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PROGRESS REPORT NO 3

PROJECT TITLE:

Changes in Cyanogenic Glycosides and Related
Enzymes in Cassava Roots During Storage
and Processing

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 936-5542-G-00-6028-00

SUBMITTED BY

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THAILAND

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15

Project Profile

Country: Thailand

Grant no.: 936-5542-G-00-6028-00

Program: Program on Science and Technology Cooperation

Project Title: Changes in Cyanogenic Glycosides and Related Enzymes in Cassava Roots During Storage and Processing

Project Leader: Professor Montri Chulavatnatol, Ph.D.

Organization: Biochemistry Department, Faculty of Science, Mahidol University, Bangkok 10400

Co-investigator: Assoc. Professor Somsak Ruchirawat, Ph.D.

Project Consultant: None

Authorized Officer: Professor Natth Bhamarapavati, M.D., D.Sc.

Total Project Budget: US\$129,670

Project Duration: May 7, 1986 - December 31, 1989

Reporting Period: May 1 - October 31, 1987 (6 months)

Budget Allocation for This Period: 434,005 Baht

Contents

1. *Background/Introduction:*

During the 6-month period covered by this report, May 1 to October 31, 1987, most equipments and supplies were received and used in the research. There were also changes in personnel. A M.Sc.-student, Miss Pennapa Kisamanonta, joined the project and started her M.Sc. thesis research on the molecular structure of cassava linamarase. Miss Pissopa Kitjahn who was employed as a technical assistant in August 1986 decided to further her advanced study elsewhere and left the position on May 31, 1987. A replacement was found to continue her work. Mr. Vasin Dhanasarnsombut, B.Sc. (Chulalongkorn) was employed as technical assistant starting June 1, 1987. Unfortunately, he was called by his father to help in their family business and resigned the position on September 30, 1987. We have been looking for a new technical assistant since then.

2. *Objectives:*

- 2.1 To characterize linamarase from cassava stem, petiole and root cortex
- 2.2 To purify glucosyltransferase from cassava leaves
- 2.3 To start tissue culture of cassava

3. *Materials and Methods:* See attached documents

4. *Results/Discussion*

4.1 Cassava linamarases from stem, petiole and root cortex were characterized. Details can be found in the abstract presented to the 13th Conference of Science and Technology of Thailand, Hay Yai, October 20-23, 1987 (Attached document no. 7.1).

4.2 Cassava glucosyltransferase was partially purified from young leaves and its catalytic properties were established. Details

can be found in the abstract presented to the 13th Conference of Science and Technology of Thailand, Hat Yai, October 20-23, 1987 (Attached document no. 7.2).

4.3 Miss Thidarat Eksittikul, Senior technical assistant, was trained by Mrs. Yupa Mongkolsook of Kasetsart University on the technique of plant tissue culture. She then set up a plant tissue culture room in the Department of Biochemistry and started the tissue culture of cassava in October 1987. The culture plant specimens will be used to study the biotransformation of linamarin. The cooperation of Mrs. Yupa Mongkolsook in her training is fully appreciated.

5. *Conclusion/Remarks:*

We have characterized cassava linamarase and partially purified cassava glucosyltransferase. Tissue culture of cassava has been established and in progress.

6. *Workplan for the Next Period:*

We shall purify and characterize cassava glucosyltransferase. We shall study the biotransformation of linamarin using plant tissue culture.

7. *Attached documents*

7.1 Eksittikul, T. and Chulavatnatol, M. (1987) Catalytic properties of linamarase from cassava. 13th Conference of Science and Technology of Thailand, Hat Yai, pp. 548-549.

7.2 Chalermisrachai, P. and Chulavatnatol, M. (1987) Properties of glucosyltransferase in cassava. 13th Conference of Science and Technology of Thailand, Hat Yai, pp. 546-547.

