การขนส่งซูโครสในมันสำปะหลัง *Manihot esculenta* Crantz.

นางสาว ธิดารัตน์ เอกสิทธิกุล

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SUCROSE TRANSPORT IN CASSAVA *Manihot esculenta* Crantz.

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ซูโครสเป็นรูปแบบของสารคาร์บอนที่พืชใช้ส่งผ่านไปยังส่วนต่าง ๆ และมีความสำคัญต่อผลิตผลของพืช ซูโครสสามารถขนส่งผ่านเยื่อเซลล์ไปยังส่วนต่างๆ ของพืชเพื่อการเจริญเติบโต การพัฒนา และการสะสมอาหารโดยอาศัยโปรตีนส่งผ่าน ในมันสำปะหลังซูโครสสะสมที่รากในรูปของแป้ง ได้ทำการศึกษาการขนส่งซูโครสในมันสำปะหลังโดยวิธี uptake ของใบและรากของมันสำปะหลังมีขนาดเส้นผ่านศูนย์กลาง 12 mm บ่มในสารละลายที่มี ^{14}C-sucrose หลังจากนั้นนำไปวัดค่าปริมาณรังสี พบว่าการ uptake ของซูโครสแปรผันโดยตรงกับเวลาในการบ่ม และมีลักษณะกราฟเป็นแบบ Michaelis-Menten มีค่า K_m = 1.13 mM, V_{max} = 3.7 nmol/hr/cm^2 ในใบ และ ในรากมีค่า K_m = 20 mM, V_{max} = 0.018 nmol/hr/cm^2 สรุปได้ว่าการขนส่งซูโครสในมันสำปะหลังมีโปรตีนตัวกลาง การ uptake ของซูโครสถูกยับยั้งด้วย N-ethylmaleimide (NEM), p-chloromercuribenzensulfonic acid (PCMBS) และ iodoacetic acid (IAA) แสดงว่าโปรตีนตัวกลางมีหมู่ไธออลเป็นองค์ประกอบ พบว่าการ uptake ในใบถูกยับยั้งอย่างสมบูรณ์ด้วย protonophore, dinitrophenol (DNP) และ carbonylcyanide m-chlorophenylhydrazone (CCCP) และถูกยับยั้งด้วยตัวยับยั้งเมแทบอลิซึม เช่น วานาเดท (vanadate), อิริโทรซิน B (erythrosin B) และไซยาไนด์ แสดงว่าการ uptake เกี่ยวข้องกับการขนส่งโปรตอน และเป็นกระบวนการที่ใช้พลังงาน

มันสำปะหลังเป็นพืชที่มีสารประกอบไซยาไนด์ในทุกเนื้อเยื่อ (ยกเว้นเมล็ด) ในรูปของ cyanogenic glucosides โดยชนิดที่พบมากที่สุด คือ ลินามารินซึ่งมีประมาณ 90% และโลทัวสตราลินประมาณ 10% ลินามารินถูกสังเคราะห์ขึ้นที่ใบ ได้ทำการศึกษาผลกระทบของไซยาโนเจนิกกลูโคไซด์ต่อการขนส่งซูโครสในใบ พบว่าในขณะที่มีลินามารินอยู่ด้วย การขนส่งซูโครสลดลงโดยมีค่า I_{50} ที่ความเข้มข้นของ ลินามารินเป็น 0.1 mM แต่ในเนื้อเยื่อของรากพบว่าลินามารินมีผลกระตุ้นการขนส่งซูโครส

จากการเตรียมเยื่อหุ้มเซลล์ของใบมันสำปะหลัง โดยวิธี aqueous two-phase system ซึ่งประกอบด้วย Dextran T500 และ PEG 3350 ได้เยื่อหุ้มเซลล์ในรูป vesicles ซึ่งสามารถ uptake ^{14}C-sucrose ในปริมาณแปรผันโดยตรงกับเวลา เมื่อนำมาแยกโปรตีนที่จับกับซูโครสได้โดยใช้ sucrose-affinity column พบว่าโปรตีนขนส่งซูโครสมีน้ำหนักโมเลกุล 62 กิโลดาลตันใน SDS-PAGE

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต……..
สาขาวิชา	ชีวเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา…….
ปีการศึกษา	2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

3972822023 : MAJOR BIOCHEMISTRY
KEY WORD: SUCROSE TRANSPORT / CASSAVA
THIDARAT EKSITTIKUL : SUCROSE TRANSPORT IN CASSAVA *Manihot esculenta* CRANTZ. THESIS ADVISOR: ASSIST. PROF. TIPAPORN LIMPASENI, Ph.D., THESIS COADVISOR : PROF. MONTRI CHULAVATNATOL, Ph.D.,
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Sucrose is a major translocated form of carbon assimilates in many plant species and plays a key role in crop production. Sucrose has been shown in many plants to be actively transported across membrane by a transport protein to heterotrophic organs for its growth, development and storage. In cassava, sucrose is stored in root as starch. Sucrose transport in cassava was studied by uptake experiment. Cassava leaf and root discs, 12 mm in diameter were incubated in the medium containing ^{14}C-sucrose. It was found that the K_m 1.31 mM, V_{max} 3.7 nmole/hr/cm^2 in leaf disc and K_m 20 mM, V_{max} 0.018 nmole/hr/cm^2 in root disc, indicating that sucrose transport in cassava was carrier- mediated. Sucrose uptake was inhibited by N-ethylmaleimide (NEM), p-chloromercuribenzensulfonic acid (PCMBS) and iodoacetic acid (IAA) suggesting that the sucrose transport protein consist thiol groups. The sucrose uptake in leaf disc was strongly inhibited by protonophore such as dinitrophenol (DNP) and carbonylcyanide m-chlorophenylhydrazone (CCCP) and inhibited by metabolic inhibitors such as vanadate, erythrosin B, and KCN, suggesting that the uptake involved proton transport and was energy-dependent.

Cassava is a known cyanophoric plant. All cassava tissues, with the exception of seeds, contain the cyanogenic glycosides, linamarin (>93% total cyanogen) and lotaustralin (<10% total cyanogen), which were synthesized in the leaves. The effect of cyanogenic glucosides on sucrose transport was studied. It was found that in presence of linamarin, sucrose transport was inhibited with I_{50} value at 0.1 mM linamarin. On the contrary, sucrose transport was significantly the activated in the root disc.

Plasma membrane of cassava leaves was purified by aqueous two-phase system consisted of Dextran T500 and PEG 3350. The membrane preparation appeared as vesicles and exhibited linear uptake of ^{14}C-sucrose with time. Sucrose binding protein was purified by using sucrose-affinity column and it showed MW of 62 kDa on SDS-PAGE.

Department	Biochemistry	Student's signature……
Field of study	Biochemistry	Advisor's signature……
Academic year	2001	Co-advisor's signature……

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LIST OF ABBREVIATIONS

$(NH_4)_2HPO_4$	di-ammonium hydrogen phosphate
$(NH_4)_2S_2O_8$	ammonium persulfate
$(NH_4)_2SO_4$	ammonium sulfate
$(NH_4)H_2PO_4$	ammonium dihydrogen phosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
Bq	bequerel
BSA	bovine serum albumin
dpm	disintegration per minute
DTT	dithiothreitol
EDTA	Ethelenediaminetetraacetic acid
g	gravitational acceleration
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetic acid
KCl	potassium chloride
KCN	potassiumcyanide
kDa	kilo Dalton
M	molar
MW	molecular weight
MW.	Molecular weight
NEM	N-Ethylmaleimide
nm	nano-metre
PCMBS	ρ-Chloromercuribenzoic acid

PEG	**polyethylene glycol 3350**
PMSF	**Phenylmethylsulfonyl fluoride**
CCCP	**Chlorocarbonyl cyanide m-chlorophenylhydrazone**
SAX	**strong anion exchanger**
SDS	**sodium dodecyl sulfate**
DNP	**ρ-Dinitrophenol**
MOPS	**3-[(N-Morpholino)propanesulfonic acid**
HEPES	**N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]**

CHAPTER I

INTRODUCTION

1.1 Major Crop Products

In nature the predominant crop products are carbohydrates such as sucrose, starch, fructan and cellulose, or hydrocarbons such as rubber and oils. They are all polymeric compounds and are all insoluble except sucrose and fructan. These crop products are synthesized by the photosynthetic tissue, usually the leaves but the sites of accumulation or deposition of the products can vary from one crop to another (Table 1.1). Table 1.2 shows various sites of crop product synthesis and the accumulation site at subcellular level (1). The biosynthetic pathway of these products usually starts from sucrose as shown in Figure 1.1.

1.2 Sucrose

Sucrose is a disaccharide, consisting of an α-D- glucopyranoside joined to a β-D-fructopyranoside by α-1,2 linkage between the anomeric carbon atoms. The structure lacks aldehyde group. Therefore, sucrose is a non-reducing sugar. Sucrose, an uncharged molecule, is non-reactive to functional groups of other biological molecules. Sucrose is highly soluble in water (1 g in 0.5 ml or 58.7 M) and does not have an inhibitory effect on biochemical processes. These properties of sucrose make it suitable for its role in transporting carbohydrate in many higher plant species (2).

