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CHANGES OF CYANOGENIC GLYCOSIDES AND RELATED ENZYMES IN CASSAVA ROOTS DURING STORAGE AND PROCESSING

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cc: Burton
Prof. Cantle

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1. Executive Summary

Cassava cyanogenesis was a much neglected subject but it remained a real threat to the health of millions of tropical poor subsisting on the root crop. To improve the safety of cassava diet, the molecular understanding of cyanogenesis and root deterioration was sought in this project. The breakdown of linamarin, the major form of the cyanogenic glucosides in cassava, was the crucial step in releasing the free and toxic cyanide. The process was catalyzed by linamarase. In this project, cassava stem and petiole were found to be rich in linamarase. The finding enabled both a rapid purification and an extensive characterization of the enzyme. The finding also suggested the possible use of the stem and petiole as convenient sources of linamarase in facilitating the removal of linamarin in cassava food processing. On the linamarin synthesis, the key enzyme, glucosyltransferase, was partially purified and characterized. This enzyme could serve as a critical target for genetic manipulation to create acyanogenic cassava. Plant tissue culture technique was also employed to study the cyanogenesis in cassava. Plantlets in tissue culture, free from pests and pathogens, were shown to contain both linamarin and linamarase. This finding casted doubt on the widely held concept about the defence role of cyanogenic glycosides. The capability of the petiole slices from the plantlets to incorporate radioactive valine precursor into linamarin further confirmed that cyanogenesis occurred in the plantlets under the tissue culture condition. Therefore, plant tissue culture should be a useful system to test factors that may alter the cyanogenesis. On the deteriorating roots, a dramatic increase in the peroxidase activities was observed and this may be associated with the tissue browning and the formation of toxic polyphenols. These findings led to a subsequent collaborative project with two laboratories in England and Denmark to work towards acyanogenic cassava. The new project just received funding from the European Economic Commission.

2. Research Objectives

The major aim of the project is to improve the safety of cassava consumption. It is known that cassava storage roots form an important source of carbohydrate for half a billion poor people in the tropical zone (1). World output of cassava storage roots totals about 150 million tons annually. In 1989, Thailand not only was the top cassava producer, yielding nearly 22 million tons, but also the top exporter of tapioca products, valued nearly 25 billion Baht. Worldwide, two-thirds of cassava is consumed as human food and one-third as

animal feed. For the human consumption, about half is eaten as cooked fresh roots and the remainder was processed into flour or meals.

As a staple diet for the tropical poor, cassava can caused various health problems due to its cyanide content (2). Cassava cyanide exist in two cyanogenic glucosides: the major form is called linamarin and the minor form is lotaustralin (3). Although cyanogenesis which includes the synthesis and degradation of the cyanogenic glucosides has been worked out in other plants such as white clover, flax and sorghum (3), limited work has been done on the molecular basis of the cassava cyanogenesis (4-6). The lack of the molecular knowledge has prevented any rational approach to decrease or abolish the chronic cyanide toxicity due to prolonged cassava consumption. So this project was designed to seek more molecular understanding of cassava cyanogenesis.

3. Methods and Results

The findings in this project have been summarized previously (see 5.2.2 and 5.2.5) and they can be grouped into 4 parts as follows.

3.1 The breakdown of linamarin to release cyanide;
3.2 The synthesis of linamarin;
3.3 The cyanogenesis of cassava plantlets in tissue culture; and
3.4 The deterioration of cassava storage roots.

3.1 The breakdown of linamarin to release cyanide

The hydrolytic breakdown of linamarin into glucose and acetone cyanohydrin is the crucial step leading to the release of free cyanide. The process is catalyzed by a specific β-glucosidase called linamarase. The unstable acetone cyanohydrin can undergo autohydrolysis to yield acetone and HCN. This step can be also facilitated by an enzyme called hydroxynitrile lyase. Thus enzymic characteristics of these reactions should be made clear.

Prior to this project, the hydrolase from cassava has been investigated (5,6). Since the hydrolysis of acetone cyanohydrin can occur enzymically or non-enzymically, the reaction may be difficult to control. On the other hand, the linamarase which catalyzes the hydrolysis of linamarin offers a more attractive possibility for future manipulation. If linamarase is inactivated or abolished, linamarin

should remain intact and non-toxic. Previous work on cassava linamarase employed the storage root cortex as the source and the purification was difficult (5).

Our findings on cassava linamarase and methods used in studying it have been described previously (see 5.1.1, 5.1.3, 5.1.5, 5.2.1, 5.2.3, 5.3.1, 5.3.3, 5.3.5, 5.3.6, 5.3.9, 5.3.11, and 5.3.12). Only a summary is provided below.

The main finding was the demonstration that cassava stem and petiole were much richer sources of linamarase than the root tissue. This led to a simple purification procedure and a possible application of cassava linamarase from stem and petiole in accelerating linamarin breakdown in processing of cassava-based food. Furthermore, additional properties of the enzyme have been established: isozymes, isoelectric points, amino acikd composition, kinetic properties, stability and recently the aggregation states from 4 to >12 subunits (see data in 5.2.5). The enzyme activity can be inhibited by substrate analogs, δ-gluconolactone and isopropyl- β-D-thioglucopyranoside. Its amino acid sequence is now being worked out. In addition, other researchers have later confirmed some of our findings (7,8).

3.2 <u>The synthesis of linamarin</u>

Based on the work on flax and white clover (3), linamarin is made from valine in two major reaction steps. The first involves the microsomal oxidation of value into acetone cyanohydrin. The second reaction is a conjugation of the acetone cyanohydrin with glucose moiety donated by UDP-glucose. The second reaction is catalyzed by a glucosyltransferase.

Prior to this project, there was a demonstration that ^{14}C-valine can be incorporated into linamarin by cassava tissues (4). No study has been carried out on the cassava microsome or the cassava glucosyltransferase.

During the initial period of the project, a great deal of effort was made in the isolation of cassava microsome fraction from young leaves. Despite various trials employing different conditions, only little activity of the microsomal oxidation of valine into acetone cyanohydrin can be obtained. Consequently, a study was performed on the second step, the glucosyltransferase-catalyzed synthesis of linamarin from acetone cyanohydrin and UDP-glucose. The study has

been described previously (see 5.1.2, 5.2.4, and 5.3.2) and is summarized below.

Cassava young leaves were found to be the richest source of glucosyltransferase. The enzyme purification from the young leaves was partially achieved by ammonium sulfate precipitation and followed by a gel filtration using a Sephacryl 200 column. A UDP-glucose hydrolysing activity was removed from the desired enzyme fraction while some sucrose synthase activity remained in the fraction of glucosyltransferase. The sucrose synthase activity was removed after further purification of the glucosyltransferase using a fast protein liquid chromatography (FPLC) and a MonoQ column. However, instability of the enzyme activity did not allow the purification to homogeneity. With the partially purified enzyme, some kinetic properties were studied. Its native molecular weight was only 46,000.

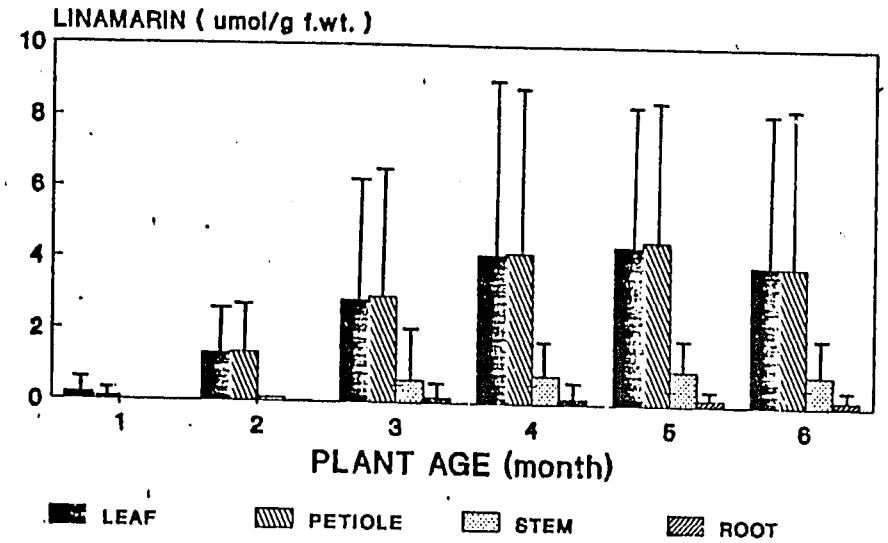
3.3 The cyanogenesis of cassava plantlets in tissue culture

Cassava plantlets can be propagated from any parts of the plant by tissue culture technique using the simple Murashige-Skoog medium. Although plant tissue culture of cassava is widely used in genetic collection and selection (for example, see 9) it has not been used to investigate the cyanogenesis in cassava before. Our work which will be soon presented in 5.1.4 will be the first attempt to use cassava plant tissue culture to help answer some issues on cyanogenesis.

Firstly, we wanted to know if cassava plantlets in tissue culture possessed linamarin. From Fig. 1, it was clear that both petioles and leaves of cassava plantlets contained linamarin. The levels increased with the age of the plantlets. Moreover, the leaves of the plantlets also possessed linamarase (Fig. 2). Thus, cyanogenesis might occur in the cassava plantlets under the sterile condition of tissue culture. This finding raised a question on the role of linamarin in defence against pests or pathogens.

Secondly, the linamarin content in the cassava plantlets was not altered significantly by the mineral levels or the presence of valine instead of glycine in MS medium unless the valine content was very high. This finding cannot confirm the field observation that the cyanide level in cassava roots varies with the soil minerals. However, it was noted that the plantlets in tissue culture did not form storage roots and hence may exist in a physiological state different from that of the cassava plants in the field.

Fig. 1 Effects of age on linamarin contents (upper panel) and growth rate (lower panel) of cassava plantlets in tissue culture. The growth rate is expressed as the length of the stem. For each condition, the data were obtained from 50 plantlets and presented as mean ± SD.



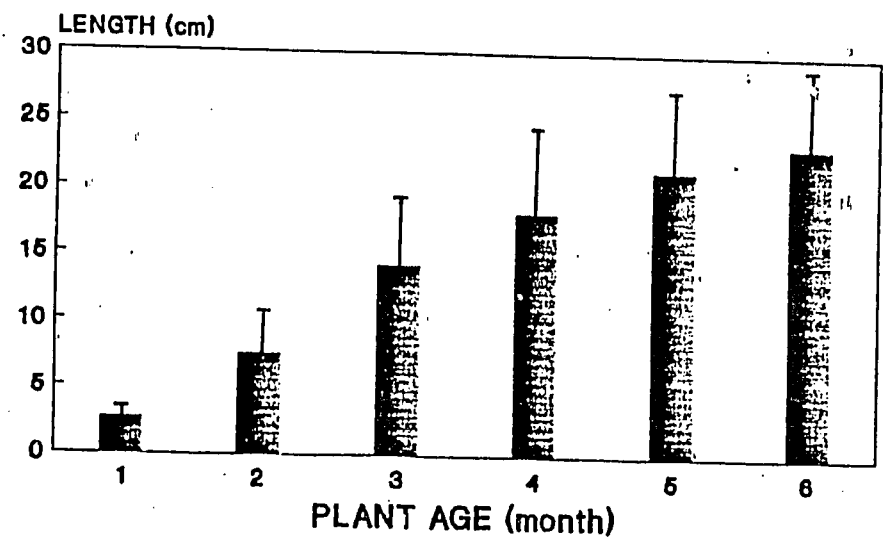


Fig. 2 Linamarase activity and protein content in the leaves of cassava plantlets from tissue culture at various ages. For each condition, the data were obtained from 50 plantlets and presented as mean ± SD.

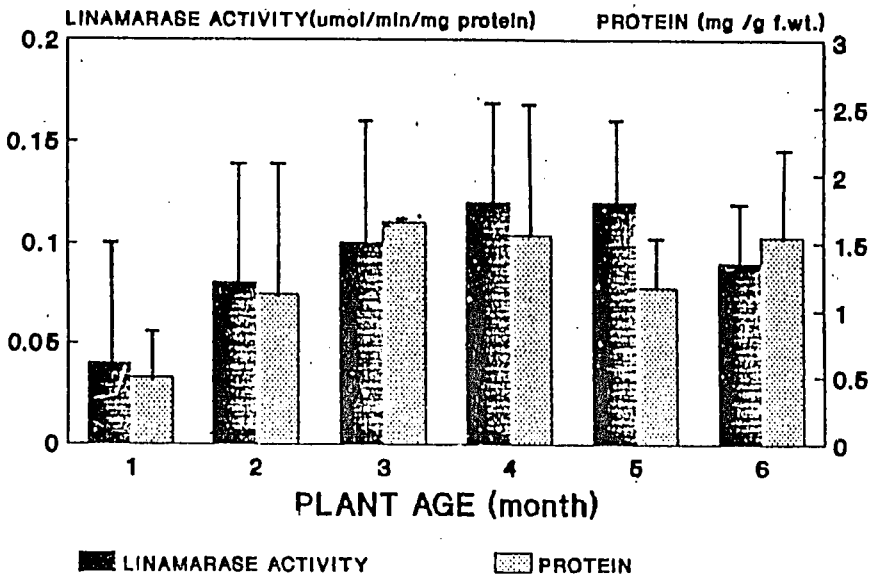
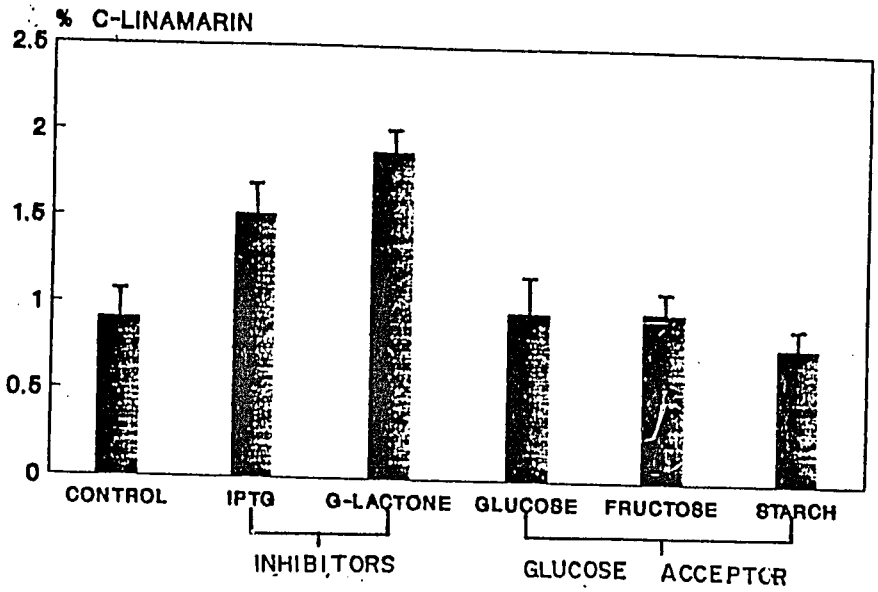


Fig. 3 Effects of linamarase inhibitors and glucose acceptors on radioactive incorporation from [^{14}C]-valine into linamarin by petiole tissue slices. The tissue splices (0.2 g) were incubated with 4.15 x 10^6 dpm of 30 μM [^{14}C]-valine in 1.8 ml MS medium for 12 hours at room temperature. The inhibitors used were 10 mM isopropyl-β-D-glucopyranoside (IPTG) and 0.2 mM δ-gluconolactone (G-lactone). The acceptors used were 10 mM glucose, 10 mM fructose and 10% (w/v) starch.



Thirdly, the petiole slices from cassava plantlets were capable of incorporating ^{14}C-valine into linamarin (Fig. 3). The level of incorporation was enhanced by the presence of an inhibitor of linamarase, either δ-gluconolactone or isopropyl-β-D-thioglucopyranoside, but not by an acceptor of UDP-glucose, ie. glucose, fructose, or starch.

3.4 The deterioration of cassava storage roots

Cassava storage roots deteriorate rather quickly. The spoiled roots are known to be toxic due to both the released cyanide and the accumulated polyphenols (10). The deteriorated roots contain discoloured tissue caused by the "browning" process. It is believed that oxidative polymerization of phenols and other components takes place in this process. Peroxidases have been shown to exist in the cassava roots and they have been suggested to be involved in the process (11,12). Our work on cassava peroxidases has been described previously (see 5.3.8 and 5.3.10).

After the cassava peroxidase activity was precipitated from the crude extract by ammonium sulfate at 40-80%, the enzyme can be separated into two forms, bound and unbound, by FPLC on a MonoQ column (see data in 5.2.5). From the analysis of specific activities (Table 1), it was clear that the cortex of the deteriorated roots possessed a large increase in both the bound and unbound forms of peroxidases. While the unbound form of the parenchyma also increased markedly, the bound form of the parenchyma did not change with the deteriorating process. Whether these dramatic changes of the peroxidases are related the development of colour in the parenchyma region next to the cortex remains to be investigated.

TABLE 1

Specific activities of peroxidases of cassava storage roots

(Unit = A_{465}/min/μg protein)

Peroxidase Fractions (From MonoQ column)	Fresh Roots		Deteriorated Roots (7 days RT)	
	Cortex	Parenchyma	Cortex	Parenchyma
Unbound	56.8	1.3	955	319
Bound	7.6	3.3	64	3

4. Impact, Relevance and Technology Transfer

The findings have provided more understanding about cassava cyanogenic system. The potential usefulness of linamarase from cassava stems and petioles left after the harvest of the roots has been recognized by the finding in this project. So it would be worthwhile for investigators in cassava food processing to try if addition of the crude extract from the cassava stems and leaves can accelerate the removal of linamarin and hence cyanide toxicity. The information gained on glucosyltransferase has suggested that the enzyme may be abolished by genetic engineering methods in the future to create acyanogenic cassava. The potential use of cassava plant tissue culture in testing the control of cyanogenesis and the physiology of acyanogenic cassava should benefit the goal of creating acyanogenic cultivar. This outcome should make the cassava consumption by the poor people in the developing countries safer.