ชื่อ-สกุล ผู้เสนอ ธิดารัตน์ เอกสิทธิ์กุล สาขาวิชา :
 นว บ.ศ. วน คร. อ. พ. พ. ก. ก.
 กษัตรา เกษตร
 ชีวภาพ วิศวกรรม
ที่ทำงาน ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล วิทย์-เทคโน ทรัพย์-แคว้น
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CATALYTIC PROPERTIES OF LINAMARASE FROM CASSAVA
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Linamarase is a glucosidase, catalysing the hydrolysis of cyanogenic glucoside, linamarin in cassava, releasing the toxic HCN. The enzyme has been partially purified from cassava root parenchyma (1) and used for determination of cyanogenic glucoside in cassava trading. Linamarases were found in abundance in cassava petiole, stem and root cortex. They were purified and their properties were reported (2). In this report, the substrate specificity of the enzyme was investigated. The substrate must contain glucose or fucose linked by β -glycosidic bond to aglycone part. The enzyme from each source was separated into three isozymes by chromatofocusing. The pH optimum, K_m and V_{max} values of these isozymes were also compared.

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คุณสมบัติของเอนไซม์ลินามาเรสจากมันสำปะหลัง
ธิดารัตน์ เอกสิทธิ์กุล และ มนรี จุฬาวัตนกุล
ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ถนนพระราม 6 กท. 10400

ลินามาเรสเป็นเอนไซม์ที่ย่อยสลายสารประกอบไซยาไนด์ที่พืชสังเคราะห์เอง เมธอรามาซาล ไดโน
ลินามาเรส ให้ก๊าซไฮโดรเจนไซยาไนด์ ซึ่งเป็นอันตรายต่อสิ่งมีชีวิตในปัจจุบัน เอนไซม์ลินามาเรส
ได้ถูกนำมาใช้ในการการค้า โดยใช้หาปริมาณของสารประกอบไซยาไนด์ในมันสำปะหลัง ได้แก่อินทามาริน
เป็นส่วนใหญ่ ซึ่งเป็นตัวกำหนดราคาของมันสำปะหลัง จากการศึกษาที่ผ่านมา พบว่า ลินามาเรส
สามารถเตรียมได้จากชิ้นส่วน ๆ ของมันสำปะหลัง โดยพบว่าจะมีปริมาณมากในก้านใบ ต้น และเปลือก
ของมันสำปะหลัง ตามลำดับ คุณสมบัติของเอนไซม์ลินามาเรสที่เตรียมได้มี นอกจากที่เตรียมงานแล้ว
ได้ศึกษาต่อพบว่าเอนไซม์ที่มีความจำเพาะต่อพันธะเคมี กล่าวคือ จะย่อยพันธะเคมีที่พันธะ
หรือพันธะเคมี เช่นพันธะ β -glycosidic bond กับ aglycone เราได้แยกเอนไซม์ที่เป็นสามไอโซไซม์
และเปรียบเทียบค่า optimum pH, K_m และ V_{max} ของเอนไซม์ทั้งสามส่วน และของไอโซไซม์ไว้ด้วย

ผู้เรียบเรียง (ไทย) : ฤกษ์ชัยดิษฐ์ อธิบายโดยย่อเกี่ยวกับงานวิจัย

ตารางที่ 1 : การเปรียบเทียบการย่อยสลายของ glycoside ต่าง ๆ โดยเอนไซม์จากเนื้อเยื่อรากและเนื้อเยื่อของ petiole และลำต้นของ cassava ที่ใช้ 10 mM substrate และเวลาปฏิกิริยาใช้ 2 mM.

Substrate	% Relative activity		
	Cortex enz.	Petiole enz.	Stem enz.
Linamarin	100	100	100
PNP-β-glucoside	100	100	100
PNP-α-glucoside	0	0	1.2
PNP-β-fucoside	93	86	100
PNP-β-mannoside	4	3	40
PNP-β-galactoside	0	3	2
PNP-β-gentiobioside	0	0	0
Amygdalin	0	0	0
Protasin	0	0	0

ตารางที่ 2 : ฤกษ์ชัยดิษฐ์ อธิบายโดยย่อเกี่ยวกับงานวิจัย

SUBSTRATE / ENZYME SOURCE	Km (mM)		Vmax (μmol/min/mg)	
	Graph	Computer	Graph	Computer
<u>PNP-β-D-GALACTOSIDE</u>				
Root cortex	16	18	377	281
Petiole	16	16	312	308
Stem	13	13	625	895
<u>Linamarin</u>				
Root cortex	1.4	1.0	102	133
Petiole	1.1	0.6	55	111
Stem	0.9	0.6	175	181
<u>Linamarin</u>				
Isozymes from petiole				
pI 4.3	2.0	1.3	166	124
pI 3.3	2.0	1.3	14	14
pI 2.9	1.3	0.5	66	135