Table 1.1 Storage tissues and organs of the major crop products

Product	Tissue	Organ
Sucrose	Storage parenchyma	Root (beet)
		Stem (cane)
Fructan	Storage parenchyma	Root and stem tubers
Starch	Endosperm	Seeds (cereals)
	Storage parenchyma	Tubers
Cellulose	Epidermal	Seed fibers (cotton)
	Secondary xylem	Stem (wood)
Oils	Cotyledons	Seeds
	Storage parenchyma	Fruit (oil palm)
Rubber	Secondary phloem latex	Stem
Protein	Endosperm	Seeds (cereals)
	Cotyledons	Seeds (legumes)

From : (1)

Table 1.2 Intracellular sites of synthesis and deposition of the crop products

Product	Site of synthesis	Site of deposition
Sucrose	Cytosol	Vacuole
Fructan	Vacuole	Vacuole
Starch	Amyloplast	Amyloplast
Cellulose	Plasma membrane	Cell wall
Oils	Plastids and endoplasmic reticulum	Oil bodies
Rubber	Cytosol	Rubber particles
Protein	Ribosomes on endoplasmic reticulum	Protein bodies

From : (1)

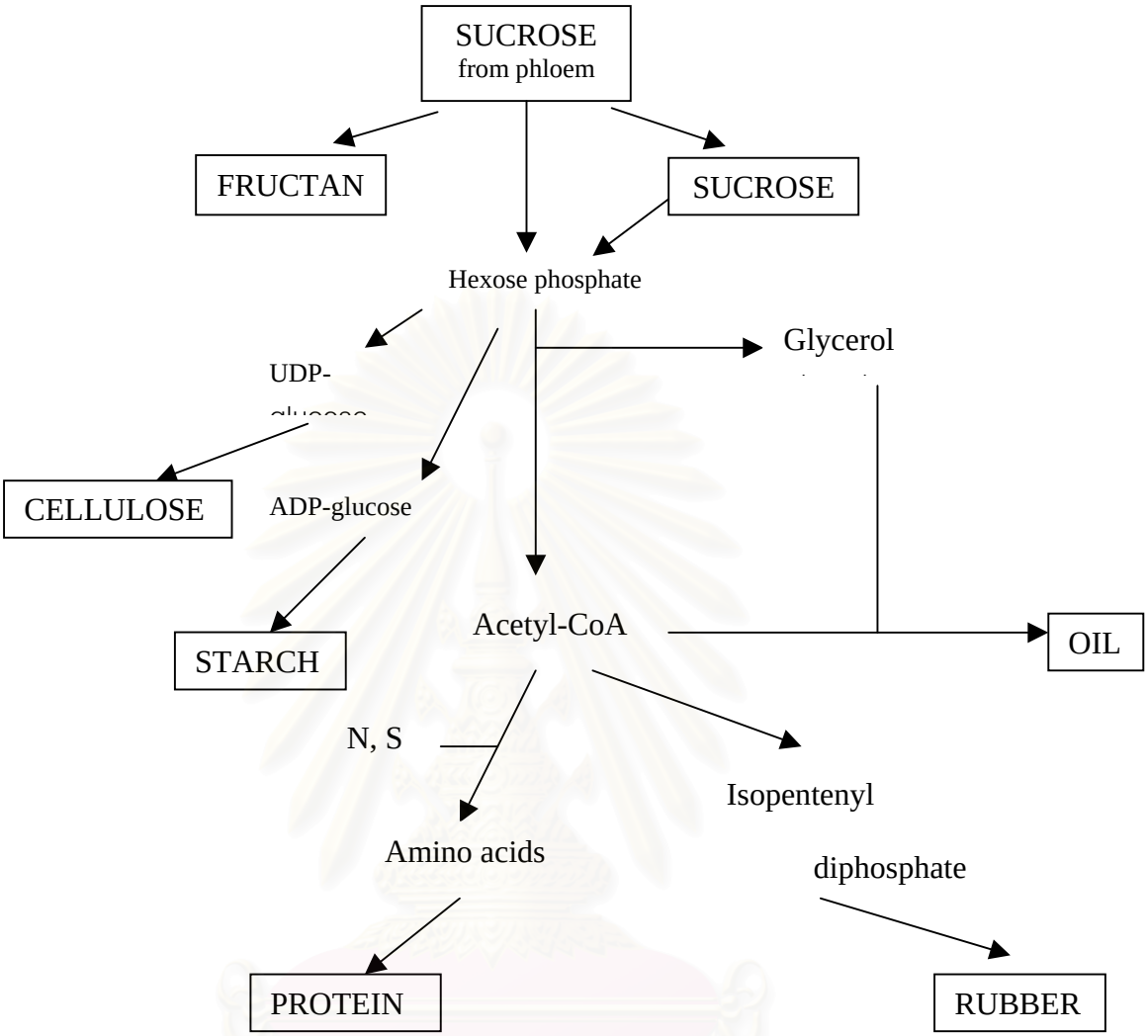


Figure 1.1 Outline of the flow of assimilate towards the different crop products

From : (1)

In 1975, Zimmerman and Ziegler used plant exudation and TLC chromatography technique to show that sucrose was the major component in phloem sap of over 500 plant species (3), one of the example was shown in Table 1.3. In plant, sucrose is the major product in photosynthesis which occurs in the cytosol of photosynthetically active source organs like leaves and delivered or transported to several non-photosynthetic tissues. Generally, 50-80 percents of the sucrose synthesized during photosynthesis is transported and used for growth and development such as synthesis of structural polysaccharides of the germinating seed, formation of flowers or fruits, and accumulation of carbohydrate reserve in seeds or tuber tissues in the form of polysaccharide, starch or fructan. Moreover, sucrose serves as intermediates of synthetic pathways for several cellular components (Figure 1.2)(4).

1.3 Sucrose synthesis

Cell fractionation and enzyme localization technique provided evidence that sucrose is synthesized in the cytosol of photosynthetic cells due to the relative impermeability of the chloroplast inner envelope to sucrose (5). Sucrose is synthesized from the pool of hexose monophosphate which is the condensation product of two triose phosphates, dihydroxyacetone phophate (DHAP) and phosphoglyceraldehyde (G-3-P), the key products of Calvin cycle in the chloroplast.

Fructose-1,6-biphosphate is subsequently converted into fructose-6-phosphate or glucose-1-phosphate. Sucrose is synthesized from the hexose

Table 1.3 The chemical compositions of xylem and phloem sap exudate from White Lupine

	Xylem Sap (tracheal) mg/l	Phloem Sap (Fruit bleeding) mg/l
Sucrose	ND[a]	154,000
Amino acids	700	13,000
Potassium	90	1,540
Sodium	60	120
Magnesium	27	85
Calcium	17	21
Iron	1.8	9.8
Manganese	0.6	1.4
Zinc	0.4	5.8
Copper	Tr[b]	0.4
Nitrate	10	ND[a]
PH	6.3	7.9

ND[a] = Not present in detectable amount.

Tr[b] = Present in trace amount.

From: (3)

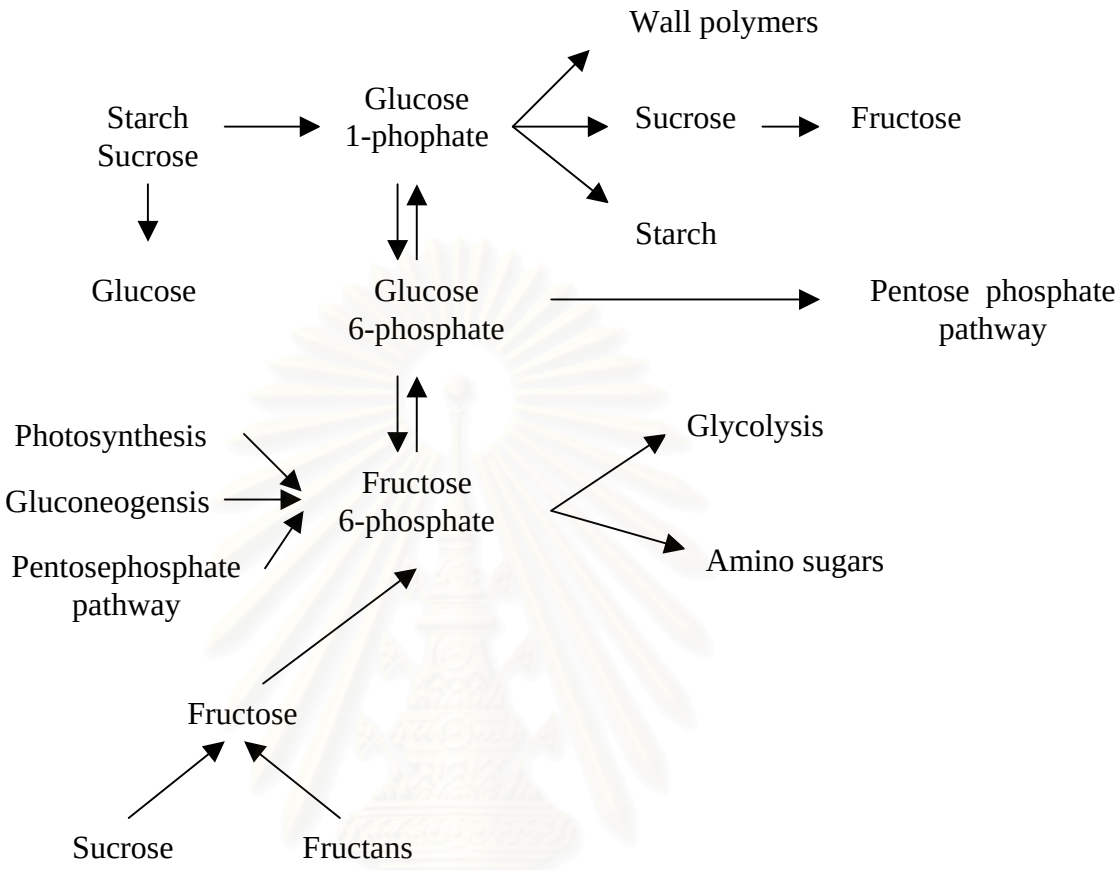


Figure 1.2 Sucrose as source of intermediates for many biological process

From : (4)