This project has offerred an opportunity to establish plant biochemistry in Mahidol University. It has made possible for staff and students to work on an interesting aspect of secondary metabolism which has a relation to an important economic crop of Thailand. It has led to the establishment of a plant tissue culture unit in the Department of Biochemistry. This facility and the equipment will be useful for further investigation into cassava cyanogenesis and studies on other plants at molecular levels.

5. Project Activities/Outputs

The project provided opportunities for training 5 graduate students at M.Sc. level in plant biochemistry dealing with secondary metabolism. Three students completed their thesis work and awarded the M.Sc. degrees while two more students were in the process of writing their thesis. The titles of these thesis are given below (5.1).

Five full papers on various aspects of the project findings were published (see section 5.2). It is anticipated that 2-3 more papers will be written up for publication in the near future.

There were 12 abstracts of presentations on the project made in scientific meetings. The titles are given in section 5.3.

5.1 Theses (abstracts 5.1.1-5.1.3 attached)

5.1.1 Eksittikul, T. (1987) Purification and characterization of linamarase from cassave (*Manihot esculents* Crantz). M.Sc. thesis, Mahidol University, Bangkok.

5.1.2 Chalermisrachai, P. (1988) Glucosyltransferase from cassava (*Manihot esculenta* Crantz). M.Sc. thesis, Mahidol University, Bangkok.

5.1.3 Kisamanonta, P. (1989) Some structural characteristics of linamarase from cassava (*Manihot esculenta* Crantz). M.Sc. thesis, Mahidol University, Bangkok.

5.1.4 Panutat, P. (in preparation) Cyanogenesis of cassava plantlets in tissue culture. M.Sc. thesis, Mahidol University, Bangkok.

5.1.5 Sermsuvitayawong, K. (in preparation) The structure and properties of the isozymes of cassava linamarase. M.Sc. thesis, Mahidol University, Bangkok.

5.2 Full papers (copies attached)

5.2.1 Eksittikul, T. and Chulavatnatol, M. (1986) Comparison of cassava linamarases from petiole, stem and root cortex. ICSU Short Reports 6, 380-381.

5.2.2 Chulavatnatol, M., Ruchirawat, S., Eksittikul, T., Prawat, U., Chalermisrachai, P., Kitjaharn, P. and Prawat, H. (1987) Changes of cyanogenic glycosides and related enzymes in cassava roots during storage and processing: Cyanogenic glycosides in cassava (*Manihot esculenta* Crantz). Proceedings of His Majesty's Fifth Cycle Commemorative Conference of USAID Science Research Award Grantees, Nakorn Pathom, Thailand, pp 191-195.

5.2.3 Eksittikul, T. and Chulavatnatol, M. (1988) Characterization of cyanogeneic β-glucosidase (Linamarase) from cassava (*Manihot esculenta* Crantz). Arch. Biochem. Biophys. 266, 263-269.

5.2.4 Chalermisrachai, P. and Chulavatnatol, M. (1988) Glucosyltransferase from cassava (*Manihot esculenta* Crantz). Proceedings of 7th Symposium of Federation of Asian and Oceanian Biochemists, Kuala Lumpur, Malaysia, POS-C-07.

5.2.5 Chulavatnatol, M., Ruchirawat, S., Svasti, J., Eksittikul, T., Chalermisrachai, P., Kisamanonta, P., Panutat, P. and Sermsuvitayawong, K. (in press) Biotransformation of cyanogenic glycosides in cassava (*Manihot esculenta* Crantz). Proceedings of Workshop on AID/SCI Funded Research in Agricultural Biotechnology, Bangkok, Thailand.

5.3 <u>Abstracts of meetings</u> (copies attached)

5.3.1 Eksittikul, T. and Chulavatnatol, M. (1986) Petiole, stem, and root cortex of cassava as new sources of linamarase. 12th Conference on Science and Technology of Thailand. 20-22 October, Bangkok, Thailand, pp. 452-453.

5.3.2 Chalermisrachai, P. and Chulavatnatol, M. (1987) Properties of glucosyltransferase in cassava. 13th Conference on Science and Technology of Thailand. 20-22 October, Songkhla, Thailand, pp. 546-547.

5.3.3 Eksttikul, T. and Chulavatnatol, M. (1987) Catalytic properties of linamarase from cassava. 13th Conference on Science and Technology of Thailand. 20-22 October, Songkhla, Thailand, pp 548-549.

5.3.4 Chulavatnatol, M., Ruchirawat, S., Eksittikul, T., Prawat, U., Chalermisrachai, P., Kitjaharn, P. and Prawat, H. (1987) Biotransformation of cyanogenic glucosides in cassava (*Manihot esculenta* Crantz). The First Princess Chulabhorn Science Congress. International Congress on Natural Products. 10-13 December, Bangkok, Thailand, p BC27.

5.3.5 Eksittikul, T. and Chulavatnatol, M. (1988) Isozyme forms of linamarase from cassava (*Manihot esculanta* Crantz). 14th Conference on Science and Technology of Thailand. 19-21 October, Bangkok, Thailand, pp. 382-383.

5.3.6 Kisamanonta, P. and Chulavatnatol, M. (1988) Structure of cassava linamarase. 14th Conference on Science and Technology of Thailand. 19-21 October, Bangkok, Thailand, pp 346-347.

5.3.7 Chulavatnatol, M., Eksittikul, T., Chalermisrachai, P. and Kisamanonta, P. (1988) Towards cyanide-free cassava. Proceedings of the Eight Symposium of the International Society for Tropical Root Crops. 30 October-5 November, Bangkok, Thailand, p. 392.

5.3.8 Eksittikul, T. and Chulavatnatol, M. (1989) Cassava peroxidases. 15th Conference on Science and Technology of Thailand. 18-20 October, Chiangmai, Thailand, pp 574-575.

5.3.9 Kisamanonta, P. and Chulavatnatol, M. (1989) Stability of cassava linamarase in sodium dodecyl sulfate. 15th Conference on Science and Technolgy of Thailand. 18-20 October, Chiangmai, Thailand, pp 592-593.

5.3.10 Eksittikul, T. and Chulavatnatol, M. (1990) Cassava peroxidases. 16th Conference on Science and Technology of Thailand, 25-27 October, Bangkok, Thailand, pp 328-329.

5.3.11 Sermsuvitayawong, K., Svasti, J. and Chulavatnatol, M. (1990) Aggregation of peptide chains in cassava linamarase. 16th Conference on Science and Technology of Thailand, 25-27 October, Bangkok, Thailand, pp 328-329.

5.3.12 Sermsuvitayawong, K., Svasti, J. and Chulavatnatol, M. (1990) Structure of cassava linamarase. International Conference on Biotechnology and Environmental Science: Molecular Approaches. 21-24 August, Bangkok, Thailand, p. 132.

6. Project Productivity

The main goal in understanding the molecular events in cyanogenesis in cassava and storage root deterioration was mostly fulfilled. However, the related goals of developing method to improve the processing of cassava roots by manipulation of gases and exposure to chemicals were not carried out. It was known that cassava roots kept in plastic bags can be stored for a longer period. Instead, the challenge on the use of cassava plant tissue culture was considered more attractive and more study was done on this aspect.

7. Future Work

One significant consequence of this PSTC-supported work was the recent collaboration developed between this laboratory and two other laboratories in Europe to pursue further work on cassava cyanogenesis. The first of the two laboratories is that of Professor B.L. Moller of the Royal Veterinary and Agricultural University in Copenhagen, Denmark. This laboratory has experience working on the plant microsome. The second laboratory is that of Dr. M.A. Hughes at University of Newcastle, England. Dr. Hughes has expertise in genetic study of cyanogenic plants and will work on the genetics of the cassava enzymes. The collaboration has just been supported by a grant from Science and Technology for Development Program of European Economic Commission to carry out the molecular and genetic studies of cassava cyanogenesis. Under this support, the microsomal enzymes will be studied in Copenhagen, the glucosyltransferase and linamarase as well as tissue culture of cassava will be conduct in Bangkok. The genetics of the enzymes will be investigated in Newcastle. The combined results should form a logical approach towards creating a cyanide-free cultivar of cassava in the near future.

8 Literature Cited

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(3) Conn, E.E. (1980) Cyanogenic compounds. Ann. Rev. Plant Physiol. 31, 433-451.

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(5) Cooke, R.D., Blake, G.G. and Battershill, J.M. (1978) Purification of cassava linamarase. Phytochemistry 17, 381-383.

(6) Kojima, M., Iwatsuki, N., Data, E.S., Villegas, C.D.V. and Uritani, I. (1983) Changes of cyanide content and linamarase activity in wounded cassava roots. Plant Physiol. 72, 186-189.

(7) Mkpong, O.E., Yan, H., Chism, G. and Sayre, R.T. (1990) Purification, characterization and localization of linamarase in cassava. Plant Physiol. 93, 176-181.

(8) Yeoh, H.-H. (1989) Kinetic properties of beta-glucosidase from cassava. Phytochemistry 28, 721-724.

(9) Szabados, L., Hoyos, R. and Roca, W. (1987) *In vitro* somatic embryogenesis and plant regeneration of cassava. Plant Cell Rep. 6, 248-251.

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(12) Plumbley, R.A. and Hughes, P.A. (1982) DEAE-cellulose separation of peroxidases from cassava (*Manihot esculenta* Crantz) root tissue. J. Food Biochem. 6, 197-206.

Purification and Characterization of Linamarases from Cassava (*Manihot esculenta* Crantz)

Thidarat Eksittikul, M.Sc. thesis, Mahidol University, 1986
Bangkok, Thailand

ABSTRACT

Stem, petiole and root cortex of cassava were found to be richer in linamarase than root parenchyma while no activity was found in the leaf.

Linamarase was purified from extract of stem, petiole or root cortex by precipitation at 65 % ammonium sulfate saturation followed by Sepharose 6 B column chromatography and Chromato focusing. The enzyme from each source was found to have the native M_r of 600,000. By SDS-PAGE, the subunit M_r was found to be 63,000. So the native structure was oligomeric. The enzyme from each source was separated by Chromatofocusing into three isozymes with the pI values of 4.2-4.3, 3.3-3.6 and 2.8-2.9. The enzyme from each source has K_m values of 1-2 mM for linamarin and 13-18 mM for p-nitrophenyl-β-D-glucopyranoside, optimum temperature 55°-65°C and pH optima 7 for linamarin, pH 8 for p-nitrophenyl-β-D-glucopyronoside. They were similarly inhibited by δ-gluconolactone and isopropyl-β-D-thioglucopyranoside. Based on these similar properties, they were likely to be the same enzyme. The isozymes showed the same K_m value of 1-2 mM for linamarin. It can be concluded that petiole, stem and root cortex of cassava were good sources of linamarase.

Panpaka Chalermisrachai, M.Sc. thesis, Mahidol University, 1988
Bangkok, Thailand

Thesis Title Gluosyltransferase from cassava (*Manihot esculenta* Crantz.)

Name Miss Panpaka Chalermisrachai

Degree Master of Science (Biochemistry)

Thesis Supervisory Committee

Mr. Montri Chulavatnatol, Ph.D.

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ABSTRACT

A glucosyltransferase which catalyzes the conjugation of acetone cyanohydrin to glucose in the last step of linamarin biosynthesis was isolated from young leaves of cassava (*Manihot esculenta* Crantz.). The glucosyltransferase was found to be a soluble enzyme and was partially purified by 40-80% ammonium sulphate fractionation followed by Sephadex G-200 column and Mono Q HR 5/5 column chromatography. These steps resulted in a purification of 6 folds. The partially purified glucosyltransferase possessed negligible linamarase and little UDP-glucose hydrolysis activity. The native molecular weight of cassava glucosyltransferase was 46,000. The partially purified preparation consisted of five major bands of 13,000; 21,000; 25,000; 31,000 and 39,000 separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The enzyme activity was unstable but could be kept at -20°C for 17 days with 50% loss of the activity.

Catalytically, the partially purified glycosyltransferase showed a pH optimum of 9 to 10 and a temperature optimum of 37°C. The substrate specificities were also analyzed. UDP-glucose was the best sugar donor with a K_m of 0.15 mM which ADP-glucose was not and UDP-galactose was only half as good as UDP-glucose. Among the sugar acceptors tested, acetone cyanohydrin was the best and better than butanone cyanohydrin. Other aliphatic alcohols were poorer sugar acceptors in the following order: isopropanol, butanol, ethanol and glycerol. Phenol was unable to serve as the sugar acceptor for this enzyme.

The enzyme was unable to catalyze the transfer of glucose from UDP-glucose to starch, linamarin or simple sugars i.e. glucose, galactose and sucrose. However, a high glucosyltransferase activity was detected in the presence of UDP-glucose and fructose with a K_m for fructose of 4.4 mM. This activity could be due either to a contaminating UDP-glucose: D-fructose-glucosyltransferase or to cassava glucosyltransferase having two activities, one for linamarin synthesis and the other for sucrose synthesis.

Pennapa Kisamanonta, M.Sc. thesis, Mahidol University, 1989
Bangkok, Thailand

Thesis Title: Some structural characteristics of linamarase from cassava (*Manihot esculenta* Crantz.)

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Date of Graduation 2 June B.E. 2532 (1989)

ABSTRACT

Three isozyme of cassava petiole linamarase were used in two types of studies: structural study and subunit interaction study. Native isozymes were insensitive to the following proteases: trypsin, pronase, thermolysin, papain, subtilisin, collagenase and bromelain. After denaturation, they became sensitive to trypsin, pronase and thermolysin and yielded similar proteolytic fragments suggesting a similar protein chain among the isozymes. Using TNBS to modify lysine residues, the native and denatured forms of the isozyme pI 4.3 were found to have 179 and 199 mole lysines/mole isozyme respectively. Similarly, the native and denatured forms the isozyme pI 2.9 contained 88 and 100 moles/mole respectively. In the presence of a competitive inhibitor, the numbers of modified lysines of the native isozymes were not changed. The data indicated that most

of lysine residues were exposed but none was localized at the catalytic site. The isozymes were probably glycoproteins since they can be retained by a Con A-Sepharose column. The anti-linamarase serum was able to recognize the modified isozymes except isozyme pI 2.9 but could not recognize the heat-denatured isozymes. Guanidine hydrochloride dissociated the enzyme into smaller forms and caused inactivation also. Complete inactivation was observed by 3M GuHCl. However, the isozymes were stable in SDS up to 20% at room temperature for 30 min. In SDS-PAGE containing 1% SDS, active forms of different sizes were found. Treatment with β-mercaptoethanol did not dissociate the enzyme into subunits but heat treatment caused the dissociation and the complete loss of the activity.

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COMPARISON OF CASSAVA LINAMARASES FROM PETIOLE, STEM AND ROOT CORTEX

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Roots of cassava (*Manihot esculenta* Crantz) are an important staple food in the tropics. However, cassava roots also contain two toxic cyanogenic glucosides, linamarin and lotaustralin, and their degrading enzyme, linamarase (or linamarin β-D-glucoside glycohydrolase; EC 3.2.1.21). The enzyme has been purified from root parenchyma (1) and used for linamarin assay in cassava trading and research. Being the second largest cassava-producer in the world (after Brazil), Thailand has a great abundance of stems, leaves and root cortex as by-products from cassava plantation and industry. So we decided to explore these by-products as possible new sources of linamarase.

Cassava tissue pieces were chopped and homogenized in ice-cold 0.1 M acetate buffer, pH 5.5, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in a Waring blender. After passing through a Miracloth to remove insoluble material, the homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant fluid was brought to 65% saturation by adding solid ammonium sulfate. Initial experiments showed that most of the enzymic activity (90%) was precipitated at this salt concentration. The precipitate was recovered by centrifugation at 10,000 g for 50 min at 4°C, redissolved in 10 mM phosphate buffer, pH 5.5 and dialyzed against the buffer (3 x 50 vol) overnight at 4°C. Linamarin was prepared from cassava root cortex as described previously (2).

Among the tissues extracted, petiole, stem and root cortex were found to have high specific activities of the enzyme (Table 1). Thus they were used in further study. By passing the ammonium sulfate preparation through a column of Sepharose 6B, the

Table 1 Distribution of linamarase in cassava tissues (mean ±SD)

Ammonium sulfate fraction from	Preparation (n)	Protein/fresh weight (mg/gm)	Specific activity (μmol/min/mg prot.)
Leaf	4	0.32 ± 0.23	0
Petiole	5	0.92 ± 0.65	2.85 ± 2.02
Stem	4	1.25 ± 0.27	1.76 ± 1.10
Root cortex	3	0.28 ± 0.14	1.56 ± 1.53
Root parenchyma	3	0.39 ± 0.01	0.05 ± 0.04

enzyme was separated from other proteins and found to have a native M_r of 600,000 (for stem and petiole enzymes). By this technique, the cortex enzyme was partially resolved into two peaks with M_r of 570,000 and 400,000. The active fractions were pooled, applied onto a chromatofocusing column and eluted with Polybuffer having pH range of 7 to 4. The enzyme from each source was separated into two isozymes: one with pI of 4.1 and another with pI <4 which was eluted with 1 M NaCl. By SDS-PAGE, all fractions contained a major band with M_r of 63,000 which was the subunit of linamarase. After the gel filtration step, most other proteins were eliminated and the enzyme preparation was about 90% pure. Using the enzyme preparation from the Sepharose column, the K_m and V_{max} were estimated (Table 2). On the basis of these values, linamarases from petiole and stem were similar but different from that of the root cortex. The findings suggested that the petiole, stem and root cortex were possible new sources of the enzyme.