เอกสารอ้างอิง

1. Cook, R.D., Bloke, G.G. and Battershill, J.M. (1978) *Phytochemistry* 17, 361-363.
2. Eksittikul, T. and Chulavatnatol, M. (1986) Comparison of cassava linamarases from petiole, stem and root cortex. *ICSU Short Reports C*, 360-361.

ชื่อ-สกุล ผู้เสนอ พารมพกา เจริญธรรชชัย สาขาวิชา: _____

ธรณี ชีวเคมี ธรณีวิทยา ธรณีวิทยา ธรณีวิทยา ธรณีวิทยา ธรณีวิทยา ธรณีวิทยา

ที่ทำงาน ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล วิทยาศาสตร์ วิทยาศาสตร์ วิทยาศาสตร์

ถนนพหลโยธิน ๕ กรุงเทพฯ 10400 โทร. 2460063 คบ แพทย์ ทวีป

252

PROPERTIES OF GLUCOSYLTRANSFERASE IN CASSAVA
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Glucosyltransferase is mainly obtained from fresh young leaf and shoot of cassava (*Manihot esculenta* Crantz). It is responsible for the last glycosylation step in biosynthetic path way of linamarin. Linamarin, α -hydroxyisobutyronitrile β -D-glucoside, is a cyanogenic glucoside widely occurring in many staple plant foods and feeds, such as cassava, flax, lima bean, peas, and white clover. Glucosyltransferase catalyzes the transfer of glucose from UDP-glucose to an acceptor, α -hydroxynitrile or acetone cyanohydrin. Using the crude extraction for glucosyltransferase. The optimum pH was found to be 9.0 where as the optimum temperature was 3^oC. The enzyme was precipitated by ammonium sulphate at 40-50% saturation and was chromatographed in Sephadex G-200 column. The native Mr was determined to be 40,000. UDP-glucose was the best glucose-donor. Among the hydroxy-containing compounds tested, the best glucose-acceptor was acetone cyanohydrin.

คุณสมบัติของกลูโคซิลทรานสเฟอเรสในต้นลำปะเท็ง
พารมพกา เจริญธรรชชัย และ มณตรี จุฬาวัตนตล
ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ถนนพหลโยธิน ๕ กทม. 10400

กลูโคซิลทรานสเฟอเรสพบมากที่สุดในอ่อนและยอดที่ผลิใหม่ของต้นลำปะเท็ง เอนไซม์นี้ทำหน้าที่ในขั้นตอนของการเดินน้ำตาลอินเป็นขั้นตอนสุดท้ายของขบวนการสังเคราะห์ลิมาาริน ลิมาารินหรืออะฟาไฮดรอกซีไอโซบิวโรไนไตรล์ เบต้า ดี กลูโคไซด์ เป็นน้ำตาลไซยาไนด์ ที่พบมากในพืชที่ใช้เป็นอาหารและเลี้ยงสัตว์ เช่น ต้นลำปะเท็ง ปอ ข้าวโพด ข้าวคั่ว และต้นไถ่ โค้ดเวอ์ กลูโคซิลทรานสเฟอเรสจะเร่งการสังเคราะห์น้ำตาลกลูโคสจาก UDP-glucose ไปให้อะฟาไฮดรอกซีไอโซบิวโรไนไตรล์ หรืออะไฮโดรไซยาไนด์อิน กลูโคซิลทรานสเฟอเรสที่สกัดได้มีค่า pH เหนือสามที่ pH 9.0 และจุดอุณหภูมิที่เหมาะสมที่สุดคือ 37^oC เอนไซม์จะถูกนำมากำหนดฤทธิ์ขึ้นโดยวิธีการตกตะกอน ด้วยแอมโมเนียมซัลเฟตที่ความเข้มข้น 40-50% และวิธีการโครมาโตกราฟีโดยวิธี Sephadex G-200 column จากการศึกษานี้พบว่าน้ำหนักโมเลกุลของเอนไซม์นี้เป็น 46,000 ส่วนของน้ำตาลกลูโคส UDP-glucose จะมีความสามารถในการสังเคราะห์กลูโคซิลโคสดีที่สุด ส่วนที่รับน้ำตาลโคสดีที่ดีที่สุดคือ อะไฮโดรไซยาไนด์อิน