monophosphate through the combined activities of three enzymes located in the cytosol as follow:

UDP-glucose pyrophosphorylase (EC 2.7.7.9): catalyses the formation of activated glucose molecule with $\Delta G^{0'}$ = -2.88 kJ/mol

glucose-1-phosphate + UTP $\longleftrightarrow$ UDP-glucose + PPi

Sucrose phosphate synthase (SPS)(EC 2.4.1.14) : catalyse a transglycosylation reaction with $\Delta G^{0'}$ = -1.46 kJ/mol

UDP-glucose + fructose 6-phosphate $\longleftrightarrow$ Sucrose 6-phosphate + UDP

Sucrose phosphatase (EC 3.1.3.24): catalyzes the hydrolysis of phosphate group wit $\Delta G^{0'}$ = -18.4 kJ/mol

sucrose-6-phosphate + H_2O $\longrightarrow$ sucrose + Pi

Moreover in most plants there is another enzyme in cytosol, a sucrose synthase (EC 2.4.1.13), which theoretically capable of catalyzing sucrose forming reaction :

UDP-glucose + fructose $\longleftrightarrow$ sucrose + UDP

This reaction has the $\Delta G^{0'}$ = 3.99 kJ/mole; therefore, it cannot occur spontaneously. Sucrose synthase tends to catalyse the cleavage of sucrose molecule in heterotrophic tissues. Moreover, the large negative $\Delta G^{0'}$ of the

reaction catalysed by sucrose phosphatase indicating that the production of sucrose is irreversible and it allows sucrose synthesis to continue even in the tissue that already contains large amount of sucrose, especially in plants like sugarcane or sugar beets which store large amount of sucrose in their storage organs.

1.4 Sucrose transport in plants

Sucrose is predominantly synthesized in a photosynthetically active organ, usually mature leaves and transported to the non-photosynthetic organs. The exporting tissue is often referred to as ‘source tissue’ and the importing tissue as ‘sink tissue’.

Sucrose in mesophyll of the leave cells is translocated via a phloem which is a vascular system of plants used for solute transport. Phloem tissue is a complex tissue composed of at least three cell types: parenchyma cells, the conducting sieve-tube cells and companion cells that are associated with the sieve-tube cells/or sieve element. The conducting sieve-tube is separated by sieve tube membrane which contains pores through which the phloem sap flows. Figure 1.3 showed the structure of phloem tissue (5). In general, all transport of materials between plants cells involves the cell wall and cell membrane. There are two main types of transport . One is apoplastic transport which involves movement through the matrix of the cell wall and cell membrane. Second types is symplastic transport which involves movement from one cell to another via plasmodesmata(Figure 1.4) (6).

There are two principal pathways for the delivery of sucrose from mesophyll cytoplasm into the minor vein sieve-element-companion cell complex

namely (a) symplastic loading, in which sucrose moved via plasmodesmata and (b) diffusion by an apoplastic loading. The extent to which plants can use either of the two pathways for sucrose delivery depends on the number of plasmodesmatal connections between the minor vein sieve-element-companion cell complex and its surrounding cells. This varies between species, or ecological condition. Plant will use the symplastic transport if the number of plasmodesmata are sufficient for transport sucrose at the required rate (7, 8). The symplastic pathway is usually elucidated by following the intercellular movement of a fluorescent dye, Lucifer Yellow (LYCH), after injection in to cytosol. It is a charged molecule which presumably cannot diffuse through membrane and moves only via plasmodermata (8, 9).

In 1974, Gieger *et al*.(10) showed that sucrose loading was via apoplastic route by exogeneous supplied of ^{14}C-sucrose in the apoplast and it was taken into sieve-element from the apoplast. Delrot's work in 1989, also showed the same result when he applied ^{14}C-sucrose to a leaf surface and finally found the ^{14}C-sucrose in the phloem(11). Other supporting evidence for apoplastic transport was the inhibition of ^{14}C-sucrose uptake by p-chloromercuribenzene sulfonic acid (PCMBS) in *Vicia faba* leaves(12). Because PCMBS is membrane-impermeable compound and remains in the apoplast, any effect it has must be localized on the apoplastic surface of the membrane. In general, PCMBS is a sulfhydryl modifying reagent, alkalating to the –SH group of cystein amino acids of proteins. Therefore, the sucrose loading by apoplastic transport in *Vicia faba* leaves and the carrier involved contains the sulfhydryl group in its active site or close to the active site of the sucrose transporter (8).

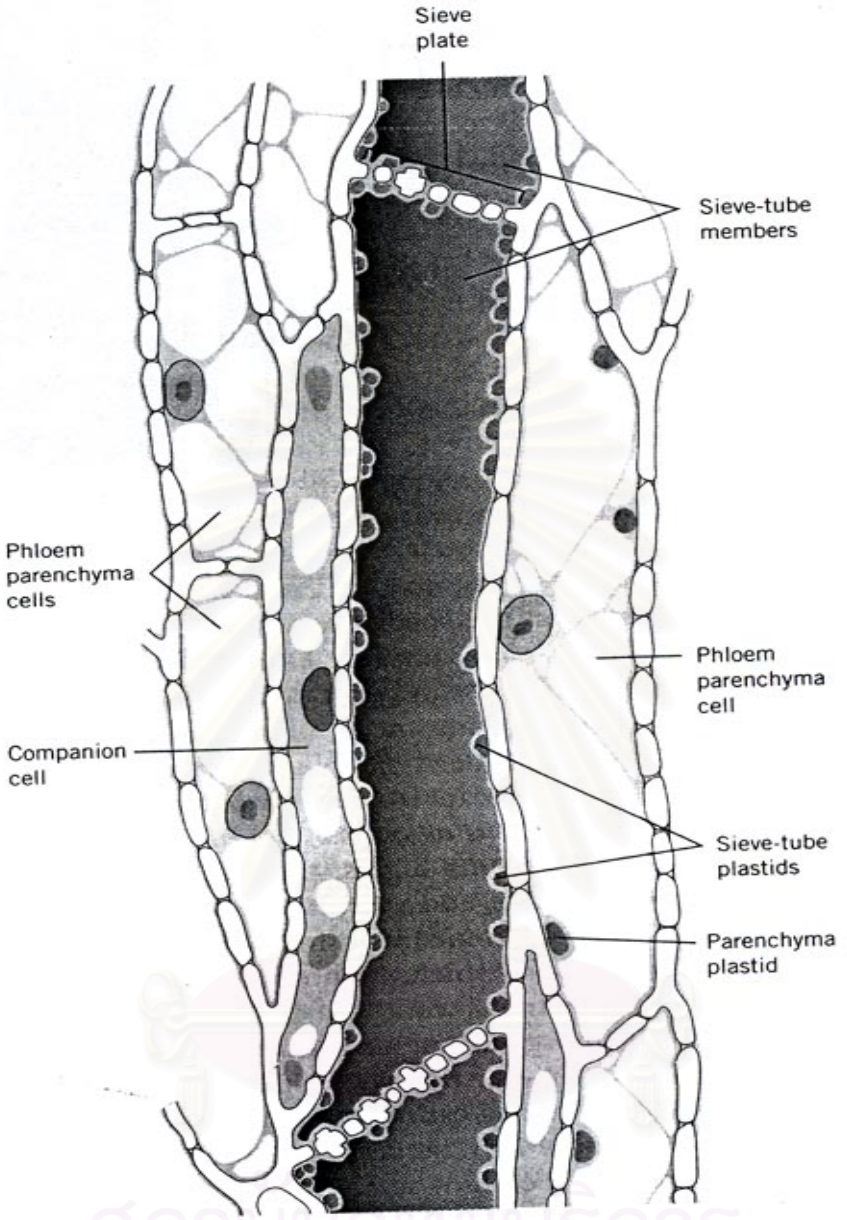


Figure 1.3 The structure of phloem of tissue from the stem of tobacco

From: (5)