Table 2 Properties of cassava linamarases

Enzyme source	Native M_r(x10^{-3})	Subunit M_r(x10^{-3})	pI value	K_m (Mx10^4)	V_{max} (nmol/min/mg)	Optimal temp (°C)
Petiole	600	63	4.1; <4	5.6	3.14	65
Stem	600	63	4.1; <4	5.0	3.33	55
Root cortex	400;570	63	4.1; <4	2.1	1.40	60

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FY 1986

CHANGES OF CYANOGENIC GLYCOSIDES AND RELATED ENZYMES IN CASSAVA ROOTS DURING STORAGE AND PROCESSING (6.404): CYANOGENIC GLYCOSIDES IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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ABSTRACT

Chronic cyanide toxicity is a health treat to the poor, who survive on cassava diet. The toxicity is attributed to the cyanogenic glucosides, linamarin and lotaustralin. In this project, the biotransformation of the glucosides was studied with the aim to lessen the toxicity. Linamarase, the linamarin-hydrolyzing enzyme, was found in abundance in cassava petiole, stem and root cortex. The enzyme was purified and characterized. Glucosyltransferase, the last enzyme in linamarin biosynthesis, was present in young leaves. It is being purified and characterized. During storage, an apparent synthesis of linamarin in cassava roots was confirmed. A procedure was established to isolate linamarin and lotaustralin from cassava root cortex for use in research and as standard in determining cyanide content in tapioca products.

INTRODUCTION

Stored roots of cassava (*Manihot esculenta* Crantz), also called tapioca or manoic, provide an important source of carbohydrates for some 500 million people in 30 tropical countries. After rice, maize and sugarcane, cassava is the fourth most important source of calories. In 1986, the world cassava production was 143 million tons. Thailand and Brazil were the top producers, each producing about 20 million tons. Thailand has been the world's largest exporter of tapioca products. In 1986, she exported about 6.5 million tons, earning approximately 19,000 million baht which was about equal to the earning from rice export in the same year. A survey last year showed that the area used for cassava cultivation in Thailand was nearly 8 million rai (equivalent to 12,800 Km^2 or 2.5% of total land of the country). World-wide, about two-thirds of cassava is consumed as human food and one-third as animal feed. For the human consumption, half is eaten as cooked fresh roots and the remainder as processed flours or meals.

Hidden in this agro-economic profile is a health problem. Cassava is one of the 200 plant species which possess cyanogenic glucosides[1]. In cassava, the cyanogenic glucosides known today are linamarin (or phaseolunatin; 2-hydroxy-isobutyronitrile- β-D-glucopyranoside) and lotaustralin (or methylinamarin; 2-hydroxy-2-methylbutyronitrile- β-D-glucopyranoside), with the former being the majority. The average content of the cyanogenic glucosides in cassava roots is about 100 mg/kg. Thus, the world production of these natural products in cassava amounted

to 14,000 tons in 1986. The same glucosides are also present in lima bean, linen flax and white clover. The cyanogenic glucosides are toxic because of the free cyanide liberated from their hydrolysis. Thus, use of cassava as a staple diet can cause chronic cyanide toxicity, which can lead to such health problems as goiter, cretinism, tropical toxic neuropathy and tropical diabetes[2]. In short, long-term consumption of cassava by man or animals obviously does not cause death but their health can be impaired.

To minimize the health treat, three approaches have been taken. (a) A limit of 100 mg HCN/kg dry weight of tapioca products has been set up as a trade standard[3]. (b) Several post-harvest processing methods have been devised to reduce the cyanide content. And (c) Selective breeding and cultivation of low-cyanide species are encouraged. Science and technology have contributed much to (b) and (c). This project is an attempt to understand the biotransformation of the cyanogenic glucosides (mainly linamarin) in cassava, using molecular and cellular approaches. Linamarin metabolism is better-known in white clover and flax than in cassava even though more men and animals are exposed to it in the latter. It is anticipated that an understanding of linamarin metabolism in cassava will lead to (a) a rational approach to prevent post-harvest deterioration of cassava roots; and (b) a logical design for create a cyanide-free species. Both are desirable but not yet possible at present.

Studies in white clover and flax have shown that valine is converted by plant microsome to acetone cyanohydrin[1]. The latter is then conjugated to glucose by reactions with UDP-glucose to form linamarin. This reaction is catalyzed by a glucosyltransferase. Linamarin hydrolysis to yield acetone cyanohydrin is catalyzed by linamarase. The unstable acetone cyanohydrin then breaks down spontaneously or enzymatically into acetone and cyanide.

This report covers the work done in the first year. It contains studies on linamarase, glucosyltransferase and linamarin in cassava.

MATERIALS AND METHODS

Cassava roots, measuring 30-35 cm in length and 6-8 cm in diameter, stems, petioles and leaves were purchased from a Bangkok market.

Linamarin was either bought from Calbiochem or prepared from cassava root cortex. Two methods were used to prepare crude linamarin from the cortex. One involved the used of ice-cold 0.1 M phosphoric acid according to Kojima *et al*[4]. The other employed 80% ethanol at 65-70°C for extraction, according to Nambisan and Sundaresan[5]. Linamarin was assayed as HCN released after treating with an excess amount of linamarase.

To isolate pure linamarin and lotaustralin, the crude ethanol extract was concentrated under reduced pressure to give a dark brown residue. The residue was chromatographed in a silica gel column and eluted with a mixture of chloroform:methanol:water in the ratio 20:3:1 to yield lotaustralin and 6:4:1 to yield linamarin. The products were checked by TLC and NMR.

Linamarase was extracted from cassava tissues in 0.1 M acetate buffer, pH 5.5, according to the method of Cooke *et al*[6]. It was further purified by ammonium sulfate precipitation at 65% saturation, followed by gel filtration using a column of Sepharose 6B and chromatofocusing in pH range of 7 to 4 or 5 to 1[7].

Linamarase activity was assayed using linamarin as substrate by a modified procedure of Locas and Sotelo[8]. Alternatively, an artificial substrate, p-nitrophenyl-β-D-glucopyranoside, was used in the assay according to the method of Evans[9].

Glucosyltransferase was extracted from fresh young leaves in 0.15 M phosphate buffer, pH 7.6, containing 5% polyvinylpolypyrolidone and 1 mM DTT[10]. After centrifugation at 100,000 x g for 1 hr, the enzyme was collected by ammonium sulfate precipitate at 30-70% saturation.

RESULTS AND DISCUSSION

Four major studies will be described below

1. Linamarase

The enzyme has been purified from cassava root parenchyma by Cooke *et al*. Among various tissue extracts, it was shown that cassava petiole, stem and root cortex possessed much more linamarase than the root parenchyma (Table 1). The enzyme was separately purified from petiole, stem and root cortex as described elsewhere. The enzyme from each source was found by SDS-PAGE to have a subunit molecular weight of 62,000. The native structure was oligomeric having a molecular weight of approx. 600,000. The purified enzyme was separated by chromatofocusing into three isozymes with pI of 4.2, 3.5 and 2.8. With linamarin as substrate, the enzyme exhibited a pH optimum of 7 and Km of 1-2 mM. With p-nitrophenyl-β-D-glucopyranoside, the enzyme showed a pH optimum of 8 and Km of 13-18 mM. The linamarase activity was inhibited by δ-gluconolactone and isopropyl-β-D-thioglucopyranoside. Thus, purified linamarase can be easily obtained from cassava petiole, stem and root cortex. The enzyme will be useful not only in research but also in cyanide determination in tapioca trade.

Table 1 Distribution of linamarase in cassava tissues (mean ± SD)

Ammonium sulfate fraction from	Preparation (n)	Protein/fresh weight (mg/ml)	Specific activity (μmol/min/mg prot.)
Leaf	4	0.32 ± 0.23	0
Petiole	5	0.92 ± 0.65	2.85 ± 2.02
Stem	4	1.25 ± 0.27	1.76 ± 1.10
Root cortex	3	0.28 ± 0.14	1.56 ± 1.53
Root parenchyma	3	0.39 ± 0.01	0.05 ± 0.04

2. Glucosyltransferase

The enzyme was found to have a pH optimum of 8. It catalyzed the transfer of the glucosyl moiety from UDP-glucose to an alcohol acceptor. Acetone cyanohydrin was the best acceptor while ethanol, glycerol, isopropanol, butanol and phenol were not. The molecular weight of the native structure was estimated to be 46,000.

3. Root deterioration

Harvested cassava roots were allowed to deteriorate at room temperature. After 3 days, the roots became soft. By 6 days, fungal growth on the surface was noted. When the root were cut open, browning of the tissue was observed after 3 days of storage and became quite marked after 6 days. Linamarin and linamarase were mostly found in the root cortex. After 3 days of storage, the root cortex linamarase, free cyanide and protein content dropped while linamarin level rose (Table 2). The finding confirmed that of Kojima *et al*[4] which suggested the possible linamarin synthesis in cassava root during storage and deterioration. Future studies will search for ways to prevent the root deterioration. If this is achieved, post-harvest storage of cassava roots will be possible and beneficial to the farmers.

Table 2 Changes in linamarin, free cyanide, linamarase and protein in cassava root cortex during storage at room temperature

Parameter (n = 8)	Section	Day 0	Day 3
Linamarin (μmol/g wet wt.)	Head	1.13 ± 0.82	1.40 ± 1.01
	Middle	0.82 ± 0.92	1.22 ± 0.79
	Tip	1.40 ± 1.05	1.82 ± 0.68
Free cyanide (μmol/g wet wt.)	Head	0.37 ± 0.36	0.21 ± 0.16
	Middle	1.12 ± 0.73	0.79 ± 0.77
	Tip	0.82 ± 0.65	0.36 ± 0.50
Linamarase (μmol/min/mg)	Head	0.95 ± 1.65	0.31 ± 0.66
	Middle	0.70 ± 1.19	0.23 ± 0.60
	Tip	0.27 ± 0.49	0.15 ± 0.39
Protein (mg/g wet wt.)	Head	0.07 ± 0.02	0.09 ± 0.03
	Middle	0.05 ± 0.04	0.07 ± 0.03
	Tip	0.05 ± 0.01	0.08 ± 0.02

4. Linamarin isolation

A procedure was worked out to isolate linamarin and lotaustralin from the crude ethanol-extract of cassava root cortex. In a preparation, 62 gm of the crude extract yielded 1.23 gm of linamarin and 74 mg of lotaustralin. Efforts were being made to improve the isolation yield and to characterize certain unknown fractions containing sugar and cyanide. Although linamarin is available commercially, it is expensive. In the case of lotaustralin, there is no commercial source at present. Both will be useful in our project and in cyanide determination in trapioca trade.

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5.2.3

Characterization of Cyanogenic β-Glucosidase (Linamarase) from Cassava (*Manihot esculenta* Crantz)[1]

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Linamarase (EC 3.2.1.21) was purified from cassava petiole, stem, and root cortex by ammonium sulfate precipitation, column chromatography on Sepharose 6B, and chromatofocusing. The last step resolved the enzyme from each source into three forms with p*I* values of 4.3, 3.3, and 2.9. Each form was found to be oligomeric, consisting of one kind of subunit, M_r 63,000. The major isozyme with a p*I* of 4.3 from petiole showed a K_m for linamarin of 0.6 mM and possessed both β-glucosidase and β-fucosidase activities. The former was sensitive to inhibition by δ-gluconolactone, isopropyl-β-D-thioglucoside, and $HgCl_2$, whereas the latter was inhibited by Tris ion. © 1988 Academic Press, Inc.

Storage roots of cassava (*Manihot esculenta* Crantz), also called tapioca or manioc, provide an important source of carbohydrates for some 500 million people in 30 tropical countries (1). However, cassava consumption, both as human food and as animal feed, is associated with certain health problems (2). In cassava, there are two known cyanogenic glucosides: linamarin (2-hydroxyisobutyronitrile-β-D-glucopyranoside) and lotaustralin (2-hydroxy-2-methylbutyronitrile-β-D-glucepyranoside), the former being in the majority (3). Their hydrolysis is catalyzed by a specific β-glucosidase (EC 3.2.1.21), linamarase, and yields glucose and an aglycone, acetone cyanohydrin or butanone cyanohydrin. Spontaneous or enzymatic breakdown of these cyanohydrins eventually releases HCN which causes chronic cyanide toxicity among populations subsisting on cassava. In addition, the linamarin-linamarase system is thought to play a role in biological defense in some plants and insects (4). Furthermore, the enzyme is employed in the determination of the cyanogenic glucosides in cassava (5-7). It is potentially useful in accelerating the detoxification process in cassava food processing (8, 9). Because of the high degree of specificity, other plant β-glucosidases can hardly catalyze the linamarin hydrolysis (10) and thus cannot substitute for the cassava linamarase in these applications. Up to now, linamarases have been isolated from few plants: white clover (*Trifolium repens* L.) (11), flax (*Linum ussitatissinum*) (12), birdfoot trefoil (*Lotus corniculatus* L.) (13), butter bean (*Phaseolus lunatus*) (14), rubber tree (*Hevea brasiliensis*) (15), and cassava (16). Although cassava root has long been a common source of linamarase (16), cassava stem and petiole were recently shown to be better sources of the enzyme (17). In this report, improved purification, properties, and multiple forms of linamarases from cassava stem, petiole, and root cortex are described.

MATERIALS AND METHODS

Linamarase preparation. Crude enzyme was prepared as described earlier (17). Fresh cassava stems,

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[2] Received a scholarship from the National Center for Genetic Engineering and Biotechnology, Ministry of Science Technology and Energy, Thailand.

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petioles, and roots were obtained from local farms, separately chopped into small pieces, and homogenized in ice-cold 0.1 M acetate buffer, pH 5.5, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF)[4] for 2 min in a Waring blender. After passage through a Miracloth to remove insoluble material, the homogenate was centrifuged at 10,000*g* for 20 min. The supernatant fluid was brought to 65% saturation by adding solid ammonium sulfate. The precipitate was recovered by centrifugation at 10,000*g* for 50 min, redissolved in 10 mM phosphate buffer, pH 5.5, and dialyzed against the buffer (3 × 50 vol) overnight. The dialyzed sample was further purified by chromatography on a Sepharose 6B column. An aliquot (50 mg) of the dialyzed preparation was applied to a column (2 × 55 cm) preequilibrated with 10 mM phosphate buffer, pH 5.5. The active fractions were pooled and lyophilized. It was further purified by chromatofocusing in the pH range 5 to 1 according to a published method (18). A column (1 × 16 cm) of Polybuffer exchanger, PBE94 (Pharmacia), was preequilibrated with 25 mM formic acid, adjusted to pH 5.0 with ammonium hydroxide. An aliquot (10 mg) of the lyophilized preparation was dissolved in 25 mM formic acid, pH 5, and then applied onto the column. The column was eluted stepwise with 25 mM formic acid, adjusted to different pH values (4.0 and 3.0) with ammonium hydroxide, and followed by 25 mM oxalic acid adjusted to pH 1.4 with HCl. The eluting buffers were 6, 4, and 4 column volumes for pH 4.0, 3.0, and 1.4, respectively. The protein profile was monitored using Coomassie brilliant blue G-250 according to a previous method (19). The active fractions were pooled and dialyzed overnight against 10 mM phosphate buffer, pH 5.5. The preparation was then kept at −20°C as a solution or in lyophilized form. All purification steps were performed at 4°C.

Linamarase assays. Linamarase activity was determined using either linamarin or *p*-nitrophenyl-*β*-D-glucoside (PNPG). With linamarin as substrate, the assay was carried out according to a method modified from that of Lucas and Sotelo (20). Briefly, the assay was performed in a scintillation vial containing a center tube and sealed with a serum cap. The reaction mixture (2 ml) held in the vial contained 0.2 M potassium phosphate buffer, pH 6.8, 10 mM linamarin (Calbiochem), and 0.02-0.025 ml of enzyme (added last to initiate the reaction). The center tube contained 0.4 ml of 0.5% picric acid in 2.5% sodium carbonate. The vial was incubated at 37°C in a shaking water bath for 30 min. The product formation was found to be linear with time up to 60 min. The reaction was stopped by injecting 0.5 ml of 0.5 N HCl through the serum cap into the reaction mixture. The vial was further incubated for 30 min to trap HCN in the picrate solution. Afterward, the center tube was removed and 0.6 ml of distilled water was added to its content. The absorbance of the diluted content was measured at 515 nm using a Shimadzu UV-120 spectrophotometer. Using a cyanide standard curve, the amount of HCN released from linamarin was estimated. The cyanide standard curve was constructed using a KCN stock solution (12.05 mg KCN/100 ml). The absorbance at 515 nm was linearly related to HCN released under the same assay conditions up to 30 μg HCN. Purified linamarin was purchased from Sigma. The linamarin [illegible] the commercial product was de[illegible] as HCN released in the presence of an excess [illegible]marase by the above method. This method was also used with other cyanogenic glycosides as substrates.

With PNPG as substrate, the *β*-glucosidase activity was assayed according to the method of Evans (21). The reaction mixture containing 50 mM Tris-HCl buffer, pH 8, 10 mM PNPG, and 0.1 ml of the enzyme was incubated for 10 min at 40°C. The reaction was then stopped by addition of 2 ml of 1 M sodium bicarbonate. The absorbance of *p*-nitrophenol released was measured at 410 nm using a Shimadzu UV-120 spectrophotometer. A linear standard curve of *p*-nitrophenol was constructed up to 0.4 μmol. This method was also employed when other PNP-glycosides were tested as substrates. With PNPF as substrate, the assay buffer was changed to 50 mM phosphate buffer, pH 7.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described (22) using a slab gel (13 × 10 × 0.1 cm) and a linear gradient of 7 to 15% (w/v) acrylamide. The electrode buffer was 0.1% (w/v) SDS in 25 mM Tris-glycine, pH 8.3, and the tracking dye was bromphenol blue. After electrophoresis, the protein bands were stained with 0.2% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid at room temperature for 2 h and made visible by destaining overnight.