สนับสนุน โดย USAID/PSTC 936-5542-G-00-6028-00
และทุนอุดหนุนการวิจัยประเภทศึกษาระดับบัณฑิตศึกษา ประจำปี ๒5๓๐ ของสภารวิจัยแห่งชาติ

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PROGRESS REPORT NO 4

PROJECT TITLE:

Changes in Cyanogenic Glycosides and Related
Enzymes in Cassava Roots During Storage
and Processing

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 936-5542-G-00-6028-00

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Rec'd In SCI. MAY 21 1996

Project Profile

Country: Thailand

Grant no.: 936-5542-G-00-6028-00

Program: Program on Science and Technology Cooperation

Project Title: Changes in Cyanogenic Glycosides and Related Enzymes in Cassava Roots During Storage and Processing

Project Leader: Professor Montri Chulavatnatol, Ph.D.

Organization: Biochemistry Department, Faculty of Science, Mahidol University, Bangkok 10400

Co-investigator: Assoc. Professor Somsak Ruchirawat, Ph.D.

Project Consultant: None

Authorized Officer: Professor Natth Bhamarapravati, M.D., D.Sc.

Total Project Budget: US\$129,670

Project Duration: May 7, 1986 - December 31, 1989

Reporting Period: November 1, 1987 - April 30, 1988 (6 months)

Budget Allocation for This Period: 471,510 Baht

Contents

1. Background/Introduction:

During this period, no change was made on the personnel. A few applicants for the vacant position of technical assistant were interviewed. However, none was found suitable and more candidates were being sought. Two graduate students, Miss Panomporn Panutat and Miss Nuntaporn Pitiguagool, undertook an advanced biochemistry project on the study of peroxidase during cassava root deterioration. The project lasted for 4 months, December 1987-March 1988. Since then, one student, Miss Panomporn Panutat, started her M.Sc. thesis work on the use of cassava tissue culture to study factors affecting cyanogenic glucoside synthesis and accumulation. In addition, the principle investigator went on a study tour for 3 weeks (April 12-30, 1988) to two institutions: Professor E.E. Conn's laboratory in the Department of Biochemistry and Biophysics, University of California at Davis, California, U.S.A. and Dr. R.D. Cooke's laboratory at the Overseas Development and Natural Resources Institute in London, England.

2. Objectives:

- 2.1 Characterization of cassava linamarase
- 2.2 Characterization of cassava glucosyltransferase
- 2.3 Peroxidase during cassava root determination
- 2.4 Cassava tissue culture
- 2.5 Tour of laboratories

3. Results/Discussion

3.1 Cassava linamarase was being studied in terms of structure and catalysis. Antibody was raised in rabbit against the purified enzyme. This was used to probe the structural differences among the isozymes. Proteolytic fragmentation of the structure was performed and the fragments would be analyzed to compare the three isozymes. The catalytic property was abolished by modification of lysine residues. These studies were being performed by Miss Pennapa Kisamanonta.

3.2 Cassava glucosyltransferase was being purified using fast-protein liquid chromatography (FPLC). Its instability was the major hindrance in its purification. The partially purified enzyme was characterized for its catalytic and structural properties. These studies were being written up as an M.Sc. thesis by Miss Panpaka Chalermisrachai.

3.3 Cassava peroxidase was studied in cassava root parenchyma and cortex. Physiological deterioration of the roots stored at room temperature was followed for 7 days. During this period, peroxidase activity of the parenchyma decreased more than that of cortex. The cortex enzyme was found to be heat-stable while that of the parenchyma was heat-labile. The parenchyma enzyme moved faster than the cortex enzyme during electrophoresis under non-denaturing condition. Both, however, showed similar subunit molecular weight by electrophoresis in the presence of sodium dodecyl sulfate. Thus peroxidase changes corresponded with the browning response during the root deterioration. The study was carried out by Miss Panomporn Panutat and Miss Nantaporn Pitiguagool.

