	
	HPLC Columums and Grade Solvon B, Glassware, General Chemicals and Reagents, etc.	$ 8,900.00
G.	Isolation and Purification of Hypoglycin A&B	$ 750.00
H.	Shipping Costs, Ackee from Jamaica to PIP	$ 500.00
I.	Office Supplies	$ 300.00
J.	PIP Administration	3,500.00
K.	Communications	$ 1,000.00
	Sub Total:	$ 73,917.00
L.	Overhead (42% excluding equipment)	24,871.00
	TOTAL, 1st INCREMENT:	$ 98,788.00

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