Protein determination. Protein concentration was determined by Lowry's method (23), with bovine serum albumin as standard.

Amino acid analysis. Amino acid composition of a protein sample was determined by a precolumn derivatization method (24, 25). The sample was first hydrolyzed in 6 N HCl at 110°C for 24 h. The phenylthiocarbamoyl (PTC) derivatives of amino acids were formed by reaction with phenylisothiocyanate (PITC). The hydrolysis and derivatization were performed with a Pico-Tag Workstation (Waters Assoc., Milford, MA). The PTC-amino acids were analyzed

[4] Abbreviations used: PMSF; phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PNPG, *p*-nitrophenyl-*β*-D-glucoside; PNPF, *p*-nitrophenyl-*β*-D-fucoside; PBE, Polybuffer exchanger; PITC, phenylisothiocyanate; PTC, phenylthiocarbamoyl; IPTG, isopropyl-*β*-D-thioglucoside.

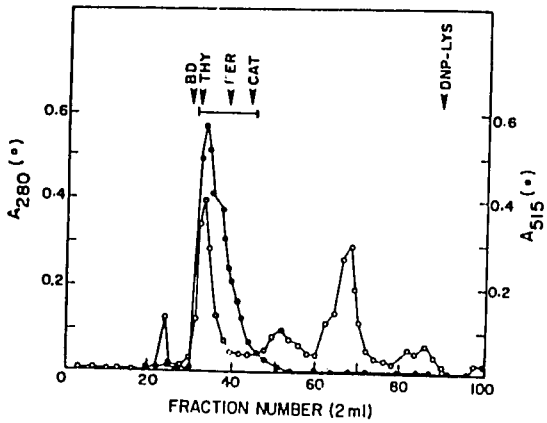

FIG. 1. Fractionation of ammonium sulfate-precipitated linamarase from cassava petiole by Sepharose 6B column chromatography. The fractions were monitored for protein at 230 nm (open circles) and linamarase activity using linamarin (solid circles). Cross-bar indicates the fractions being pooled. Arrowheads indicate the peak positions of the size markers: BD, blue dextran; THY, thyroglobulin (M_r 669,000); FER, ferritin (M_r 440,000); CAT, catalase (M_r 320,000); DNP-LYS, dinitrophenyl-lysine.

with a Pico-Tag amino acid analyzer (Waters Assoc.). This unit consisted of two pumps (Model 510), a gradient controller (Model M680), a column (Waters Pico-Tag reversed-phase), an injector (Model M710B WISP Autosampler), a detector (Model M440) set at 254 nm, an integrator (Model M740), and a column heater set at 40°C. The buffer system consisted of buffer A (0.1 M ammonium acetate, pH 5.70) and buffer B (acetonitrile:water = 60:40). The flow rate was 1 ml/min.

Kinetic analysis. K_m and V_{max} were calculated from the substrate saturation curve using a computer program for nonlinear regression (ENZFITTER; Elsevier-Biosoft).

RESULTS

Purification of linamarases from cassava petiole, stem, and root cortex. Cassava linamarases were purified from three tissues of the plant (petiole, stem, and root cortex) by the procedure described under Materials and Methods. From the petiole, the crude enzyme was found to have a high specific activity of 3.0 μmol/min/mg. Most of the activity was precipitated at 65% saturation of ammonium sulfate. When the precipitate was redissolved, dialyzed, and applied to a column of Sepharose 6B, the linamarase activity eluted near the void volume (Fig. 1), showing a native molecular weight of approximately 600,000. The enzyme obtained from the Sepharose 6B column was separated into three forms by passage through a chromatofocusing column in the pH range 5 to 1 (Fig. 2). The three forms exhibited p*I* values of 4.3, 3.3, and 2.9. By SDS-PAGE, each form was found to consist of a single protein of M_r 63,000 (Fig. 3). Table I summarizes the purification and the yield. The most predominant form from the cassava petiole was found to be one with a p*I* of 4.3 that was purified 16.8-fold with 52% yield. Similarly, the stem enzyme was purified and yielded the same three forms, the one with a p*I* of 4.3 being predominant. The enzyme from the root cortex could also be purified and separated into three forms by the above procedure. However, the three forms from the root cortex were equally distributed.

Prior to the above chromatofocusing step, the stability of the enzyme was tested in 25 mM formic acid adjusted to pH 3, 4, and 5. Under these pH conditions, the enzyme activity was mostly (80%) retained up to 7 days at 4°C.

Amino acid compositions of cassava linamarases. Table II shows the amino acid

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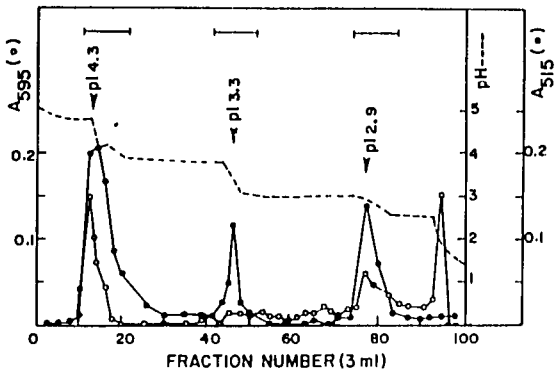

FIG. 2. Chromatofocusing column chromatography of the linamarase obtained from the Sepharose 6B column (Fig. 1). The fractions were monitored for protein by binding to Coomassie brilliant blue G-250 (open circles) and linamarase activity using linamarin (solid circles). Cross-bars indicate fractions being pooled, arrows indicate p*I* values of the active peaks, and the line without symbols represents the pH gradient.

composition of each form of linamarase from petiole, stem, or root cortex. Based on the amino acid compositions, all forms of the enzymes from these sources were similar. With the precolumn derivatization method, cysteine and methionine tended to be degraded and thus were not quantified. It was also difficult to quantify PTC-proline because it was not properly separated

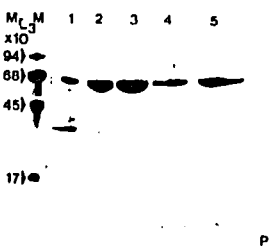

FIG. 3. SDS-PAGE of linamarase from cassava petiole during purification. Lane 1, 50 μg of ammonium sulfate precipitate at 65% saturation; lane 2, 30 μg of pooled active fractions from Sepharose 6B; lanes 3, 4, and 5, 20 μg of pooled active fractions from charomatofocusing column with p*I* values of 4.3, 3.3 and 2.9, respectively. Lane M contained the molecular weight markers: phosphorylase b (M_r 94,000); bovine serum albumin (M_r 68,000); ovalbumin (M_r 45,000); myoglobin (M_r 17,000).

from phenylthiourea which was formed from ammonia (26).

Catalytic properties of cassava linamarase. Since the purified major isozyme with a p*I* of 4.3 was obtained in sufficient quantity from petiole (Fig. 2), it was used in further investigations of catalytic properties. It was found to catalyze the hydrolysis of linamarin but not that of prunasin or amygdalin. It also catalyzed the hydrolysis of PNPG and PNPF but *p*-nitrophenyl-β-D derivatives of galactose, mannose, and gentiobiose cannot be used as its substrate. Since PNPG was a good substrate but *p*-nitrophenyl-α-D-glucoside was not, the enzyme was considered to have specificity for a β-glycosidic linkage. Among The three suitable substrates showed some different characteristics. First, the pH optima for the substrates were different: pH 7-8 for linamarin, pH 8 for PNPG, and pH 7 for PNPF (Fig. 4). Second, the K_m value for linamarin was 0.6 mM while that for PNPG was about 10-fold higher and that for PNPF was half this value (Table III). Third, the V_{max} value for linamarin was about one-quarter of that for PNPG or PNPF (Table III). Furthermore, either PNPG or PNPF could increase the K_m value for linamarin (Table III). For the linamarase activity, the K_m but not the V_{max}

TABLE I

PURIFICATION OF LINAMARASE FROM CASSAVA PETIOLE

Step	Protein (mg)	Activity[a] (μmol/min)	Specific activity (μmol/min/mg)	Fold	Yield (%)
Crude	678	2034	3.0	(1)	(100)
Ammonium sulfate	266	2879	10.8	3.6	142
Sepharose 6B	29	1425	49.1	16.4	70
Chromatofocusing					
$pI = 4.3$	21	1056	50.3	16.8	52
$pI = 3.3$	4	47	11.8	3.9	2
$pI = 2.9$	6	39	6.5	2.2	2

[a] Measured using linamarin as substrate.

was raised in the presence of δ-gluconolactone and IPTG. With PNPG as the substrate, Hg^{2+} was a strong inhibitor, causing a marked increase in K_m. In contrast, Mn^{2+} was less inhibitory while Mg^{2+}, Ca^{2+}, and Cu^{2+} were ineffective at 10 mM. EDTA at 10 mM was not itself inhibitory but could reverse the Hg^{2+} inhibition. The Hg^{2+} effect was also abolished by the presence of phosphate. In addition, Tris ion was found to inhibit the fucosidase activity by decreasing the V_{max} and increasing the K_m (Table III) but did not affect glucosidase activity.

DISCUSSION

There has been only one previous report describing the isolation and properties of cassava linamarase from root parenchyma (16). Briefly, the root parenchyma enzyme has to be purified 350-fold to reach the specific activity of 1.6 μmol/min/mg. It exhibits a native molecular weight of 600,000, a K_m for linamarin of 1.45 mM, and an optimal pH of 6. Using cassava petiole, stem, or root cortex, which are richer in the linamarase activity, we need only purify the enzyme 17-fold to reach a much higher spe-

TABLE II

AMINO ACID COMPOSITIONS (mol/100 mol RECOVERED) OF MULTIPLE FORMS OF CASSAVA LINAMARASE

Amino acid	Root cortex			Stem			Petiole		
pI:	2.9	3.3	4.3	2.9	3.3	4.3	2.9	3.3	4.3
Asx	12.22	9.17	13.51	9.07	12.36	12.55	13.54	14.89	14.86
Glx	8.38	7.51	9.80	8.34	10.76	9.81	9.49	9.88	10.40
Ser	6.07	5.66	6.84	5.78	6.35	5.77	6.45	5.91	7.94
Gly	8.52	7.32	6.91	10.00	8.80	9.09	8.01	8.19	9.73
His	1.05	0.81	1.11	0.89	1.36	1.55	1.18	1.35	1.37
Arg	2.83	1.55	1.74	1.79	2.77	2.39	3.06	3.28	4.58
Thr	3.86	2.42	2.96	2.76	3.69	4.08	3.69	3.99	4.99
Ala	6.64	4.55	5.10	5.89	6.24	6.94	6.12	6.91	7.57
Tyr	4.64	2.95	3.44	3.53	4.96	5.20	5.24	5.52	6.87
Val	3.05	1.90	3.16	2.33	3.19	3.04	3.25	3.41	4.16
Ile	2.64	1.64	2.37	1.29	2.15	3.04	2.22	2.37	3.63
Leu	5.32	3.70	4.74	3.42	4.43	6.91	4.75	5.24	6.75
Phe	4.17	2.92	3.84	1.97	3.32	5.83	3.90	4.20	5.27
Lys	2.25	1.44	1.74	0.97	1.59	2.10	1.82	1.82	3.71
Pro + NH_3	28.35	46.45	32.71	41.95	28.06	21.69	27.26	23.04	8.17

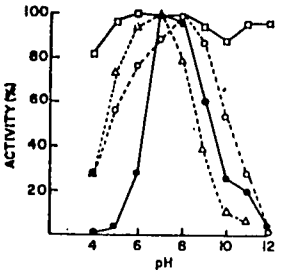

FIG. 4. pH profiles of linamarase from cassava petiole. Activity was assayed in universal buffer (30) in the pH range 4 to 12, using 10 mM substrate: linamarin (solid circles); PNPG (open circles); PNPF (triangles). The stability of the enzyme under experimental conditions was tested by assaying the activity at pH 8 using PNPG after exposure of the enzyme to various pH for 30 min (squares).

cific activity (Table I). The enzymes from these sources show native molecular weight (Fig. 1), K_m for linamarin (Table III), and optimal pH (Fig. 4) similar to those of the root parenchymal enzyme. In addition, it is the first time that the subunit molecular weight (Fig. 3), the existence of the multiple forms (Fig. 2), and the amino acid compositions (Table II) of the cassava linamarases have been established. From these data, it may be proposed that cassava linamarase is a homopolymer with a subunit molecular weight of 63,000. The molecular basis of the observed charge differences among the three forms (Fig. 2) remains to be investigated.

In comparison with other plant linamarases, the cassava enzyme exhibits both similarities and dissimilarities. The cassava linamarase is similar structurally to those of white clover (11), flax (12), butter bean (14), and rubber tree (15) in being oligomeric with a similar subunit molecular weight. However, only flax linamarase has been shown to exist in two forms (12) in comparison with the three forms of the cassava enzyme. Furthermore, the amino acid compositions of the cassava linamarase (Table II) compare favorably with that of the butter bean enzyme (14). Catalytically, the cassava linamarase shows a lower K_m for linamarin than the other plant linamarases. In contrast to the low K_m for PNPG of cassava root parenchymal linamarase (16), the enzyme from cassava petiole exhibits a much higher K_m for the artificial substrate (Table III). The K_m for PNPG is also low for the enzymes of butter bean (14) and of rubber tree (15).

Although cassava linamarase exhibits β-glucosidase activity (Table III), it lacks β-galactosidase activity, which is known to be associated with some β-glucosidases (27). Instead, the purified major isozyme of cassava linamarase displays β-fucosidase activity (Table III). Since PNPF inhibits β-glucosidase activity (Table III), it is possible that both β-glucosidase and β-fucosidase activities share a common active site. The active site of the cassava linamarase has not been fully characterized. Even though some plant β-glucosidases show high aglycone specificity (10), lack of appropriate glucosides has limited our attempt to investigate the aglycone specificity of the active site of this enzyme. However, it is expected that the active site should bind a presumed reaction intermediate, δ-gluconolactone, and a substrate analog, IPTG (Table III). The inhibition by Hg^{2+} (Table III) may suggest the importance of a sulfhydryl group in the catalysis. However, the presence of cysteine in cassava linamarase needs to be verified by

TABLE III

KINETIC PARAMETERS OF THE MAJOR ISOZYME (pI = 4.3) OF LINAMARASE FROM CASSAVA PETIOLE

Substrate (+ inhibitor)	K_m (mM)	V_{max} (μmol/min/mg)	Assay for
Linamarin	0.57	58	HCN
Linamarin + 0.15 mM δ-gluconolactone	1.04	50	HCN
Linamarin + 10 mM IPTG	2.21	65	HCN
Linamarin + 2 mM PNPG	5.31	84	HCN
Linamarin + 0.5 mM PNPF	1.02	58	HCN
PNPG	5.05	256	PNP
PNPG + 0.25 mM $HgCl_2$	15	288	PNP
PNPF	0.23	266	PNP
PNPF + 25 mM Tris-HCl	1.11	130	PNP

amino acid analysis using a method that does not destroy this amino acid. The mode of action of Tris ion appears more complex since it inhibits only β-fucosidase activity and it does so by increasing K_m and decreasing V_{max}. Further work on the active site may lead to a satisfactory explanation for this observation.

Since petioles and stems are abundant agricultural by-products of cassava plantation, they should serve as economical sources of linamarase. The ease with which reasonably pure enzyme is isolated by the procedure described in this report (up to the Sepharose 6B column step) should facilitate such usage. Thus, cassava linamarases from petioles and stems may be interesting alternatives to the fungal linamarases (28, 29) in detoxifying cassava food products (8, 9).

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GLUCOSYLTRANSFERASE FROM CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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Cassava which is a very cheap source of calories play an important role in the nutrition of the people of the developing countries. However, cassava diet also causes cyanide toxicity since cassava roots contain two cyanogenic glucosides, linamarin and lotaustalin. Their biosynthesis in the leaves utilize precursor amino acids, valine and isoleucine. Microsomal enzyme system converts these precursors into aldoximes, nitriles and then α-hydroxynitriles. Glucosyltransferase catalyzes the last biosynthetic step in which glucosyl residue from UDP-glucose is conjugated to a α-hydroxynitrile to give a cyanogenic glucoside. The paper presents the purification and properties of cassava glucosyltransferase.

(I) PURIFICATION OF CASSAVA GLUCOSYLTRANFERASE

Glucosyltransferase was isolated from fresh young leaves and shoots of cassava. It was a soluble enzyme because its activity was detected in the supernatant fraction after centrifugation of the tissue homogenate at 100,000 x g for 1 hr. The enzyme was purified by ammonium sulphate fractionation and was found to be enriched in the precipitate at 40-80% saturation. The precipitate was further chromatographed on Sephadex G-200 column. The native molecular weight was determined to be 46,000 (Fig. 1). A summary of the purification is given in Table 1.