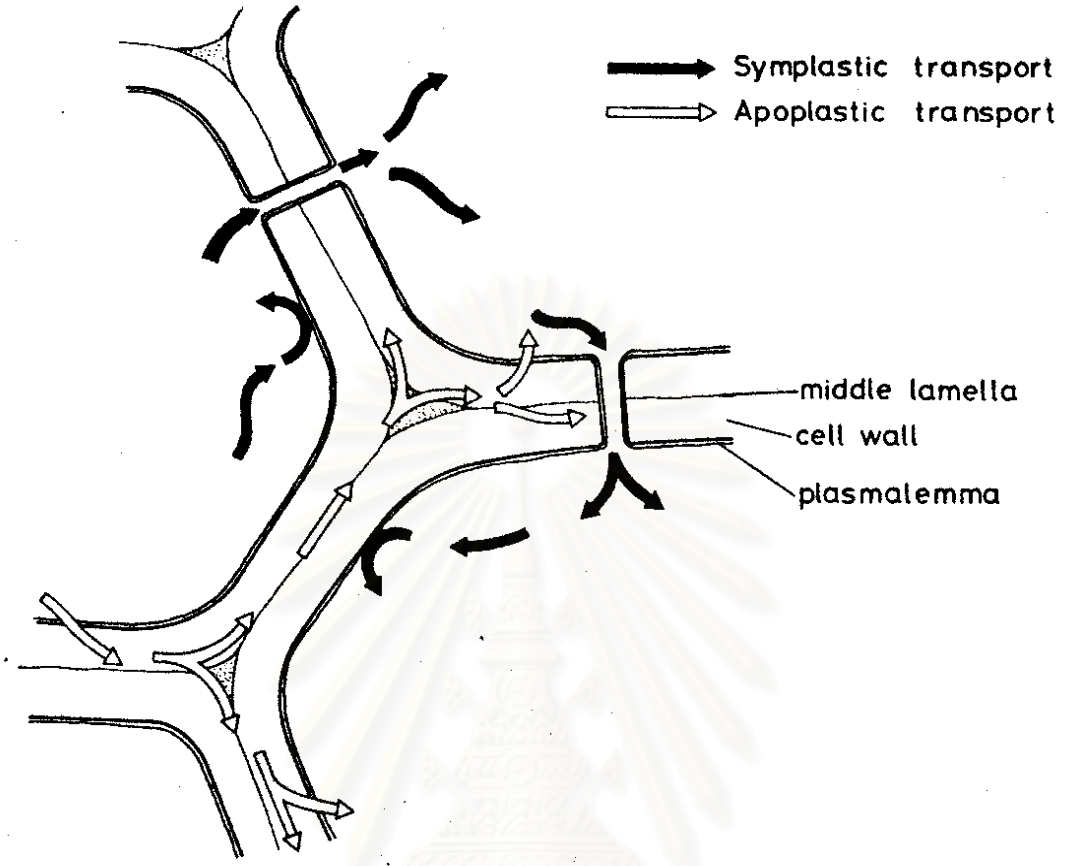


Figure 1.4 A schematic of apoplastic and symplastic transport between cells.

From : (6)

Apoplast loading of sucrose has been demonstrated in several species such as sugar beet, broad beam, potato and spinach (8). While symplastic loading of sucrose is shown in tobacco and potato (12). There has been also evidence indicating an involvement of a sucrose-H^+ co-transport by an observed decrease of sucrose uptake with an increasing pH (9 and 13) and the system is an active, energy-dependent transporter (14,15, 16). The energy for this transport is derived from the proton gradient established by a H^+/ATPase which is located in the plasma membrane.

Riesmeier *et al* (17) has reported the importance of sucrose transporter in phloem loading in potato by using antisense RNAs technique to reduce the level of the sucrose carrier SUT1 in the phloem. The result shows a retardation of growth, a change in phenotype and a dramatically reduction of tuber yield in the transgenic potato plants.

Recently, Lemoine has reviewed and postulated the existence of carrier in the sucrose transport system as shown in Figure 1.5 (18). It has been suggested that the first transmembrane event is the transport of sucrose in the vacuole. Then, sucrose exits the mesophyll cell (step 2) and, from the apoplast, enters the phloem cell (step 3). To exit the long distance pathway, several routes are possible depending on different situations encountered among species (apoplastic and symplastic unloading). When sucrose is unloaded into the apoplasmic space (step 4), it can be taken up as sucrose into the sink cells (step 5). Then sucrose is used for sink growth or development or stored as sucrose in the vacuole of the storage cells (step 6) as in sugar beet and sugar cane. Therefore, sucrose transporters in plant exist as three types: Firstly, proton / sucrose symporter which is an influx

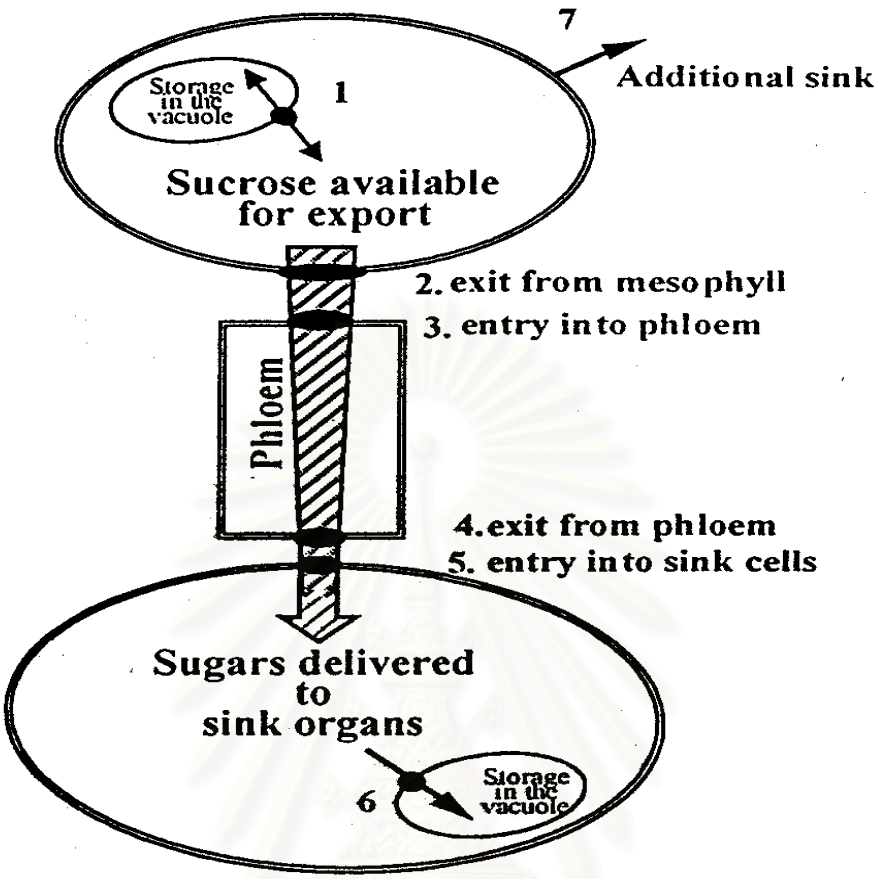


Figure 1.5 Schematic postulate of sucrose transport.

The flow of sucrose from source organ (upper part) to the sink organ (lower part) through the phloem to the different events of membrane transport discussed in the text.

From : (18)

carrier responsible for the entry of sucrose into cells. Secondly, sucrose/proton antiporters which are a tonoplast carrier. Thirdly, facilitators or antiporters which are efflux carriers responsible for unloading of sucrose in sink organs or for sucrose exit from mesophyll cells to the phloem. Up to now, over twenty different genes for disaccharide transporters have been identified from various plant species. The genes are named either SUT or SUC for sucrose transporter, or SCR for sucrose carrier (Table 1.4). The genes were successfully expressed in yeast. All of them found to belong to the same gene family, which encodes highly hydrophobicity amino acids. From the nucleotide sequences, all of them has the molecular mass around 55 kDa.

1.5 Cassava

Cassava (*Manihot esculenta* Crantz.) is the most important food crop which feeds around 750 million people in the world. Cassava is a native plant of Brazil. During the 16th and 17th centuries it was dispersed widely by the Portuguese to tropical and sub-tropical areas of Africa, Asia and the Caribbean. Cassava can grow and produce dependable yields in places where cereals and other crops cannot grow or produce well. It can tolerate drought and can be grown on soils with low nutrient capacity. Cassava has a high yield and high resistant to the damage from serious pests and diseases. Because cassava has no definite maturation point, it can be field-stored for several months or more and harvested when market, processing or other conditions are more favorable (19).