3.4 Cassava tissue culture facility was improved by the addition of a laminar flow hood in the plant tissue culture room. More plantlets were produced and they were found to possess both linamarin and linamarase. So the plantlets produced under the sterile condition of the tissue culture bottles offered an ideal opportunity to study environmental effects on the biosynthesis of the cyanogenic glucosides. Miss Thidarat Esittikul was in charge of the operation of the plant tissue culture room.

3.5 Tour of laboratories had two major objectives: to learn more about other cyanogenic glycosides and to make known our studies on cassava cyanogenic glucosides. The P.I. learned much more about other plant cyanogenic glycosides in Professor E.E. Conn's laboratory which was a strong center where the work in this area had been carried out for over three decades. In Dr. R.D. Cooke's institute, much was learned about the deterioration of cassava roots and other tropical root crops. Also there was a major effect on the biotechnology of cassava food processing. Our work was made known when the P.I. gave seminars in both laboratories. As a result, the P.I. was contacted by scientists from England and Denmark to do collaborative research with the aim to develop cyanide-free cassava.

4. Conclusion/Remarks:

We have made further progress in understanding the cassava linamarase, glucosyl-transferase, peroxidase and cassava root deterioration. Cassava tissue culture has been set up and is ready for more definitive study.

5. Workplan for the Next Period:

- 5.1 Biotransformation of linamarin using plant tissue culture.
- 5.2 Biochemical changes during cassava root deterioration.

6. Attached document:

- 6.1 Montri Chulavatnatol, Somsak Ruchirawat, Thidarat Eksittikul, Uma Prawat, Panpaka Chalermisrachai, Pissopa Kitjaharn and Hansa Prawat (1987) Biotransformation of cyanogenic glucosides in cassava (*Manihot esculenta* Crantz). Abstract presented in the First Princess Chulabhorn Science Congress, Bangkok, 10-13 December 1987.

BIOTRANSFORMATION OF CYANOGENIC GLUCOSIDES IN CASSAVA
(*MANIHOT ESCULENTA* CRANTZ)

Montri Chutavatratol†, Somsak Ruchirawat††,
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Pissopa Kitjahn† and Hansa Prawat††

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Storage roots of cassava are the main source of carbohydrates for 500 million poor people in 30 tropical countries. In 1986, the world cassava production was 143 million tons with Thailand and Brazil as the top producers, each producing 20 million tons. In the same year, Thailand earned 19,000 million baht from exporting 1/3 of her cassava products. Hidden in this agro-economic profile is a health problem. Cassava possesses two known cyanogenic glucosides: linamarin and lotaustralin, with the former being the majority. These glucosides are toxic because of HCN liberated upon their hydrolysis. With the aim to lessen the toxicity, biosynthesis and degradation of linamarin in cassava were studied. Valine was converted by plant microsome into acetone cyanohydrin. Then the latter was conjugated with glucose to form linamarin in the reaction catalyzed by a glucosyltransferase. The glucosyltransferase was found in young cassava leaves. Its native molecular weight was 46,000. Uridine 5'-diphosphate-glucose was the preferred donor of the glucosyl moiety and acetone cyanohydrin was the best glucose-acceptor. The hydrolysis of linamarin, catalyzed by linamarase, yielded glucose and acetone cyanohydrin. The latter broke down spontaneously into acetone and HCN. In addition to cassava root parenchyma as the known source of linamarase, cassava petiole, stem and root cortex were found to be richer sources of the enzyme. Thus, the enzyme was purified from these sources. It was an oligomeric enzyme, exhibiting a native molecular weight of 600,000 and the subunit molecular weight of 63,000. It was separated into three isozymes by chromatofocusing. It was specific for the hydrolysis of a β -glycosidic bond between glucose or fructose and an aglycone. An aliphatic aglycone was preferred over an aromatic one. From the root cortex, both linamarin and lotaustralin were isolated by hot ethanol extraction, followed by a silica gel column chromatography. Both linamarin and linamarase were essential for the cyanide determination in cassava trade. (Supported by USAID/PSTC 936-5542-G-00-6028-00.)