Table 1 Purification of glucosyltransferase from young leaves of cassava

Purification	Protein (mg)	Activity (nmole/hr)	Spec.act. (nmole/hr/mg)	Fold	Yield %
SN100,000	761.3	85.03×10^2	1.12×10^2	1.0	100
40-80% $AmSO_4$	438.88	90.25×10^2	2.06×10^2	1.84	106
G-200 #GTF	127.68	42.20×10^2	3.3×10^2	2.95	50

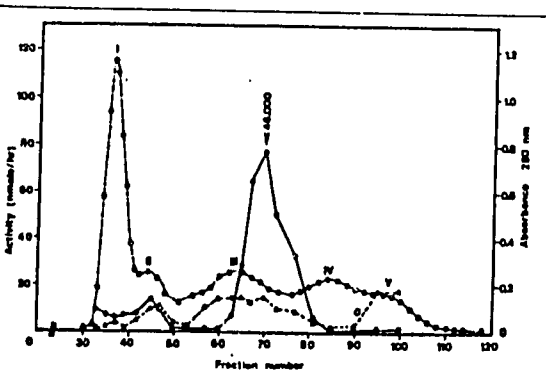
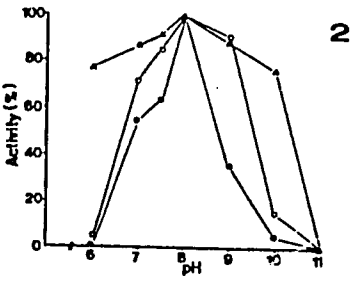


Fig. 1 Chromatograph of cassava glycosyltransferase in 40-80% ammonium sulphate fractionation on a Sephadex G-200 column

(II) PROPERTIES OF CASSAVA GLUCOSYLTRANSFERASE

The partially purified enzyme was characterized. The preparation was found to catalyze the transfer of glucosyl residue from UDP-glucose to cyanohydrins and to fructose. Its pH optimum was 8.0 for both activities (Fig. 2). However, the activity for cyanohydrins was more thermo-labile than that for fructose (Fig. 3). Furthermore the K_m values for UDP-glucose when cyanohydrins were used as acceptors were less than the value obtained with fructose as acceptor (Table 2). Thus the cyanohydrin glucosyltransferase was probably a different enzyme from sucrose synthetase but both were yet to be separated by further purification.



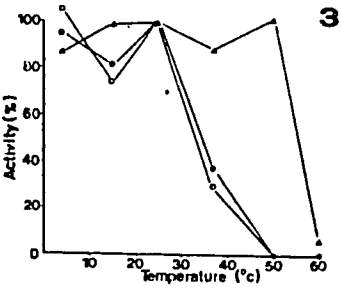


Fig. 2 The effect of pH on the enzyme activity. The enzyme was assayed using UDP-glucose as sugar donor and acetone cyanohydrin (●), butanone cyanohydrin (o) and fructose (▲) as sugar acceptors

Fig. 3 The effect of temperature on the enzyme activity. The enzyme was incubated at different temperatures for 1 hr before being assayed at 37°C, using acetone cyanohydrin (●), butanone cyanohydrin (o) and fructose (▲) as sugar acceptors.

Table 2 Kinetic parameters of cassava glucosyltransferase

Sugar acceptor	K_m of UDP-glucose (mM)	V_{max} (nmole/hr/mg)
Acetone cyanohydrin	0.15	60.1
Butanone cyanohydrin	0.08	45.5
Fructose	0.39	246.3

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IN AGRICULTURAL BIOTECHNOLOGY, 16-18 AUG. 90, BANGKOK THAILAND

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

5.2.5

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ABSTRACT

Key enzymes involved in the cyanogenesis in cassava were studied. Cassava linamarase was purified and its structural and catalytic properties were investigated. Due to its instability, cassava glucosyltransferase was partially purified and characterized. Changes in the forms of peroxidases in the cassava root parenchyma during root deterioration were observed and this may explain the browning of the spoiled root tissue. Cassava plantlets in tissue culture were found to possess the capabilities for linamarin synthesis and cyanogenesis.

Keywords: Cassava, linamarin, linamarase, glucosyltransferase, peroxidase.

Grant no: PSTC 936-5542-G-00-6028-00 "Changes in cyanogenic glycosides and related enzymes in cassava roots during storage and processing".

INTRODUCTION

Storage roots of cassava (*Manihot esculenta* Crantz) is an important source of carbohydrate for half a billion poor people in the tropical zone (1). World cassava production reached 143 million tons in 1986 (2). Thailand was not only the top cassava producer, yielding nearly 22 million tons in 1989, she was also the top exporter of tapioca products, valued nearly 25 billion Baht. About two-thirds of cassava is consumed as human food and one-third as animal feed. For the human consumption, about half is eaten as cooked fresh roots and the remainder as processed flour or meals.

Using cassava as a staple diet is associated with chronic cyanide toxicity which may cause such health problems as goiter, cretinism, tropical toxic neuropathy and tropical diabetes (1,3). Like 200 other plants, cassava is known to contain cyanogenic glycosides which can break down to release the toxic cyanide. Cassava cyanogenic glucosides are linamarin and lotaustralin. Although traditional food processing and industrial extraction of cassava starch can effectively remove the toxic cyanogens, they add cost to this caloric source of the poor. So understanding of the biotransformation of these cyanogens in cassava is essential for future manipulation to develop a cyanide-free cassava cultivar.

Linamarin and lotaustralin are secondary metabolites derived from valine and isoleucine respectively. Since linamarin is the major component in cassava, its biotransformation will be outlined in the following steps. (a) Valine is converted into acetone cyanohydrin by a microsomal enzyme system. (b) The unstable acetone cyanohydrin is conjugated using UDP-glucose into linamarin in a reaction catalyzed by a glucosyltransferase. (c) Upon tissue damage, linamarin is hydrolyzed by linamarase to release acetone cyanohydrin. (d) On autohydrolysis above pH 5 or hydrolysis catalyzed by hydroxynitrile lyase, acetone cyanohydrin releases HCN. In this project, three aspects of the biotransformation were investigated: (i) key cassava enzymes (linamarase and glucosyltransferase) were characterized, (ii) peroxidase involved in cassava root tissue damage was studied and (iii) cassava tissue culture was employed to study the role of linamarin.

MATERIALS AND METHODS

Cassava roots, stems, petioles and leaves were obtained from a Bangkok market. Cassava plantlets from tissue culture were propagated from the plantlets kindly supplied by the Central Research Laboratory, Kasetsart University. Linamarin was bought from Calbiochem. Crude linamarin was prepared from cassava root cortex according to a previous method (4). Acetone cyanohydrin and related compounds were synthesized and supplied by the Department of Chemistry, Mahidol University. Other biochemicals were purchased from Sigma. [^{14}C]-Valine and [^{14}C]-UDP-glucose were from Amersham.

Linamarase isolation and assay were performed as described previously (4). Glucosyltransferase was extrcted and assayed according to a previous report (5). Peroxidase activity was assayed using a known method. Cassava tissue culture was carried out in MS medium by a normal procedure.

RESULTS AND DISCUSSION

(i) Cassava linamarase and glucosyltransferase

The key enzymes in the cyanogenesis were linamarase and glucosyltransferase. This work started with the finding that the good sources of cassava linamarase were the stem and petiole rather than the cassava root as reported earlier (2,6). Having found an abundant source, the task of the purification of linamarase was simplified (7). the enzymic properties, both structural and catalytic, were then established. Cassava linamarase was found to be a polymeric enzyme, consisting of one single type of subunit. The subunit M_r was 63,000 (7). The polymeric forms existed in various sizes consisting of 4, 6, 8, 10 or more subunits (Table 1). These forms may be related to the observed isozymes of linamarase (7,8). The purified cassava linamarase was found to possessed both ß-glucosidase and ß-fucosidase activities. The activity was inhibited by substrate analogs, δ-gluconolactone and isopropyl-ß-D-thioglucoside (7). Dissociation of the polymeric enzyme into monomer was achieved by guanidine HCl, but the activity was also lost. On the contrary, the activity was stable in 1% SDS (9, 10). The findings suggested that the control of the association of the subunits could alter the linamarase activity in the cassava tissue, hence regulating the breakdown of linamarin.

Table 1 Molecular weights of cassava linamarase

Mol. Wt.	Estimated no. of subunits
272,400	4
353,800	6
507,700	8
624,100	10
828,400	12-14

The purification of the glucosyltransferase from young leaves was possible using ammonium sulphate precipitation and gel filtration (5, 11). The enzyme was rather unstable and still contaminated with sucrose synthetase activity. Being a small enzyme with native M_r of 46,000, it may be a target for future genetic manipulation to eliminate the enzyme. This will be a logical approach to create a cyanide-free cassava by genetic engineering.

(ii) Cassava peroxidases

Several changes took place when cassava roots were stored at room temperature for several days. The spoiled roots became unedible because of the toxic HCN released and the formation of toxic brownish polyphenols. The tissue softening and damage during storage caused the release of several enzymes. The enzymes known to cause the polymerization of phenolic compounds were peroxidases. By fast protein liquid chromatography (FPLC), the cassava root peroxidases were separated into two forms, bound and unbound forms (12). In the fresh root parenchyma, the bound form was the major component while the unbound form was the minor one. The contrary was true for the fresh root cortex (Fig. 1). After storage for 7 days, the parenchyma became brownish but not the cortex. Correlating with this change was a decrease of the bound form with an increase of the unbound form of parenthyma peroxidases (Fig. 2). In the cortex, there was change in neither the forms of peroxidases nor the color of the tissue. Thus, the unbound form of the parachyma peroxidase may be responsible for the polyphenol formation. Inhibiting the peroxidase activity by soaking the roots in some reagent may delay the browning of the stored roots. In addition, the finding also implied that cassava roots may be a good source of peroxidase, a useful enzyme in biotechnological techniques.

(iii) Cassava tissue culture

Plant tissue culture was employed to study the synthesis of linamarin. It was widely believed that the toxic cyanide released from the cyanogenic glucosides serve to repel attacking pests and plant pathogens. This suggested role could explain the lack of need for perticides in cassava plantation. If this was the case, cassava plantlets in tissue culture bottles, sterile and protected from any pests, may not need to synthesize linamarin. The cassava tissue culture was set up to test this hypothesis. The contrary was found. The plantlets were found to contain linamarin, particularly high in the leaf and petiole (Table 2). The tissues also possessed linamarase activity and can convert [^{14}C]-valine into linamarin. Thus, it may be concluded that linamarin synthesis in cassava was an intrinsic property of the plant and that linamarin may serve other useful functions in the plant.

Table 2 Linamarin contents in cassava plantlets

Age	Linamarin (μmol/g fresh wt)			
(Month)	Leaf	Petiole	Stem	Root
1	0.20 ± 0.34	0.09 ± 0.24	0.01 ± 0.02	0
2	1.32 ± 1.25	1.36 ± 1.36	0.10 ± 0.02	0.02 ± 0.04
3	2.85 ± 3.34	2.96 ± 3.56	0.64 ± 1.41	0.14 ± 0.35
4	4.15 ± 4.86	4.20 ± 4.69	0.81 ± 0.86	0.17 ± 0.37
5	4.42 ± 3.86	4.57 ± 3.89	0.97 ± 0.92	0.19 ± 0.24
6	3.91 ± 4.24	3.89 ± 4.38	0.88 ± 1.03	0.20 ± 0.31

ACKNOWLEDGEMENTS

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Fig. 1 FPLC separation of cassava peroxidases from fresh roots using a Mono Q column. P = parenchyma, C = cortex.

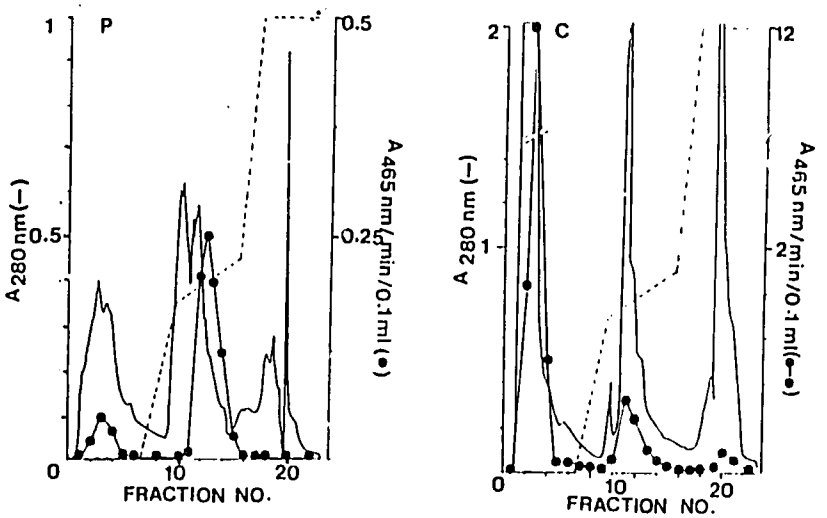
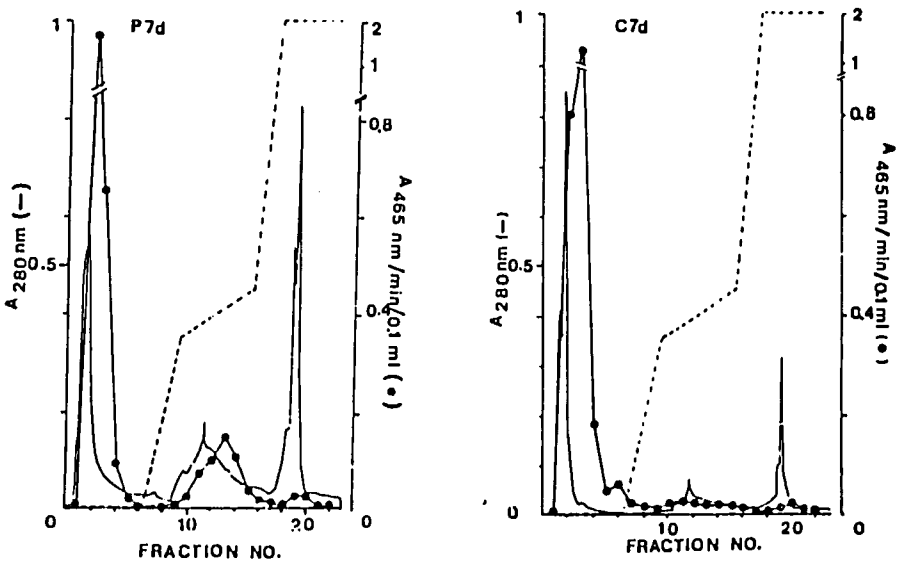


Fig. 2 FPLC separation of cassava peroxidases from 7-day stored roots using a Mono Q column. P = parenchyma, C = cortex.



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ชื่อ-สกุล ผู้เสนอ ธิดารัตน์ เอกสิทธิกุล

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PETIOLE, STEM AND ROOT CORTEX OF CASSAVA AS NEW SOURCES OF LINAMARASE

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Cassava (Manihot esculenta Crantz) is an important food in the developing world. However extensive consumption of cassava roots may run the risk of cyanide toxicity. Cassava root contains two cyanogenic glucosides, linamarin as main component and lotaustalin, and their degrading enzyme linamarase (or β-D-glucoside glycohydrolase E.C. 3.2.1.21). Hydrolysis of these glucosides by linamarase releases hydrogen cyanide when the roots are damaged. The enzyme has been partial purified from cassava root parenchyma (1) and used for determination of cyanide in cassava trading and research. Thailand is the second largest cassava-producer in the world. Much stems and leaves are by-products from cassava plantation. So we decided to look for linamarase in these by-products. The specific activities of the enzyme in petiole, stem and root cortex were found to be higher than that in the root parenchyma. The enzyme was separated from other proteins by Sepharose 6B column and found to have a native M_r of 600,000 (for petiole and stem). By this technique, the root cortex enzyme was partial resolved into two peaks with M_r of 570,000 and 400,000. By SDS-PAGE, the subunit M_r of each enzyme preparation was found to be 63,000. From chromatofocusing column in the pH range of 5 to 1, the enzyme from each source was separated into three isozymes with pI 4.3, 3.3 and 2.9. The pH optimum, K_m and V_{max} values of these enzyme preparations were also compared. The findings suggested that petiole, stem and root cortex of cassava were possible new sources of linamarase.