Table 1.4 List of the sucrose transporter sequences available in databases

Name	Species	Length (a.a.)	Acces	Functional expression/K_m
AgSUT1	Celery	512	4091891	Yes/0.14 mM
AtSUC1	**Arabidopsis**	513	481132	Yes/0.45 mM
AtSUC2	*Arabidopsis*	512	407092	Yes/0.53 mM
AtSUTX1	*Arabidopsis*	474	2160188	No
AtSUTX2	*Arabidopsis*	513	3287687	No
AtSUTX3	*Arabidopsis*	492	3810593	No
AtSUTX4	*Arabidopsis*	594	3461813	No
BvSUT1	Sugar beet	523	633172	No
DcSUT1a/b	Carrot	501	2969889,2969887	Yes/0.5 mM
DcSUT2	Carrot	515	2969884	Yes/0.7 mM
LeSUT1	Tomato	511	575299	No
NtSUT1	Tobacco	507	575351	No
NtSUT3	Tobacco	520	149981	No
OsSUT1	Rice	537	2723471	Yes/ND
PmSUC1	**Plantago**	510	1086253	Yes/0.3 mM
PmSUC2	**Plantago**	510	415988	Yes/1 mM
RcSCR1	**Ricinus**	533	542020	Yes/2 mM
SoSUT1	Spinach	525	549000	Yes/1.5 mM
StSUT1	Potato	516	542087	Yes/1 mM
VfSUT1	***Vicia faba***	523	Z93774	Yes/1.4 mM

Sequences are listed in alphabetical order and, when they are successfully expressed in yeast, the K_m value for sucrose is indicated. ND, not determined.
From : (18)

Cassava is a shrubby, tropical, perennial and a dicotyledoneous plant belonging to the botanical family Euphorbiaceae. All *Manihot* species, the normal cassava plants, have 36 chromosomes. Genetically, cassava is highly heterozygous. Both cross and self-pollination occur naturally. As a consequence of the very large variations found in cassava, both among the plants and within plants (as heterozygosity), the shrub, root shape and size, color and pigmentation of the petioles varied (20).

In addition to being used for human consumption, dried cassava is used as animal feed. Cassava has entered the modern market economy and there is a growing demand for it use in processed food such as glucose, high fructose syrup, dextrin. Specialty markets for cassava include baby foods and non-allergenic products. Cassava starch can be modified to provide characteristics that are required for more specialized food and industrial products. Modified cassava starch can be used for the production of alcohol, starch for sizing paper and textiles, glues, monosodium glutamate (MSG), sweeteners, pharmaceutical, bio-degradable products, butanol and acetone, manufacture of explosives, and coagulation of rubber latex.

Although the caloric content of cassava food product is high, an important drawback leading to a limited use for human and animal consumption is its cyanogenic potential or ability to generate HCN from the hydrolysis of its cyanogenic glucosides which are linamarin (93%) and lotaustralin (7%) (21, 22). They are accumulated in cellular vacuoles which are compartmentalized from their

biodegradative enzyme, a β-glucosidase called linamarase which is localized in the apoplastic space (23). Linamarin is accumulated in high content in leaves up to 5g/kg fresh leaves (24) while in fresh root linamarin content is in the range 100-500 mg/kg root. It is known to be synthesized in leaves and translocated throughout the plant and accumulated in root like sucrose . L-valine amino acid is a precursor of linamarin synthesis which is catalyzed by the enzyme in microsome (cytochrome P_{450} dependent enzyme) and the glucosyltransferase in cytosol (25).

In Thailand, cassava is produced in large scale and most of it is exported widely in the world. The production of the root is average about 18 million tons in each crop year from 1.5 million hectares (9.3 million rais) About 70 % of fresh roots are processed into pellets and chips for use as animal feed and they exported to European Community.

It is the interest of our research group to study the enzymes and systems involved in the cyanogenesis and starch accumulation in cassava which would lead to a clear overall picture of the functions of there systems in cassava. Enzymes involved in the cyanogenesis of cassava have been studied. Several enzymes involving sucrose and starch synthesis such as sucrose synthase, invertase, starch synthase and starch branching enzymes have also been purified.(26, 27, 28)

1.6 Objectives

Sucrose has been shown to play major roles in translocation and storage of carbohydrate. Cassava is an important root crop which accumulates carbohydrate in its tubers and sucrose transport in cassava has not yet been reported. This dissertation aims at gaining a better understanding of sucrose transport in cassava by

1) Characterization of cassava transport system in cassava leaves and roots.
2) Preliminary identification of a sucrose carrier protein in cassava leaves.

CHAPTER II

MATERIAL AND METHODS

2.1 Plant materials

The sweet variety cassava was used in this study. Fresh and fully expanded cassava leaves, usually the 4^{th} and the 5^{th} leaves (from the shoot), were from the plants grown in the experimental fields of Mahidol University, and Ramkhamhaeng University Bangkok.

Cassava tubers were purchased from Pak Klong Ta Lad market, Bangkok, Thailand.

2.2 Chemicals

Chemical	Company
Radioactive sucrose (U-^{14}C)	*Amersham Life Science*
Ammonium dihydrogen phosphate	May&Baker, England
di-Ammonium hydrogen phosphate	May&Baker, England
Ammonium sulphate	Merck, Germany
Amygdarin	Sigma, USA
CHAPS (3-[(3-Cholamiddopropy)-dimethylammonio]-1-propanylsulfonate)	Sigma, USA
ρ-Chloromercuribenzensulfonic acid (PCMBS)	Sigma, USA
Chlorocarbonyl cyanide m-phenylhydrazone (CCCP)	Sigma, USA
CHAPS (3-[(3-Cholamiddopropy)-dimethylammonio]-1-propanylsulfonate)	Sigma, USA
Dextran T 500	Pharmacia Biotech, USA
ρ-Dinitrophenol (DNP)	Sigma, USA
Dithiothreitol (DTT)	Sigma, USA
Epoxy activated Sephrose 6 B	Pharmacia Biotech, USA
Erythrosin B	Sigma, USA

Ethanolamine	Sigma, USA
Ethelenediaminetetraacetic acid (EDTA)	Sigma, USA
N-Ethylmaleimide (NEM)	Aldrich, USA
Fructose	Sigma, USA
Glucose	Sigma, USA
HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])	Sigma, USA
Iodoacetic acid (IAA)	Sigma, USA
Isopropylthioglucoside (IPTG)	Sigma, USA
Linamarin	Sigma, USA
Maltose	Sigma, USA
Mannitol	Sigma, USA
β-Mercaptoethanol	Sigma, USA
MES (2-[(N-Morpholino)ethanesulfonic acid])	Sigma, USA
MOPS (3-[(N-Morpholino)propanesulfonic acid]	Sigma, USA
ρ-Nitrophenylglucoside (PNPG)	Sigma, USA
Palatinose	Sigma, USA
Perchloric acid	Aldrich, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, USA
Phloridzin	Sigma, USA
Polyethylene glycol 4000	Sigma, USA
POPOP	Sigma, USA
PPO	Sigma, USA
Prunasin	Sigma, USA
Sucrose	Sigma, USA
Toluene	BDH, England
Triton X-100	Sigma, USA

The other common chemicals were of reagent grade from BDH, Carlo-Erba, Fluka, Merck, and Sigma.

2.3 Equipments

Equipment/Model	Company
Centrifuge/ J2-21	Beckman, USA
L8-70 Ultracentrifuge	Beckman, USA
Avanti J-30I High performance centrifuge	Beckman, USA
Cork borer	Local made, Thailand
Electrophoresis Unit/ Mighty Small II	Hoefer Pharmacia Biotech, USA
Fraction collector/ Redi Frac	Pharmacia LKB, Sweden
HPLC/ LC 1050 series	Hewlett-Packard, USA
Lyophilyzer / Flexi-Dry μP	FTS Systems, USA
Liquid Scintillation Counter LS56000	Beckman, USA
Spectrophotometer/ DU650	Beckman, USA
Super-slicer (UK. Patent No. 22556579)	UK.

2.4 Sucrose uptake of cassava plant

One classical procedure for study of solute uptake in plant was performed using discs form tissue of interest. Therefore, sucrose uptake in cassava was studied by using the leaf discs and root discs.

2.4.1 Preparation of tissue discs

In this study, the sucrose uptake was followed in both leaves and roots of cassava by using tissue discs prepared from both tissues.

2.4.1.1 Preparation of leaf discs

The mature leaves of cassava which were fully expanded, usually the 4th or 5th leaf from the shoot were freshly collected and briefly immersed in 70% ethanol for surface sterilization and reduction of surface tension of the leaves. The leaves were then washed in sterile-water to remove ethanol before punched out to prepare leaf disc using cork-borer (12). The leaf discs with different surface area (6 mm and 12 mm diameter) were prepared and immediately used in the experiments. In preparing peeled cuticle leaf discs, the leaves which were washed in sterile water. The thin cuticle at lower epidermis of cassava leaves which cannot be peeled off easily was partially removed by light brushing with a fine-toothbrush. The brushing technique was adapted from that used in preparing protoplast (29). Leaf discs of different surface area of 6 mm and 12 mm diameters were then punched out using cork-borer. The fresh leaf discs were immediately used in the experiments to prevent them from drying out.