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ก้านใบ ลำต้น และเปลือกของหัวมันสำปะหลัง เป็นแหล่งใหม่ของเอ็นไซม์ลินามาเรส

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หัวมันสำปะหลังเป็นอาหารหลักของประชากรเป็นจำนวนมากในประเทศที่กำลังพัฒนา การบริโภคมันสำปะหลังปริมาณมาก ๆ อาจมีอันตรายจากสารพิษไซยาไนด์ ซึ่งเกิดขึ้นเองตามธรรมชาติ ในรูปของ linamarin(ส่วนใหญ่) และ lotaustalin (ส่วนน้อย) ในหัวมันจะมีเอ็นไซม์ลินามาเรสที่ย่อยสลายสารดังกล่าวให้ไฮโดรเจนไซยาไนด์ เอ็นไซม์นี้มีประโยชน์ในการหาปริมาณไซยาไนด์ในการซื้อขายมันสำปะหลังและผลผลิตจากหัวมันสำปะหลัง มีผู้สามารถเตรียมเอ็นไซม์นี้ได้ทั้งบริสุทธิ์จากเนื้อของหัวมันสำปะหลัง (1) เนื่องจากประเทศไทยเป็นผู้ผลิตมากเป็นที่สองของโลก และมีใบและลำต้นจากไร่มันสำปะหลังเป็นปริมาณมาก จึงน่าจะวิเคราะห์หาเอ็นไซม์ลินามาเรสในส่วนต่าง ๆ ของมันสำปะหลัง จากการศึกษาพบว่าก้านใบ ลำต้น และเปลือกของหัวมันมีเอ็นไซม์ลินามาเรสสูง และสามารถแยกจากโปรตีนอื่น ๆ ได้ โดย gel filtration column เอ็นไซม์จากก้านใบและลำต้นมีน้ำหนักโมเลกุลเป็น 600,000 ส่วนเอ็นไซม์จากเปลือกของหัวมันมีน้ำหนักโมเลกุลเป็น 570,000 และ 400,000 เอ็นไซม์จากทั้งสามส่วนนี้มีน้ำหนักโมเลกุลของหน่วยย่อยเป็น 63,000 เอ็นไซม์จากแต่ละส่วนประกอบด้วยสามไอโซไซม์ มีค่า pI เท่ากับ 4.3, 3.3 และ 2.9 ได้เปรียบเทียบค่า optimum pH, K_m และ V_{max} ของเอ็นไซม์ทั้งสามส่วน ดังนั้นสรุปได้ว่าทั้งก้านใบ ลำต้น และเปลือกของหัวมันสำปะหลัง เป็นแหล่งใหม่ของเอ็นไซม์ลินามาเรส

ชื่อเรื่อง (ไทย) ก้านใบ ลำต้น และเปลือกของหัวมันสำปะหลัง เป็นแหล่งใหม่ของเอ็นไซม์ลินามาเรส

วิธีเตรียมเอ็นไซม์ลินามาเรส นำเนื้อเยื่อมาสับและทำให้แตกละเอียดใน 0.1 M อะซิเตทบัฟเฟอร์ pH 5.5 ที่มี 0.1 mM phenylmethylsulfonyl fluoride แล้วกรองผ่านผ้ากรอง นำน้ำสกัดไปปั่นด้วยแรงเหวี่ยง 10,000 g เป็นเวลา 20 นาที ที่ 4°C สารละลายที่ได้จะเติมเกลือแอมโมเนียมซัลเฟต ให้ได้ความอิ่มตัว 65% เอ็นไซม์เกือบทั้งหมดจะตกตะกอน แยกตะกอนออกโดยการปั่นที่ 10,000 g เป็นเวลา 30 นาที ที่ 4°C แล้วนำมาละลายใน 10 mM ฟอสเฟตบัฟเฟอร์ pH 5.5 แล้วไดอะไลส์ ค้างคืนที่ 4°C ต่อจากนี้จะนำเอ็นไซม์ที่ได้ไปแยกโดยวิธีโครมาโตกราฟีในหลอดแก้วที่บรรจุ Sepharose 6B เอ็นไซม์ที่แยกได้จะนำไปแยกไอโซไซม์โดยวิธี chromatofocusing ในช่วง pH 5-1

ตารางที่ 1 ปริมาณลินามาเรสในส่วนต่าง ๆ ของมันสำปะหลัง

Ammonium sulfate fraction from	Preparation (n)	Protein/fresh weight (mg/gm)	Specific activity (μmol/min/mg prot.)
Leaf	4	0.32 ± 0.23	0
Petiole	5	0.92 ± 0.65	2.85 ± 2.02
Stem	4	1.25 ± 0.27	1.76 ± 1.10
Root cortex	3	0.28 ± 0.14	1.56 ± 1.53
Root parenchyma	3	0.39 ± 0.01	0.05 ± 0.04

ตารางที่ 2 คุณสมบัติของลินามาเรสจากส่วนต่าง ๆ ของมันสำปะหลังที่ได้จาก Sepharose 6B column และใช้ 4-nitrophenyl-β-D-glucopyranoside เป็นสับสเตรท

Enzyme source	Native M_r (x 10^{-3})	Subunit M_r (x 10^{-3})	pI value	K_m (Mx10^4)	V_{max} (μmol/min/mg)	Optimal pH
Petiole	600	63	4.2,3.3,2.9	5.6	100	8
Stem	600	63	4.3,3.6,2.8	6.3	125	8
Root cortex	400;570	63	4.3,3.35,2.95	4.3	39	8

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Songkhla, Thailand

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ชื่อ-สกุล ผู้เสนอ พรรณผกา เฉลิมอิสระชัย

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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 occuring 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 37°C. The enzyme was precipitated by ammonium sulphate at 40-80% saturation and was chromatographed in Sephadex G-200 column. The native Mr was determined to be 46,000. UDP-glucose was the best glucose-donor. Among the hydroxy-containing compounds tested, the best glucose-acceptor was acetone cyanohydrin

คุณสมบัติของกลูโคซิลทรานเฟอเรสในมันสำปะหลัง

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กลูโคซิลทรานเฟอเรสพบมากที่ส่วนใบอ่อนและยอดที่สดใหม่ของต้นมันสำปะหลัง เอนไซม์นี้ทำหน้าที่ในขั้นตอนของการเติมน้ำตาลอันเป็นขั้นตอนสุดท้ายของขบวนการสังเคราะห์ลินามาริน ลินามารินหรือแอลฟาไฮดรอกซีไอโซบิวไทโรไนไตรล์ เบต้า ดี กลูโคซายด์ เป็นน้ำตาลไซยาโนค์ที่พบมากในพืชที่ใช้เป็นอาหารและเลี้ยงสัตว์ เช่น มันสำปะหลัง ป อ ถั่วเขียว ถั่วต่าง ๆ และต้นไวท์โคล์เวอร์ กลูโคซิลทรานเฟอเรสจะเร่งการส่งตัวน้ำตาลกลูโคสจาก UDP-glucose ไปให้แอลฟา-ไฮดรอกซีไนไตรล์ หรืออะซิโตนไซยาไนไฮดริน กลูโคซิลทรานเฟอเรสที่สกัดได้มี pH เหมาะสมที่ pH 9.0 และอุณหภูมิที่เหมาะสมที่สุดคือ 37°ซ เอนไซม์จะถูกนำมาทำให้บริสุทธิ์ขึ้นโดยวิธีการตกตะกอนด้วยแอมโมเนียมซัลเฟตที่ความเข้มข้น 40-80% และวิธีการโครมาโตกราฟีโดยใช้ Sephadex G-200 column จากการศึกษาพบว่าน้ำหนักโมเลกุลของเอนไซม์นี้เป็น 46,000 ส่วนของน้ำตาลจ่องไว UDP-glucose จะมีความสามารถในการส่งต่อกลูโคสได้ดีที่สุด ส่วนตัวที่รับน้ำตาลได้ดีที่สุดได้แก่ อะซิโตนไซยาโนไฮดริน

ผลงานนี้ได้รับทุนจาก USAID/PSTC 936-5542-G-00-6028-00
และทุนอุดหนุนการวิจัยประเภทนักศึกษาระดับบัณฑิตศึกษา ประจำปี 2530 ของสภาวิจัยแห่งชาติ

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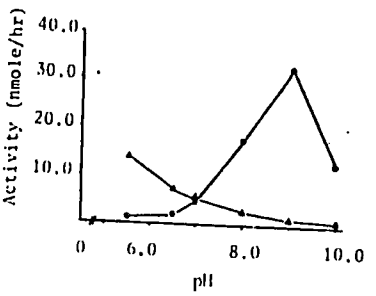
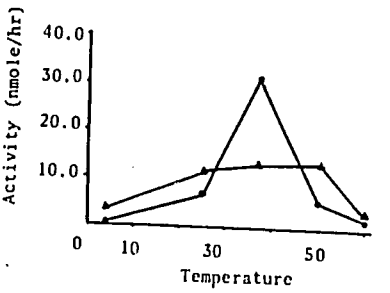
ชื่อเรื่อง (ไทย) คุณสมบัติของกลูโคซิลทรานเฟอเรสในยันสำปะหลัง

ผลจากการสกัดกลูโคซิลทรานเฟอเรสอย่างหยาบในขั้นต้น นำมาวิเคราะห์หา pH ที่เหมาะสมต่อกิจกรรมของเอนไซม์ (รูปที่ 1) และหาอุณหภูมิที่เหมาะสมด้วย (รูปที่ 2)



รูปที่ 1 แสดงกิจกรรมของกลูโคซิลทรานเฟอเรสที่ pH ต่าง ๆ กัน พบว่าที่ pH 9.0 จะให้กิจกรรมสูงสุด



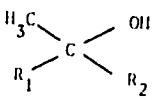
รูปที่ 2 แสดงกิจกรรมของกลูโคซิลทรานเฟอเรสที่อุณหภูมิต่าง ๆ กัน พบว่าที่อุณหภูมิ 37°C จะให้ผลดีที่สุด

กลูโคซิลทรานเฟอเรสที่นำมาทำให้บริสุทธิ์ขึ้นโดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต และ Sephadex G-200 column นำมาหาความจำเพาะของตัวให้น้ำตาล (ตารางที่ 1) และความจำเพาะของสับสเตรท (ตารางที่ 2)

ตารางที่ 1 แสดงการเปรียบเทียบความสามารถในการให้น้ำตาลกลูโคสของ UDP-glucose ADP-glucose และ UDP-galactose

Sugar-donor	Activity (nmole/hr	Relative conversion (%)
UDP-glucose	122.46	100%
ADP-glucose	0	0
UDP-galactose	33.38	27.2

ตารางที่ 2 จากโครงสร้างของอะซิโตนไซยาโนไฮดริน ซึ่งเป็นสับสเตรทของกลูโคซิลทรานเฟอเรส เมื่อนำสารประกอบอื่นที่มีโครงสร้างใกล้เคียงกันมาเปรียบเทียบความสามารถในการรับน้ำตาลกลูโคสดังแสดงในตาราง



สูตรโครงสร้างของสารประกอบที่นำมาหาความจำเพาะของสับสเตรท

Substrate	R1	R2	Activity (nmole/hr)	Relative conversion (%)
Acetone cyanohydrin	CH_3	CN	75.15	100
Glycerol	CH_2-OH	H	5.70	7.6
Ethanol	H	H	20.33	27.0
Isobutanol	CH_3	H	42.00	55.9
Butanol	CH_3CH_2	H	31.26	41.6
Phenol			6.10	8.1

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ชื่อ-สกุล ผู้เสนอ อิศาวัศน์ เอกฤทธิกุล

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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 chromato-focusing. The pH optimum, K_m and V_{max} values of these isozymes were also compared.

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คุณสมบัติของเอ็นไซม์ลินามาเรสจากมันสำปะหลัง

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ลินามาเรสเป็นเอ็นไซม์ที่ย่อยสลายสารประกอบไซยาไนด์ที่เกิดขึ้นเองตามธรรมชาติ ได้แก่ ลินามาริน ให้ก๊าซไฮโดรเจนไซยาไนด์ ซึ่งเป็นอันตรายต่อสิ่งมีชีวิตในปัจจุบัน เอ็นไซม์ลินามาเรสได้ถูกนำมาใช้ในทางการค้า โดยใช้หาปริมาณของสารประกอบไซยาไนด์ในมันสำปะหลัง ได้แก่ลินามารินเป็นส่วนใหญ่ ซึ่งเป็นตัวกำหนดราคาทางมันสำปะหลัง จากการศึกษาที่ผ่านมา พบว่า ลินามาเรสสามารถเตรียมได้จากส่วนต่าง ๆ ของมันสำปะหลัง โดยพบว่าจะมีปริมาณมากในก้านใบ ต้น และเปลือกของหัวมันสำปะหลัง ตามลำดับ คุณสมบัติของเอ็นไซม์ลินามาเรสที่เตรียมได้นี้, นอกจากที่เคยรายงานแล้วได้ศึกษาต่อมาพบว่าเป็นเอ็นไซม์ที่มีความจำเพาะต่อสับสเตรท กล่าวคือ จะย่อยสับสเตรทที่มีกลูโคสหรือฟิวโคส เชื่อมด้วย β-glycosidic bond กับ aglycone เราได้แยกเอ็นไซม์นี้เป็นสามไอโซไซม์ และเปรียบเทียบค่า optimum pH, K_m และ V_{max} ของเอ็นไซม์ทั้งสามส่วน และของไอโซไซม์ไว้ด้วย

วิถีเรื่อง (ไทย) คุณสมบัติของเอ็นไซม์ลินามาเรสจากมันสำปะหลัง

ตารางที่ 1 การเปรียบเทียบการย่อยสลายของ glycoside ต่าง ๆ โดยเอ็นไซม์ลินามาเรส ความเข้มข้นของ glycoside ที่ใช้คือ 10 mM ยกเว้นสามตัวสุดท้ายใช้ 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	106
PNP-β-mannoside	4	3	40
PNP-β-galactoside	0	3	2
PNP-β-gentiobioside	0	0	0
Amygdalin	0	0	0
Prunasin	0	0	0

ตารางที่ 2 คุณสมบัติของลินามาเรสและไอโซไซม์ของเอ็นไซม์จากมันสำปะหลัง

SUBSTRATE / ENZYME SOURCE	Km (mM)		Vmax (umol/min/mg)	
	Graph	Computer	Graph	Computer
PNP-β-glucoside				
Root cortex	16	18	377	281
Petiole	16	16	312	308
Stem	13	13	625	895
Linamarin				
Root cortex	1.4	1.0	102	133
Petiole	1.1	0.6	55	111
Stem	0.9	0.6	175	181
Linamarin				
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

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

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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 fucose 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.)*

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☐ แพทย์ ☐ ทั่วไป

ISOZYME FORMS OF LINAMARASE FROM CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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Cassava linamarase is an important enzyme, catalyzing the hydrolysis of linamarin and hence causing cyanide toxicity of cassava tubers. The enzyme exists in cassava as three isozyme forms with pI of 4.3, 3.3 and 2.9. In this report, the isozyme distribution and amino acid composition of each form from petiole, stem or root-cortex of cassava were investigated. The most predominant form in the petiole and the stem was found to be one with pI of 4.3. In the root cortex, the three isozyme forms existed in approximately equal amounts. Based on the amino acid compositions, all isozyme forms from these sources were similar. The major isozyme with pI 4.3 from petiole was used in further investigation on catalytic properties. It was found to catalyze the hydrolysis of linamarin, p-nitrophenyl-β-D-glucoside (PNPG), and p-nitrophenyl-β-D-fucoside (PNPF). Among the three substrates, there were some different characteristics. Firstly, the pH optima for the substrates were different: pH 7-8 for linamarin, pH 8 for PNPG and pH 7 for PNPF. Secondly, the K_m value for linamarin was 0.6 mM while that for PNPG was about 10-fold higher but that for PNPF was half this value. Thirdly, the V_{max} value for linamarin was about one quarter of that for PNPG or PNPF. Furthermore, either PNPG or PNPF could increase the K_m value for linamarin, suggesting that three substrates share the same catalytic site. However, the glucosidase and the fucosidase activities were differentiated by Tris inhibition of the latter but not the former.

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คุณสมบัติของไอโซไซม์ของเอ็นไซม์ลินามาเรสจากมันสำปะหลัง

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ลินามาเรสในมันสำปะหลังเป็นเอ็นไซม์ที่ย่อยสลายสารประกอบไซยาไนด์ลินามาริน ทำให้เกิดความเป็นพิษของไซยาไนด์ในหัวมันสำปะหลัง เอ็นไซม์ลินามาเรสจากมันสำปะหลังประกอบด้วย 3 isozyme ที่มีค่า pI 4.3, 3.3 และ 2.9 ตามลำดับ (1) ในรายงานนี้ได้เปรียบเทียบปริมาณของ isozyme และส่วนประกอบของกรดอะมิโนของทั้ง 3 isozyme ในส่วนของก้านใบ ลำต้น และเปลือกหัวมันไว้ด้วย พบว่าทั้ง 3 isozyme จากทั้ง 3 ส่วนมีองค์ประกอบ amino acid เหมือนกัน จากการศึกษาทางคุณสมบัติทางจลศาสตร์ของ isozyme ที่มีค่า pI 4.3 จากส่วนของก้านใบ พบว่านอกจากจะย่อยสลายลินามารินแล้ว ยังสามารถย่อยสลายสับสเตรทที่มีกลูโคส หรือฟิวโคส เชื่อมด้วย β-glycosidic bond กับ glycone ได้ และได้เปรียบเทียบค่า pH optimum, K_m และ V_{max} ของสับสเตรทเหล่านี้ไว้ด้วย นอกจากนี้ยังพบว่าสับสเตรทที่มีกลูโคสหรือฟิวโคสดังกล่าว ทำให้ค่า K_m ของลินามารินเพิ่มขึ้น แสดงว่าสับสเตรททั้ง 3 มี catalytic site ร่วมกัน และพบว่า Tris-ion สามารถยับยั้งการย่อยสลายของสับสเตรทน้ำตาลฟิวโคส

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ชื่อเรื่อง (ไทย) คุณสมบัติของไอโซไซม์ของเอ็นไซม์ลินามาเรสจากมันสำปะหลัง

ตารางที่ 1 กรดอะมิโนองค์ประกอบของ isozyme ของเอ็นไซม์ลินามาเรสจากส่วนต่างๆ ของมันสำปะหลัง

Amino acid	Root cortex			Stem			Petiole		
pI=	2.9	3.3	4.3	2.9	3.3	4.3	2.9	3.3	4.3
Asx	12.22	9.17	13.51	9.07	12.36	12.55	13.54	14.89	14.86
Glx	8.38	7.51	9.80	8.34	10.76	9.81	9.49	9.88	10.40
Ser	6.07	5.66	6.84	5.78	6.35	5.77	6.45	5.91	7.94
Gly	8.52	7.32	6.91	10.00	8.80	0.09	8.01	8.19	9.73
His	1.05	0.81	1.11	0.89	1.36	1.55	1.18	1.35	1.37
Arg	2.83	1.55	1.74	1.79	2.77	2.39	3.06	3.28	4.58
Thr	3.86	2.42	2.96	2.76	3.69	4.08	3.69	3.99	4.99
Ala	6.64	4.55	5.10	5.89	6.24	6.94	6.12	6.91	7.57
Tyr	4.64	2.95	3.44	3.53	4.96	5.20	5.24	5.52	6.87
Val	3.05	1.90	3.16	2.33	3.19	3.04	3.25	3.41	4.16
Ile	2.64	1.64	2.37	1.29	2.15	3.04	2.22	2.37	3.63
Leu	5.32	3.70	4.74	3.42	4.43	6.91	4.75	5.24	6.75
Phe	4.17	2.92	3.84	1.97	3.32	5.83	3.90	4.20	5.27
Lys	2.25	1.44	1.74	0.97	1.59	2.10	1.82	1.82	3.71
Pro + NH_3	28.35	46.45	32.17	41.95	28.06	21.69	27.26	23.04	8.17

ตารางที่ 2 คุณสมบัติทางจลนศาสตร์ของ isozyme ลินามาเรสที่มี pI 4.3 จากส่วนก้านใบของมันสำปะหลัง

Substrate (+ inhibitor)	Km (mM)	Vmax (μmol/min/mg)	Assay for
Linamarin	0.57	58	HCN
Linamarin + 0.15 mM δ-gluconolactone	1.04	50	HCN
Linamarin + 10 mM IPTG	2.21	65	HCN
Linamarin + 2 mM PNPG	5.31	84	HCN
Linamarin + 0.5 mM PNPF	1.02	58	HCN
PNPG	5.05	256	PNP
PNPG + 0.25 mM $HgCl_2$	15	288	PNP
PNPF	0.23	266	PNP
PNPF + 25 mM Tris HCl	1.11	130	PNP

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STRUCTURE OF CASSAVA LINAMARASE

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Linamarase catalyzes the hydrolysis of linamarin, a cyanogenic glucoside, to give an unstable acetone cyanohydrin which further breaks down to yield HCN. Thus, the linamarin/linamarase system is responsible for the toxicity of cassava tubers. Since cassava linamarase is a polymeric enzyme that exists in three isozyme forms, its structure should be investigated. This was performed using three approaches : enzymic digestion, chemical treatment and antibody neutralization. Native linamarase was resistant to trypsin, pronase, collagenase, bromelain and thermolysin. However, after heat denaturation, it became sensitive to pronase, trypsin and thermolysin. After heat denaturation, isozyme with pI 4.3 was more sensitive to trypsin than the other two isozymes. The enzyme was completely inactivated by incubation in 3M guanidine hydrochloride (GHCl). At 0.5M GHCl, the enzyme was converted into multiple forms of varying sizes as shown by chromatography on a Sepharose 6B column. These forms still possessed enzymic activity. The three isozyme forms were equally sensitive to neutralization by antibody raised in rabbits. These findings suggested some unusual structure of cassava linamarase.

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โครงสร้างของเอนไซม์ลินามาเรสจากหัวมันสำปะหลัง

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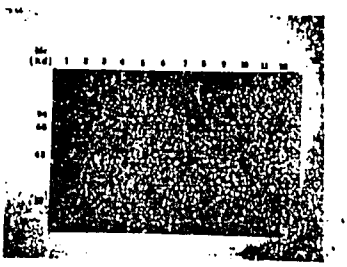
ลินามาเรสเป็นเอนไซม์ที่เร่งการสลายของสารลินามาริน ซึ่งเป็นสารประเภทไซยาโนจินิก กลูโคไซด์ แล้วได้สารไม่คงตัวอะซิโตนไซยาโนไฮดริน ซึ่งจะสลายต่อไปให้กาซไฮโดรเจนไซยาไนด์ จากความสัมพันธ์ระหว่างลินามาริน และ เอนไซม์ลินามาเรสนี้เอง ก่อให้เกิดความเป็นพิษขึ้นในหัวมันสำปะหลัง เนื่องจากเอนไซม์ลินามาเรสจากหัวมันสำปะหลังเป็นเอนไซม์ขนาดใหญ่ และปรากฏอยู่ในรูปไอโซไซม์สามชนิดดังนั้น โครงสร้างของเอนไซม์ดังกล่าวจึงเป็นสิ่งที่น่าทำการศึกษา การศึกษาจึงได้ถูกกระทำไปในสามหัวข้อดังนี้คือการย่อยโดยใช้เอนไซม์ การให้ทำปฏิกิริยากับสารเคมี และการทำให้การทำงานของเอนไซม์ไม่สามารถแสดงออกโดยแอนติบอดี้ พบว่าลินามาเรสในสภาพธรรมชาติมีความทนทานต่อไพทีเลสชนิดทริปซิน โพรเนส คอลลาจิเนส โบรมิเลน และเทอร์โมไลซิน อย่างไรก็ตามหลังการทำให้เสียสภาพธรรมชาติโดยความร้อน เอนไซม์จะเปลี่ยนสภาพมามีคุณสมบัติที่ไวต่อการย่อยด้วยโพรเนส ทริปซิน และเทอร์โมไลซิน และยังพบว่าหลังการเสียสภาพโดยความร้อนนั้น ไอโซไซม์ชนิด pI 4.3 จะมีความไวต่อการย่อยด้วยทริปซินมากกว่าไอโซไซม์อีกสองชนิด นอกจากนี้พบว่าเอนไซม์จะถูกทำให้อยู่ในสภาพที่ไม่สามารถทำงานได้อย่างสิ้นเชิง โดยการให้อยู่ใน 3M กัวนิดีนไฮโดรคลอไรด์ และที่ความเข้มข้น 0.5 M ของกัวนิดีนไฮโดรคลอไรด์ พบว่าเอนไซม์จะถูกเปลี่ยนให้อยู่ในหลายรูปแบบ ซึ่งมีขนาดแตกต่างกันดังแสดงในรูปแบบการแยกเอนไซม์ด้วย Sepharose 6B คอลัมน์ ซึ่งแต่ละรูปแบบของเอนไซม์นั้นยังคงมีนิยมทริซ์อยู่ นอกจากนี้ยังพบว่าไอโซไซม์ทั้งสามชนิดมีความไวเท่ากันต่อการห้ามการทำงานด้วยแอนติบอดีจากกระต่าย จากข้อมูลดังกล่าวนี้แสดงถึงความแตกต่างจากเอนไซม์ทั่วไปของโครงสร้างของเอนไซม์ลินามาเรสจากหัวมันสำปะหลัง

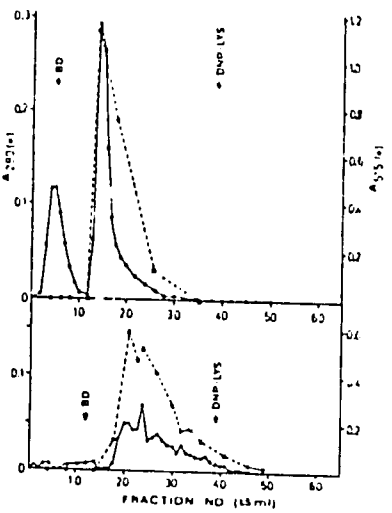
ชื่อเรื่อง (ไทย) โครงสร้างของเอนไซม์ฉีนามาเรสจากหัวมันสำปะหลัง

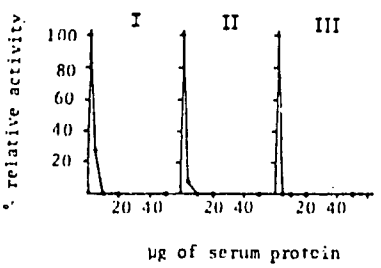
ไอโซไซม์ที่ใช้ในการทดลองเตรียมมาจากการเอาเอนไซม์ที่ได้จาก Sepharose 6B column ไปผ่าน Chromatofocusing column ชนิด step gradient ในช่วง pH 1.4-5(1) จะได้ไอโซไซม์ออกมาสามชนิด ที่ pI 4.3, 3.3 และ 2.9



รูปที่ 1 SDS-PAGE ของไอโซไซม์ทั้งสามชนิดของเอนไซม์ฉีนามาเรสที่เสียสภาพธรรมชาติแล้วโดยความร้อน ถูกย่อยด้วยทริปซินที่ 37°C ในอัตราส่วน 1:50 แถวที่ 1 Standard protein แถวที่ 2,3, และ 4 คือไอโซไซม์ pI 4.3 ในภาวะไม่มีทริปซินนาน 90 นาที และมีทริปซินนาน 0 และ 90 นาที แถวที่ 5,6,7,8 คือไอโซไซม์ pI 3.3 ในภาวะที่ไม่มีทริปซิน นาน 0 และ 90 นาที กับภาวะที่มีทริปซิน นาน 0 และ 90 นาที แถวที่ 9,10,11,12 คือ ไอโซไซม์ pI 2.9 ในภาวะไม่มีทริปซินนาน 0 และ 90 นาที กับภาวะที่มีทริปซินนาน 0 และ 90 นาที

รูปที่ 2 เปรียบเทียบรูปแบบการแยกเอนไซม์ฉีนามาเรสด้วย Sepharose 6B คอลัมน์ ชะโดยเอนไซม์ด้วย 10 mM โปรแตสเซียมฟอสเฟต pH 5.5 (ภาพบน) กับชะโดยด้วย 0.5 M ทัวนิดีนไฮโดรคลอไรด์ ละลายใน 10 mM โปรแตสเซียมฟอสเฟต pH 5.5 (ภาพล่าง)





รูปที่ 3 การนิวเทอร์ไลเซ่ของแอคทิวิตี้ของไอโซไซม์ทั้งสามของฉีนามาเรสโดยแอนติบอดี้ (I) ไอโซไซม์ pI 4.3 (II) ไอโซไซม์ pI 3.3(III) ไอโซไซม์ pI 2.9

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TOWARDS CYANIDE-FREE CASSAVA

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Abstract :

Despite years of search and breeding, no cyanide-free cassava has been made available to farmers. Thus, chronic cyanide toxicity remains a health threat to populations subsisting on cassava. Although food processing effectively removes most of the toxic cyanide, it adds costs to this stable food of the poor. Cassava cyanide exists in three forms : the stable form (linamarin and lotaustralin), the unstable form (acetone and butanone cyanohydrins) and the free form (HCN). Being volatile, the free cyanide is most easily lost. On the other hand, the breakdown of the cyanohydrins into ketones and HCN is pH-dependent. Above pH 5, they decompose by themselves. Between pH 3.5 and 5 their hydrolysis is catalyzed by a hydroxynitrile lyase. In contrast, the stable cyanogenic glucosides are most difficult to remove. They are formed from the labile cyanohydrins in the reaction of glucosyltranferase and their hydrolysis back to the cyanohydrins is catalyzed by linamarase. Both enzymes from cassava have been studied. Based on their properties and cassava physiology, three approaches may be suggested to produce cyanide-free cassava: (i) eliminating the synthesis of glucosyl-transferase, (ii) increase the amount of linamarase, and (iii) interupting the transport and storage of the cyanogenic glucosides in cassava roots.

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ชื่อ-สกุลผู้เสนอ ธิดารัตน์ เอกสิทธิกุล

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CASSAVA PEROXIDASES

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Peroxidase is a plant enzyme widely used in many biological and immunological assays. In plants, peroxidases are believed to be involved in numerous processes ranging from metabolism of hormone to defense mechanism to oxidative deteration of plant tissues. Cassava roots begin to deteriorate soon after harvest and the process may involved peroxidases. In this report, peroxidases could be extracted from root cortex and root parenchyma and were both precipitated at 40-80% ammonium sulfate. These peroxidases were seperated by fast-protein liquid-chromatography using a Mono Q column. The major form of root cortex was eluted as an unbound peak while the major form of parenchyma peroxidase was eluted at 0.19-0.2 M KCl. The major form (unbound peak) of the root cortex was further applied to a Superose 12 column. The native molecular weight was found to be 28,000. The enzyme activity was detected on a polyacrylamide gel electrophoresis by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB).

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เปอร์ออกซิเดสจากมันสำปะหลัง

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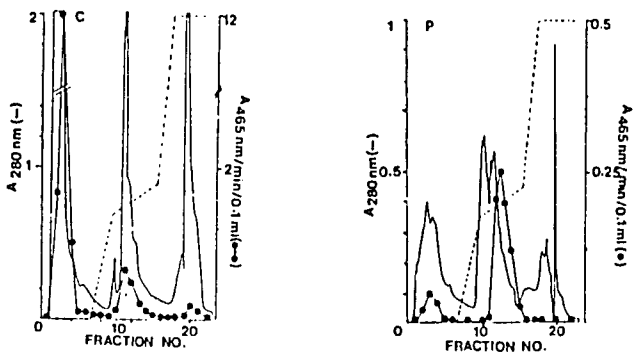
เปอร์ออกซิเดส เป็นเอ็นไซม์จากพืชที่นิยมใช้กันอย่างกว้างขวางในการวิเคราะห์ทางชีวเคมีและอิมมูโน ในพืชเชื่อกันว่าเอ็นไซม์นี้เกี่ยวข้องกับขบวนการต่างๆ อย่างมากมาย เช่น ในขบวนการย่อยสลายของฮอร์โมนในพืช ในขบวนการการ defense ตลอดจนขบวนการเน่าเสียของพืช ในมันสำปะหลังซึ่งเป็นพืชที่เกิดการเน่าเสียอย่างรวดเร็วหลังการเก็บเกี่ยว ก็หมายว่าในขบวนการดังกล่าวมีเปอร์ออกซิเดสเกี่ยวข้องอยู่ด้วย ในรายงานนี้สามารถเตรียมเปอร์ออกซิเดสได้จากส่วนของเปลือกและเนื้อมันสำปะหลัง และสามารถแยกจากโปรตีนอื่นได้ด้วยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตที่ความเข้มข้น 40-80% และด้วยโครมาโตกราฟีแบบเร็วด้วยระบบ FPLC โดย Mono Q คอลัมน์ พบว่าเปอร์ออกซิเดสจากส่วนเปลือกและเนื้อมันสำปะหลังจะแตกต่างกัน และพบว่าเปอร์ออกซิเดสจากส่วนของเปลือกมันสำปะหลัง มีน้ำหนักโมเลกุล 28,000 และสามารถจะๆ activity stain ใน PAGE โดยใช้โดอะมิโนเบนซิดีน

ชื่อเรื่อง (ไทย) เปอร์ออกซิเดสจากมันสำปะหลัง

ผลการทดลอง

ตารางที่ 1 แสดงการตกตะกอนเอ็นไซม์เปอร์ออกซิเดสด้วยแอมโมเนียมซัลเฟต

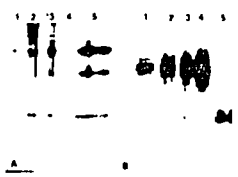
Fraction	Protein (mg)	Total activity A_{465} nm/min	Specific activity A_{465} nm/min/mg
Cortex			
Crude	917	9	0.3
40% $(NH_4)_2SO_4$	13	51	4.1
80% $(NH_4)_2SO_4$	79	1160	20.2
Parenchyma			
Crude	399	120	0.3
40% $(NH_4)_2SO_4$	6	2	0.24
80% $(NH_4)_2SO_4$	11	29	2.53



รูปที่ 1 แสดงการแยกเอ็นไซม์เปอร์ออกซิเดสที่ตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตที่ความเข้มข้น 80% โดยคอลัมน์ Mono Q ในระบบ FPLC ที่ถูกชะล้างด้วยเกลือ KCl ที่ความเข้มข้น 0-0.5 M (----)

C = จากส่วน root cortex

P = จากส่วน root parenchyma



รูปที่ 2 แสดงแถบเปอร์ออกซิเดสใน PAGE เมื่อยังไม่ผ่านขั้นตอนการแยก (1) ตกตะกอนด้วย 0-40% และ 40-80% เกลือแอมโมเนียมซัลเฟตตามลำดับ (2 และ 3) เมื่อแยกด้วย Mono Q คอลัมน์ (unbound และ bound = 4 และ 5)

A เมื่อย้อมด้วย Coomassie blue R

B เมื่อย้อม activity ด้วย DAB

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ชื่อ-สกุลผู้เสนอ เพ็ญภา กิษามานนท์

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STABILITY OF CASSAVA LINAMARASE IN SODIUM DODECYL SULFATE

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Linamarase is a β-glucosidase catalysing the hydrolysis of linamarin to liberate hydrogen cyanide which is responsible for toxicity in cassava tubers. In this report, the stability of linamarase in SDS was studied. The enzyme was found to retain about 80-90% of its activity after incubation with SDS up to 20% at room temperature for 30 min. Hence the enzyme activity-structure relationship was study by PAGE in the presence of 1% SDS, followed by staining for activity with a fluorescent substrate, 4-methylumbelliferyl β-D-glucoside. Five different active forms of closely related high molecular weights were observed in PAGE with 1% SDS or without SDS. This finding suggested that in cassava the enzyme may exist in multiple forms with varying numbers of subunits. Since the presence of β-mercaptoethanol (1 mM) in SDS did not dissociate enzyme into the monomer, the subunit interaction probably did not involve disulfide bonding. The monomer was obtained only after heating the enzyme which also inactivated the enzyme.