2.4.1.2 Preparation of cassava root discs

The periderm and cortex of fresh root was peeled off and the parenchyma was cross section sliced into 1.5 mm thick. The thickness was controlled by using the super slicer (UK. Patent No. 22556579). Figure 2.1 showed the cross section of cassava root (30) and the section A was used in this study. The 12 mm diameter root discs were punched using cork-borer (12). The root discs were washed three times in osmoticum solution; 0.3 M mannitol in 20 mM MES/NaOH pH 5.0 , 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$, in order to remove the starch

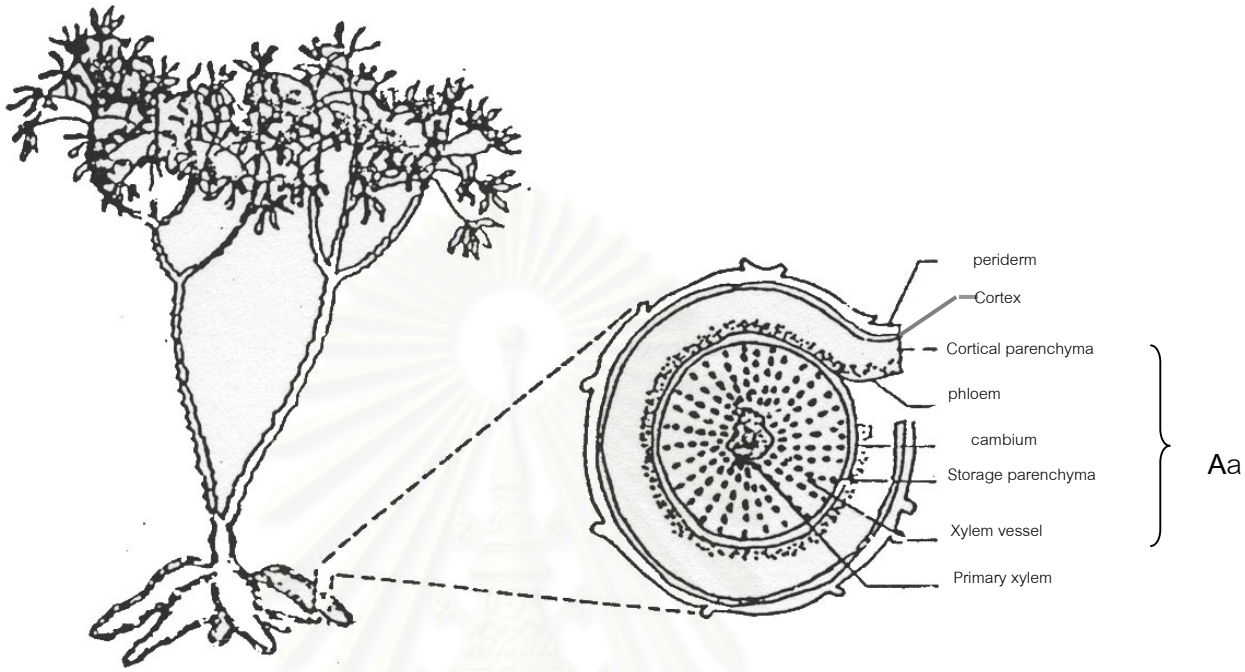


Figure 2.1 Schematic illustration of cross-section of cassava root.

The root disc were punched out from section A as a 12 mm diameter disc with cork-borer.

From : (30)

leakage from the cell during punching. The root discs were immediately used in the experiment.

2.4.2 The sucrose uptake of cassava

Seven to ten freshly prepared leaf discs or root discs were floated with lower epidermis or the brushed side down in 5 ml of medium in 47 mm diameter petri-dish . The medium contained 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 M mannitol in 20 mM MES / NaOH pH 5 and 1 mM sucrose with (U-^{14}C) sucrose (7.4 Bq/ml or 22.8 MBq / mmole) (31). The dish was incubated at room temperature, illuminated with light intensity of 2,000 luxes using fluorescence lamp. After appropriate incubation time, each leaf disc was removed from the medium and blotted on filter paper before washing three times in 20 ml of medium without sucrose for 3 minutes with gentle agitation to remove the free space label. After the last wash, each disc was blotted on filter paper again before transferred into a scintillation vial.

2.4.3 Measuring the radioactivity in leaf discs

The leaf disc in scintillation vial was digested and decolorized the chlorophyll by addition of 0.1 ml of 65% $HClO_4$ (v/v), 0.1 ml of 0.2% Triton X-100 and 0.3 ml of 35% H_2O_2 into the vial, tightly capped then incubated at 75°C for 8-12 hrs with occasional agitation (23). In order to achieve the minimum quench effect of chlorophyll the vial was incubated at room temperature for 2 hrs before incubation at 75°C, 8-12 hrs. Each vial was added with 5 ml of scintillation fluid containing 0.03% POPOP (w/v), 0.5% PPO (w/v) and 50% Triton X-100

(v/v) in toluene. The radioactivity was measured as “dpm” using Beckman (LS 6000) liquid scintillation counter. The total dpm of the incubation medium before staring the experiment was also counted. Each washing medium was also counted (100 μl) to check the release of sucrose during washing. Figure 2.2 showed the schematic of sucrose uptake experiment with cassava leaf discs (32). The uptake was calculated as dpm per disc or as the relative % uptake or nmole / disc otherwise indicated.

Calculation of sucrose uptake

Knowing the counts in dpm for each treatment and the concentration of sucrose in the reaction mixture, the mole of sucrose taken up by each disc can be calculated. For example in 5ml of reaction mixture with 1 mM sucrose, if 10 μl of reaction mixture contained 1,000 dpm was used so;

The total dpm = (5 ml x 1000dpm) / 0.01 = 5×10^5dpm

amount of sucrose in reaction = (1 mM x 5 mlx1000) / 1000 ml

= 5 μmole = 5000nmole

If the average observed dpm for the leaf discs at 4 h were 4,000 dpm. So that the sucrose uptake of the leaf disc was

= (4000 dpm x 5000 nmole) / 5×10^5

= 40 nmole sucrose / 4 h.

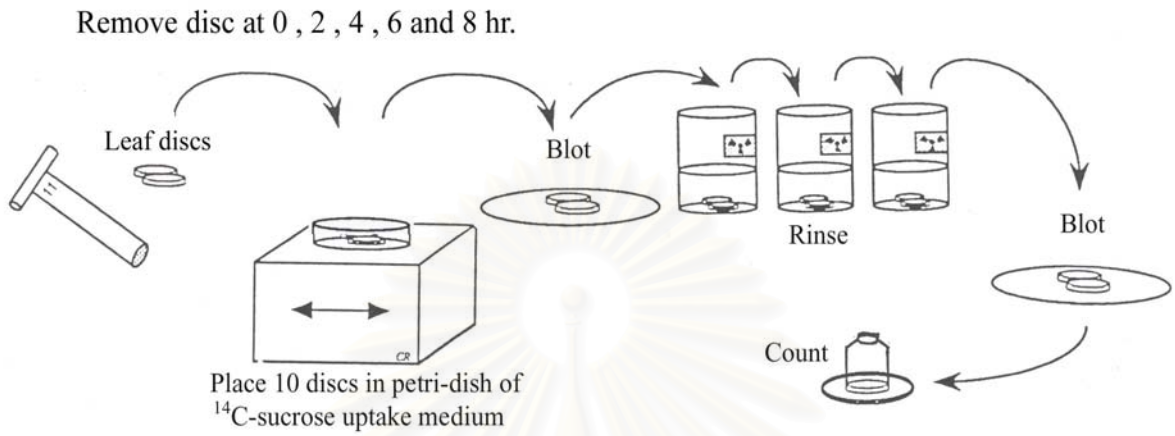


Figure 2.2 Schematic illustration of ^{14}C-sucrose uptake by cassava leaf discs Experiment.

Modified from : (32)

2.4.4 Autoradiography of labeled leaf discs

The path of ^{14}C – sucrose movement in cassava leaf discs were observed by autoradiography. The labeled leaf discs were washed 3 times as described in section 2.4.2. The labeled leaf discs were gently pressed between four layers of filter paper, tightly taped and made sure that the leaf discs did not bend. The package was inserted into the silica gel box and was dried in an oven at 40-50°C for 72 hrs. The dried leaf discs were placed in a clear plastic bag. The labeled-dried leaf discs were exposed to X-ray film in the darkroom by placing a piece of X-ray film over the plastic bag containing labeled leaf discs. The preparation was then sandwiched with the following materials : one sheet of foam , the labeled leaf discs and X-ray film ,and one piece of heavy carboard (33) (Figure 2.3). The sandwiched material was wrapped with black plastic sheet. The package was left in the dark at room temp for several weeks. During this time the ion produced by radiation will chemically alter the X-ray emulsion. The distribution of ^{14}C-sucrose appeared as dark areas on the developed film. Photographs of the films were prepared as negative print which showed the radioactive path as white track against black background.

To enhance the autoradiography process, intensifying screen was used in this study the plastic bag with labeled leaf discs was placed inside the cassette with intensifier screen with the X-ray film on top .The cassette was left in the darkroom for four weeks. Then, the film was developed and the dark area was observed.