ความคงทนของเอนไซม์ลินามาเรสจากมันสำปะหลังในสารละลายโซเดียมโดเดซิลซัลเฟต

เพ็ญภา กิษามานนท์ และ มนตรี จุฬาวัฒนกล
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ลินามาเรสเป็นเอนไซม์เบต้ากลูโคไซเดสที่เร่งการสลายลินามาริน ทำให้เกิดไฮโดรเจนไซยาไนด์ ซึ่งก่อให้เกิดความเป็นพิษในหัวมันสำปะหลัง ในการศึกษาความคงทนของเอนไซม์ลินามาเรส พบว่าเมื่อเอนไซม์อยู่ในสารละลาย SDS ความเข้มข้นสูงถึง 20% ที่อุณหภูมิห้อง เป็นเวลา 30 นาที เอนไซม์ยังคงสภาพเร่งปฏิกิริยาประมาณ 80-90% จากการศึกษาความสัมพันธ์ของโครงสร้างกับการเร่งปฏิกิริยาของเอนไซม์โดย PAGE ที่มี 1% SDS และติดตามสภาพเร่งปฏิกิริยาโดยการย้อมด้วย 4-methylumbelliferyl-β-D-glucoside ซึ่งเป็นสารตั้งต้นเรืองแสงพบว่าเอนไซม์มี 5 รูปแบบที่มีขนาดใหญ่ใกล้เคียง ทั้งใน PAGE ที่มีและไม่มี 1% SDS ผลการทดลองนี้แสดงว่า ในหัวมันสำปะหลัง เอนไซม์อาจปรากฏอยู่ในหลายรูปแบบที่ประกอบด้วยจำนวนของหน่วยย่อยที่ต่างกัน และเมื่อมี 1 mM β-mercaptoethanol ในสารละลาย SDS พบว่าเอนไซม์ไม่แตกออกเป็นหน่วยย่อย แสดงว่าการจับกันของหน่วยย่อยอาจไม่เกี่ยวข้องกับพันธะไดซัลไฟด์ แต่เอนไซม์จะแตกออกเป็นหน่วยย่อยได้เมื่อนำไปต้ม ซึ่งจะทำให้เอนไซม์เสียสภาพเร่งปฏิกิริยาด้วย

ชื่อเรื่อง (ไทย) ความคงทนของเอนไซม์อินนามาเรสจากมันฝรั่งในสารละลายโซเดียมไดเดซิลซัลเฟต

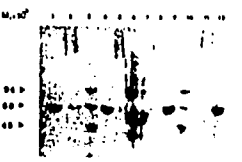
ผลการศึกษาต่อไปนี้แสดงให้เห็นถึงความสัมพันธ์ของโครงสร้างกับคุณสมบัติในการเร่งปฏิกิริยาของเอนไซม์อินนามาเรส

ตารางที่ 1 ผลของ SDS ต่อสภาพเร่งปฏิกิริยาของเอนไซม์อินนามาเรส เมื่อบ่มเอนไซม์ใน 10 mM โปรแตสเซียมฟอสเฟต pH 5.5 ที่มี SDS ความเข้มข้นต่าง ๆ ที่อุณหภูมิห้องนาน 30 นาที

ความเข้มข้นของ SDS (%)	relative activity (%)	จำนวนครั้งของการทดลอง
0	100	5
0.1	98	5
1	77	5
2	80	2
4	96	2
5	84	2
10	98	2
20	83	2

รูปที่ 1 การวิเคราะห์เอนไซม์อินนามาเรสโดย SDS-PAGE ในภาวะที่มี 1% SDS เมื่อมี (A 1, 2,11,12,B4,5) หรือไม่มี β-mercapto-ethanol (A 4,5,8,9,B 1,2) เปรียบเทียบกับเมื่อต้ม (A 1,4,9,12,B 2,5) และไม่ต้ม (A 2,5,8,11,B 1,4) ก่อนหยอดใส่เจล (A) ย้อมโปรตีนด้วย Coomassie brilliant blue R-250 (B) ย้อมด้วย 4-methylumbelliferyl-β-D-glucoside

(A)



(B)

รูปที่ 2 การวิเคราะห์เอนไซม์อินนามาเรส โดย PAGE

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2. Kisamanonta, P. M.Sc. Thesis in Biochemistry. Faculty of Graduate Studies, Mahidol University, 1989.

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B-040

ชื่อ-สกุล ผู้อภิปรายงานวิจัย ธิดารัตน์ เอกสิทธิกุล
☐ นาย ■ นส. ☐ นาง ☐ ศ. ☐ ร. ☐ รศ. ☐ ผ. ☐ ค.
ที่ทำงาน ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
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☐ วิทย์-ศึกษา ☐ ทรัพย์-แวดล้อม
☐ แพทย์ ☐ ทั่วไป

CASSAVA PEROXIDASES

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In cassava, peroxidase is believed to be involved in the oxidative deterioration process of the root. In this study, peroxidase was extracted from the fresh root and 7 days after harvested root (deteriorated root). In the previous report, most of the peroxidases activity were precipetated at 40 - 80 % ammonium sulfate saturation. By using Mono Q column in FPLC system, the major form of root parenchyma peroxidase from deteriorated root differed from the fresh root, while the major form of root cortex peroxidase from both fresh and deteriorated root were eluted as unbound peak. From the spectra analysis the major form of root cortex and parenchyma peroxidase from fresh root were haemoproteins. The peroxidase activity was detected by diaminobenzedine hydrochloride and the pH optimum of these enzyme were 5.5.

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เปอร์ออกซิเดสจากมันสำปะหลัง

ธิดารัตน์ เอกสิทธิกุล และ มนตรี จุฬาวัฒนาถล
ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ถนนพระราม 6 กท. 10400

ในขบวนการเน่าเสียของหัวมันสำปะหลัง ซึ่งเป็นพืชเศรษฐกิจของประเทศเรา เชื่อว่ามีเอ็นไซม์เปอร์ออกซิเดสเกี่ยวข้องด้วย ในการศึกษานี้ ได้เปรียบเทียบเปอร์ออกซิเดสที่เตรียมจากส่วนเปลือกและเนื้อของหัวมันสำปะหลังสด และที่ทิ้งไว้ เจ็ดวันหลังการเก็บเกี่ยว (รากไม่สด) ได้เคยรายงานไว้แล้วว่า เปอร์ออกซิเดสสามารถแยกจากโปรตีนอื่นได้ด้วยการตกตะกอนที่ 40 - 80 % ของเกลือแอมโมเนียม ซัลเฟต อิ่มตัว และโดย Mono Q คอลัมน์ ในระบบ FPLC ในการศึกษานี้พบว่าเปอร์ออกซิเดส จากส่วนของเนื้อ - มันสำปะหลัง ระหว่างรากสด และรากไม่สด จะมีคุณสมบัติในการจับกับคอลัมน์ต่างกัน ในขณะที่เปอร์ออกซิเดสจากส่วนของเปลือกมันสำปะหลัง ระหว่างรากสด และรากไม่สด จะมีคุณสมบัติในการจับกับคอลัมน์เหมือนกัน และ major form ของเปอร์ออกซิเดสจากเปลือกและเนื้อมันสำปะหลังของรากสด เป็น haemoprotein และมี optimum pH ที่ 5.5 เมื่อใช้ไดอะมิโนเบนซิดีน ไฮโดรคลอไรด์ เป็นสับสเตรท

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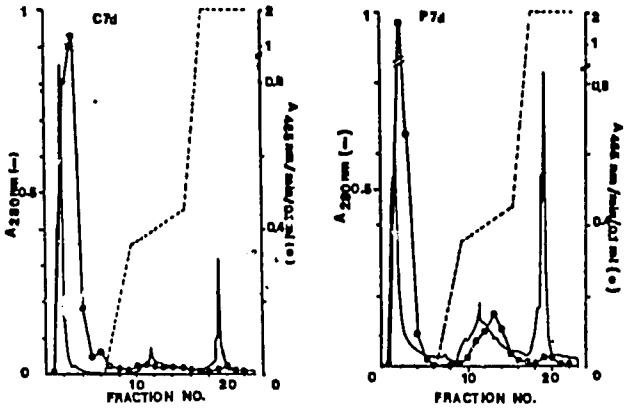
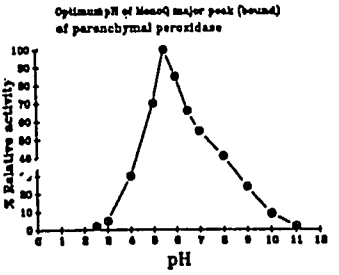
ชื่อเรื่อง (ไทย) เปอร์ออกซิเดสในมันสำปะหลัง

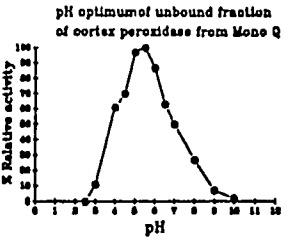
ผลการทดลอง

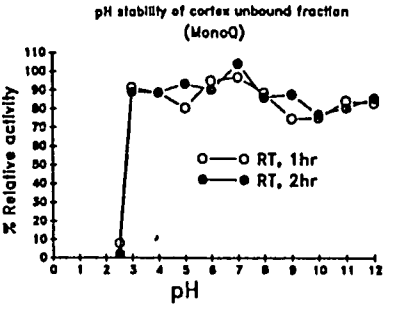


รูปที่ 1 แสดงการแยกเอ็นไซม์เปอร์ออกซิเดสที่ตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตที่ความเข้มข้น 40-80% โดยคอลัมน์ Mono Q ในระบบ FPLC ที่ถูกชะล้างด้วยเกลือ KCl ที่ความเข้มข้น 0-0.5 M (------)

C7 d = root cortex ของหัวมันไม่สด P7d = root paranchyma ของหัวมันไม่สด







รูปที่ 2 แสดงผลของ pH ต่อการทำงานของเปอร์ออกซิเดส จากส่วนต่างๆ ของหัวมันสำปะหลังสด

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ชื่อ-สกุล ผู้ขอรับทุนวิจัย กรรณิกา เสริมสุวิทยวงศ์

☐ นาย ■ นส. ☐ นาง ☐ ศ. ☐ ร. ☐ รศ. ☐ ผ.

ที่ทำงาน ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
ถนนพระราม ๖ กทม. ๑๐๔๐๐ โทร. ๒๔๖๐๐๖๓ ต่อ ๒๕๒

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■ ชีวภาพ ☐ วิศวะ-เทคโน
☐ วิทย์-ศึกษา ☐ ทรัพย์-แวดล้อม
☐ แพทย์ ☐ ทั่วไป

โปรดพิมพ์ให้เต็มกรอบ

AGGREGATION OF PEPTIDE CHAINS IN CASSAVA LINAMARASE

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Linamarase, a β-glycosidase enzyme involved in the process of cyanogenesis, was purified from the stem, petiole and root cortex of cassava by ammonium sulphate precipitation and gel filtration.

Under native conditions, linamarase from all three sources showed an apparent molecular weight of 600,000 on Sepharose 4B chromatography and gave a ladder pattern in polyacrylamide electrophoresis, indicating that at least part of the difference in the molecular forms observed was due to aggregation. However, under denaturing conditions, linamarase appeared to consist of a single type of subunit of appearent molecular weight 63,000 as judged by SDS, acid-urea and triton-acid-urea polyacrylamide gel electrophoresis.

การจับกลุ่มของสายเปปไทด์ในลินามาเรสจากมันสำปะหลัง

กรรณิกา เสริมสุวิทยวงศ์*, ม.ร.ว.ชิษณุสวรรค์ สวัสดิวัตน์* และมนตรี จุฬาวัฒนาถล*

*ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

ลินามาเรส เอนไซม์ β-glycosidase ที่เกี่ยวข้องในกระบวนการที่ก่อให้เกิดไซไคเจนไซยาไนด์ในมันสำปะหลัง ถูกสกัดจากส่วนลำต้น, ก้านใบ และเปลือกชั้นนอกของรากหัว และถูกทำให้บริสุทธิ์โดยผ่านการตกตะกอนด้วย แอมโมเนียมซัลเฟต และกรองโดยวุ้น (gel filtration)

ลินามาเรสจากเนื้อเยื่อทั้ง 3 แหล่ง ถูกศึกษาโครงสร้างพื้นฐานระดับโมเลกุลด้วยวิธีวิเคราะห์ต่าง ๆ ในสภาวะที่ไม่เสียสภาพ ลินามาเรส ปรากฏขนาดโมเลกุล > 600,000 ดาลตัน โดยใช้ Sepharose 4B โครมาโตกราฟี และถูกพบว่าส่วนหนึ่งของความแตกต่างของรูปแบบของโครงสร้าง มาจากการมีระดับการจับกลุ่มของสายเปปไทด์ของลินามาเรสในจำนวนที่ต่างกัน เมื่อศึกษาลินามาเรสที่ถูกทำให้เสียสภาพโดยใช้ SDS , ยูเรีย และ triton พบว่า ขนาดโมเลกุลของหน่วยย่อย เท่ากับ 63,000 ดาลตัน และหน่วยย่อยของลินามาเรสนั้น น่าจะประกอบด้วยสายโพลีเปปไทด์เพียง 1 ชนิด

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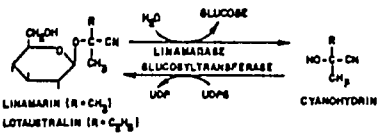
รื่นเรื่อง (ใหม่) การจับกลุ่มของสายเปปไทด์ในลินามาเรสจากมันสำปะหลัง

ลินามาเรสในมันสำปะหลัง สามารถเร่งปฏิกิริยา การสลายตัวของลินามารินไปเป็นกลูโคส และอะซีโตน ไซยาไนไฮดริน ซึ่งสามารถสลายตัวต่อไปได้ ไฮโดรเจนไซยาไนด์ ก่อให้เกิดปัญหาในการส่งออกผลิตภัณฑ์จากมันสำปะหลัง

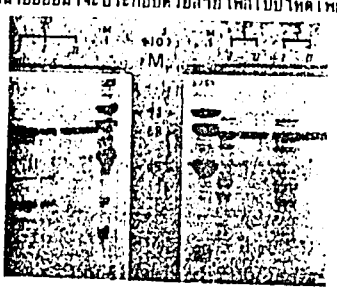


ลินามาเรสเนื้อเยื่อส่วนลำต้น, ก้านใบ และเปลือก ชั้นนอกของรากหัว ถูกสกัดแยก และทำให้บริสุทธิ์

รูปที่ 1 แสดงปฏิกิริยาการเกิดไฮโดรเจนไซยาไนด์ใน มันสำปะหลัง

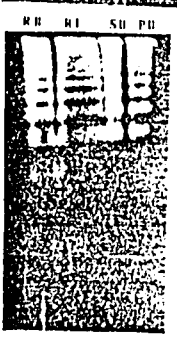
ลินามาเรสในสภาวะไม่เสียสภาพปรากฏขนาดโมเลกุล >600,000 ดาลตัน โดยเซฟาโรส 4 บี โครมาโตกราฟี และผลจากอัลดาไล ไฟลีอะคริลาไมด์ เจลอิเลคโตรโฟเรซิส และเซลลูโลส อะซีเตต อิเลคโตรโฟเรซิส ให้ข้อเสนอแนะว่า ลินามาเรสมีส่วนหนึ่งของความแตกต่างที่มาจากภาวะการจับกลุ่ม (aggregation state) ของสายโมเลกุลที่ต่างกัน

เมื่อศึกษาโครงสร้างของลินามาเรสในสภาวะที่ถูกทำให้เสียสภาพโดย SDS และความร้อน หรือโดยยูเรียที่มีและไม่มี triton X-100 ร่วม พบว่า ขนาดโมเลกุลของหน่วยย่อย เท่ากับ 63,000 ดาลตัน และหน่วยย่อยน่าจะประกอบด้วยสายโพลีเปปไทด์เพียง 1 ชนิด



(ค)

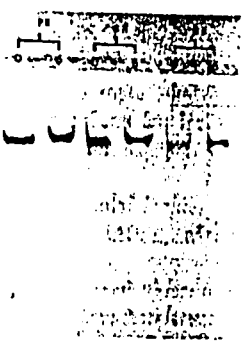
รูปที่ 2 แสดงผลการหลลงที่สังเกตได้เมื่อลินามาเรสอยู่ในสภาวะไม่เสียสภาพ (ก: อัลดาไล ไฟลีอะคริลาไมด์ เจล อิเลคโตโฟเรซิส, ข: เซลลูโลส อะซีเตต อิเลคโตรโฟเรซิส และเมื่อลินามาเรส อยู่ในสภาวะที่เสียสภาพ (ค: SDS-ไฟลีอะคริลาไมด์ เจล อิเลคโตโฟเรซิส, ง: ACID-UREA-TRITON เจล อิเลคโตโฟเรซิส)



(ก)

P S R P S R
II I II II II II

(ข)



(ง)

P = ลินามาเรสที่สกัดมาจาก petiole หรือก้านใบ, S = ลินามาเรสที่สกัดมาจาก stem หรือ ลำต้น
R = ลินามาเรสที่สกัดมาจาก root cortex หรือเปลือกของรากหัว
I = ลินามาเรสที่ถูกทำให้บริสุทธิ์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต
II = ลินามาเรสที่ถูกทำให้บริสุทธิ์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต และกรองโดยวุ้น (gel filtration)

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STRUCTURE OF CASSAVA LINAMARASE

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Cassava produces cyanogenic glycosides which can release the highly toxic HCN, that can cause health risks for consumers and create problems in export of cassava products.

Linamarase, the beta-glycosidase enzyme catalyzing the hydrolysis of cyanogenic glucosides, has been reported to exist in three molecular forms in the cassava, separable by chromatofocusing. We have purified linamarase from the stem, petiole and root cortex of cassava by ammonium sulphate precipitation and gel filtration. The purified enzyme from all three sources gave a single band in SDS-PAGE with M_r 63,000 and also yielded one major band in isoelectric focusing. Under native conditions, linamarase showed a broad peak of activity with apparent molecular weight of >600,000 in Sepharose 4B chromatography and gave a single broad band in cellulose acetate electrophoresis. However, a ladder pattern consisting of 1, 2, 3, 4, 5, 6 and higher multiples was observed in non-denaturing PAGE, suggesting that at least part of the difference in molecular forms was due to aggregation. Under denaturing conditions, a single band was also obtained in acid-urea and triton-acid-urea electrophoresis, suggesting that the aggregates consisted of a single type of polypeptide chain. This aggregation is probably an *in vivo* phenomenon, because the ladder pattern in non-denaturing PAGE is observed even when fresh crude extracts are used.

Further studies are in progress to define the precise structural organization of linamarase enzyme and the physiological significance of this aggregation.

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