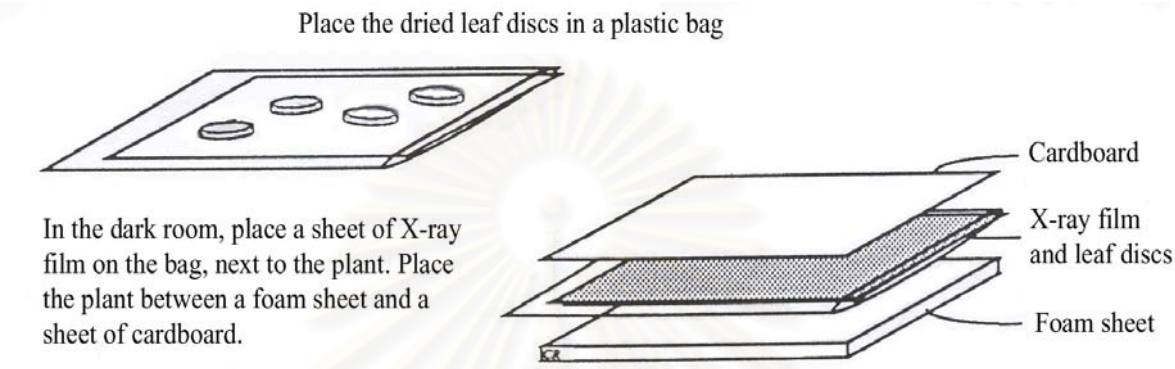


Figure 2.3 Schematic illustration of autoradiography process of cassava leaf discs

Modified from : (33)

2.4.5 The pH optimum of sucrose uptake in cassava leaf

The peeled leaf discs with 12 mm diameter was incubated in 1 mM ^{14}C-sucrose in the medium as described in section 2.4.2. The pH of MES / NaOH buffer was varied between 4 to 6. After 4 hrs of incubation, the leaf discs were prepared for measuring the radioactivity uptake as described in section 2.4.3.

2.4.6 Time course of sucrose uptake by cassava leaf

The peeled/un-peeled leaf discs were incubated in the medium as described in section 2.4.2 at the optimal pH, determined in section 2.4.5. Five petri-dishes each containing 7-10 leaf discs were prepared and incubated with 1 mM ^{14}C-sucrose at room temperature at intervals of 0, 2, 4, 6 and 8 hrs. The time course of sucrose uptake of 6 mm and 12 mm in diameter leaf discs were compared.

2.4.7 The kinetic of sucrose uptake in cassava leaf discs

The kinetic characteristics of sucrose uptake was determined by incubation of ten peeled leaf discs 12 mm diameter in each petri-dish with varying sucrose concentrations (with constant amount of (U-C^{14}) sucrose or total dpm). The sucrose concentration used in the experiment was 0.1, 0.5, 1, 2, 5 and 10 mM. The mannitol concentration of the medium was decreased in the experiments which the concentration of sucrose exceeded 1 mM in order to keep the osmolarity in the experimental system constant. All the experimental dishes were incubated for 4 hrs, under illuminated condition as described in section 2.4.2 and the radioactivity measured as described in section 2.4.3.

2.4.8 Effect of saccharides on sucrose uptake of cassava leaf discs

Effect of other sugars on the sucrose uptake were performed by adding the sugar to be tested in the incubation medium together with sucrose and incubated as performed in section 2.4.7. The concentration of the tested sugars used was varied, while ^{14}C -sucrose concentration was fixed at 1 mM. When the total concentration of sugar and sucrose in the incubation medium exceeded 1 mM, the mannitol concentration was decreased in order to keep the osmolarity constant. Seven leaf discs were incubated in each experiment, The sucrose uptake of cassava leaf discs incubated with 1 mM ^{14}C -sucrose in absence of test sugar was used as control set. The result was expressed as % relative activity to the control set.

2.4.9 The effect of sulfhydryl modifying reagents on sucrose uptake in cassava

Three sulfhydryl modifying reagents, N-ethylmaleimide (NEM), p-chloromercuribenzensulfonic acid (PCMBS) and iodoacetic acid (IAA) were tested for their effect on sucrose uptake of cassava leaf discs. The leaf discs were incubated in the sucrose uptake medium in the presence of these reagents in separate petri-dish. Ten leaf discs were incubated at each concentration of the reagent which was added at the start of incubation time. The experiments were performed as described in section 2.4.2 and the radioactivity was measured as described in section 2.4.3.

The effect of these agents on sucrose uptake of the root discs was also studied. The experiment was performed as above replacing the leaf discs with prepared as described section 2.4.1.2.

2.4.10 The effect of metabolic inhibitors on the sucrose uptake of cassava tissue

The well known metabolic inhibitors such as vanadate, KCN and erythrosin B were tested for their effects on sucrose uptake of cassava in leaves tissue. The experiment was performed by incubation of 7-10 leaf discs in 1 mM ^{14}C –sucrose in the presence of one of these reagents at the added start of incubation time, under the condition that described in section 2.4.2. V_2O_5 was prepared in 0.1 N NaOH as concentrate stock solution, then adjusted to pH 8 with HCl then diluted to working concentration with 20 mM MES / NaOH pH 5.0.

The effect of uncoupler or protonophore, chlorocarbonyl cyanide phenylhydrazone (CCCP) and p-dinitrophenol (DNP) on sucrose uptake was tested. They were prepared in 95% ethanol at concentration 20 mM , then diluted to 4 , 2 and 1 mM with 50% ethanol as second stock solution. They were further diluted with 20 mM MES / NaOH pH 5.0 to the working concentration (in μM). To correct the solvent effect, a control experiment containing the same amount of ethanol was included.

2.4.11 The effect of cyanogenic glycosides on sucrose uptake in cassava

Cyanogenic glycosides such as linamarin, lotaustralin, amygdalin and prunasin were tested for their effect on sucrose uptake in cassava leaf discs. Seven to ten leaf discs were incubated in 1mM ^{14}C –sucrose in the presence of these glycosides as described in section 2.4.10 and the sucrose uptake was measured as described in section 2.4.2. The effect of bioflavonoid glycoside, phoridzin was also tested.

All of the glycosides were dissolved in distilled water as stock solution and diluted to working concentration with 20 mM MES / NaOH pH 5.0.

2.5 Purification of sucrose binding protein

Sucrose binding protein from cassava leaves was isolated according to the following method :

2.5.1 Preparation of microsomal fraction

Mature cassava leaves (250 g.) were homogenized four times for 30 seconds each with a Braun homogenizer in 450 ml of 50 mM MOP/NaOH pH 7.5 contain 0.33 M sucrose, 5 mM EDTA, 5 mM DTT, 5 mM ascorbic acid, 0.5 mM PMSF, 0.2 % casein (enzymatic hydrolyzate boiled for 10 min) and 0.2 % BSA (protease free) and 0.6 % PVPP (34 and 35). The homogenate was filtered through 240 μ nylon net , the supernate was centrifuged at 10,000xg for 10 min. The supernatant was further centrifuged at 50,000xg for 30 min. The pellet or microsomal fraction was collected and suspend in 10 ml. of buffer A (5 mM potassium phosphate buffer pH 7.8 containing 0.33 sucrose , 5 mM KCl , 1 mM DTT and 0.1 mM EDTA). This microsomal fraction was used for further isolation of plasma membrane.

2.5.2 Isolation of plasma membrane by aqueous two phase system

The two phase system requires two water soluble polymers that will be dissolved in water without phase separation at moderate concentration. In this study, Dextran T500 (Dx: predominantly poly (α-1,6,-glucose) and polyethylene

glycol (PEG-3350) were used.

Phase system was made up by weighing from stock solution of 20% (w/w) Dextran T 500 and 40% (w/w) PEG 3350 and concentrated salt solution (Table2.1). The final weight of 4 g. or 36 g. phase system contained 6.5% (w/w) each of Dextran T 500 and PEG 3350 was usually yielded about 90% of plasma membranes partitioned to upper phase of PEG (36).

The stock solution was added in decreasing order of their densities : Dx, PEG, salt and water respectively. The 36 g. final weight of phase system was used in the isolation of cassava leaves plasma membrane.

The microsomal fraction was added to the preweighed phase mixture then mixed and left to settle (Figure 2.4). The PEG upper phase containing plasma membrane fraction was collected and diluted at least 3-fold with buffer A and centrifuged at 50,000xg for 1 hr to collect the plasma membrane. The membrane was suspended in small volume of buffer. The fraction was assayed for ATPase which was marker of plasma membrane described by Thom *et al.* (37) as the phosphate release (38) or ADP formation by HPLC as described by Hirunyapaisarnsakul (27).

Table 2.1 Phase mixture and phase systems used in plasma membrane purification

Component	Weight of phase mixtures	
	3.0 g	27.00 g
20% (w/w) Dextran T500[a]	1.10-1.30 g	11.16
40% (w/w) Poly(ethylene glycol) 3350[a]	0.55-0.65 g	5.58 g
Sucrose (solid)	0.339 g	3.05 g
0.2 M Potassium phosphate, pH 7.8	0.075 ml	0.675 ml
2 M KCl	0-0.0075 ml	0.068 ml
0.5 M DTT in 50 mM EDTA[b]	0.008 ml	0.072 ml
Add water to a final weight of	3.00 g	27.00 g
Sample	1.0 ml	9.0 ml
Final weight of phase system	4.0 g	36.0 g

[a] Preparation of polymer stock solutions, see 32.
[b]DTT was added fresh from a concentrated stock solution in EDTA, pH=7.

Modified from : (36)

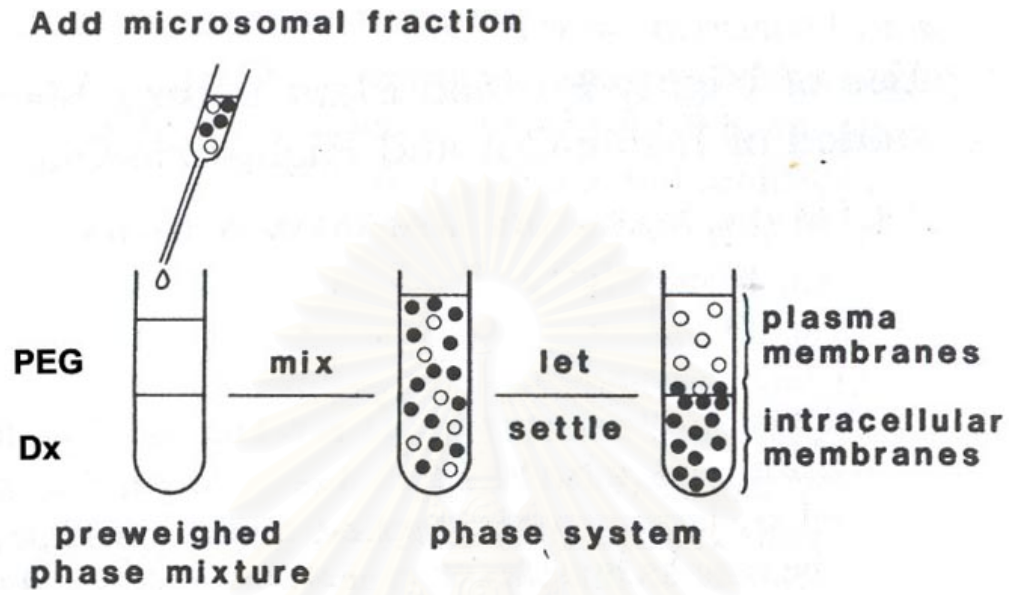


Figure 2.4 Schematic of plasma membrane isolation in aqueous two-phase system

From : (36)

2.5.2.1 Assay of ATPase

ATPase activity was assayed by monitoring its hydrolytic reaction of ATP which released phosphate and ADP was formed according to Ames (38) and Hiranyapaisanrsakul (26). The reaction mixture of 240 l containing 40 g plasma membrane preparation, 0.33 M sucrose, 3 mM ATP, 4 mM $MgSO_4$, 50 mM KNO_3 in 30 mM HEPES pH 7.5 was incubated for 30-60 min at 37 °C in the presence or absence of 0.1 mM vanadate.

The ATPase activity was monitored as ADP formation. The reaction mixture was heated for 10 min to stop the reaction, then centrifuged at 10,000 rpm for 30 min. The supernatant was monitored for ADP formation by HPLC systems with Spereclone-SAX column; (10 , 250x4.6 mm : Phenomenex, USA.) Mobile phases were (A) 10 mM ammonium phosphate, pH 3.0 and (B) 450 mM ammonium phosphate, pH 4.5. The sample was injected and run at the flow rate of 1 ml per minute. After injection, the gradient of mobile phase was established as followed : isocratic 0% B for 2 min and linear increase to 100% B for 14 min, isocratic 100 % for 2 min The detector was set at 254 nm. Under this condition ATP and ADP were eluted at 8.9 and 6.7 min respectively.

2.5.3 Sucrose affinity resin preparation

Sucrose affinity column was prepared of purification of sucrose binding protein by ligand immobilized on epoxy-activated Sepharose 4B according to the following procedure (39). Epoxy-activated Sepharose 6B were swollen in deionized water for 1 hr after which it was washed extensively with deionized water following by 400 ml of 0.1 M NaOH. From 2 gm of epoxy-activated bead,

6 ml of wet gel was obtained. The bead was incubated in 50 ml of 50 mM Sucrose in 0.1 M was filtered on sinter glass funnel to remove uncoupled sucrose. To stop the cross-linking reaction, the coupled gel was washed and incubated in 1 M ethanolamine for 4 hrs at room temperature. The coupled gel was further washed in 200 ml of 0.1 M borate buffer pH 8 containing 0.5 M NaCl, followed by 200 ml of 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl. The coupled gel was stored in 50 mM Tris-HCl buffer pH 7.5 containing 0.1 M sucrose and 0.01 % sodium azide at 4°C until further use.

2.5.4. Membrane solubilization and chromatography procedures

Plasma membrane from section 2.5.2 was solubilized with 1% CHAPS by dropwise addition from 10% stock solution, then the solution was acidified with NaH_2PO_4 pH 5.2 and incubated on ice bath with continuous stirred for 45 min. The solution was centrifuged at 50,000xg for 1 hr. The supernatant containing solubilized membrane fraction was subjected to chromatography on the sucrose affinity column. The column was equilibrated with the starting buffer(50 mM sodium phosphate buffer pH 5.2 containing 0.1% CHAPS, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$). The unbound protein was eluted with the starting buffer at the flow rate of 0.2 ml/min. The bound protein was eluted with the starting buffer containing 0.1 M sucrose at the flow rate 0.5 ml/min. The column was finally washed to remove other proteins remained. The fractions were collected with time mode at 5 min/fraction. The bound fractions were pooled, dialyzed against 50 mM phosphate buffer pH 7.5 containing 0.1% CHAPS using a membrane with cut-off size 5,000, then lyophilized and kept frozen at –20 °C for

further used or suspended in 20 mM potassium phosphate buffer pH 7.5 for further analysis.

2.6 Sucrose uptake of native plasma membrane vesicle

Plasma membrane vesicle purified from section 2.5.2 was used to study sucrose uptake. Preliminary uptake experiment was performed with 32 μg protein of plasma membrane in the reaction mixture containing 0.3 M sorbital, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM DTT in 50 mM potassium phosphate buffer pH 7.8 in the presence of ^{14}C-sucrose at 37 °C at varying times. The reaction was terminated by adding 1.75 ml of chilled rinsing buffer containing 0.3 M sorbital, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM DTT in 50 mM potassium phosphate buffer pH 7.5 and 5 mM $HgCl_2$ with vigorous mixing. The mixture was rapidly filtered on Nitrocellulose Millipore filter (with 0.45 μ pore size, 25 mm diameter) pre-wetted with rinsing buffer. The filter was further rinsed with 1.5 ml of rinsing buffer, then removed from the filter unit and placed in scintillation vial. After drying in an oven at 50 °C for 1 hr, 5 ml of scintillation fluid was added and measured for the radioactivity (40).

A control experiment was run for determination of non-specific binding of radioactivity and plasma membrane vesicle and retained on the filter. The rinsing buffer was added to the reaction mixture in the presence of ^{14}C-sucrose which incubated on ice then 32 μg protein was added and immediately filtering and processed as mention above, this reaction tube designated as zero time or blank.

2.7 Determination of protein concentration

Protein concentration was determined by the method of Bradford (41) using bovine serum albumin as standard (see Appendix B). The 100 μl of sample was mixed with 1 ml of coomassie blue reagent and left for 5 minutes before measured absorbance at 595nm. The 1 liter of coomassie blue reagent was the mixture of 100 mg coomassie blue G 250, 50ml ethanol, 100 ml 85% phosphoric acid, and 850 ml distilled water.

2.8 SDS-polyacrylamide gel electrophoresis

The SDS-polyacrylamide gel electrophoresis system was performed according to the modified method described by Laemmli (42). The slab gel system consisted of a stacking gel (10 x 2 x 0.1cm) of 3% (w/v) acrylamide and a separating gel (10 x 8.6 x 0.1cm) of 10% (w/v) acrylamide. The gel preparation was described in Appendix C. The SDS-polyacrylamide gel electrophoresis was performed at a constant current of 20 mA. The standard molecular weight markers used were phosphorylase b (MW = 94 kDa), bovine serum albumin (MW = 67 kDa), ovalbumin (MW = 43 kDa), carbonic anhydrase (MW = 30 kDa), soybean trypsin inhibitor (MW = 20.1 kDa) and α-lactalbumin (MW = 14.4 kDa). Following electrophoresis, the gel was stained for protein to determine molecular weight of sucrose binding protein as described in section 2.8.1.

2.8.1 Determination of protein pattern on polyacrylamide gel by silver stain

The gel from electrophoresis methods was fixed in 50% methanol for 10

minutes and then washed with excess ultrapure water and stained in staining solution containing 0.8%, W/V, silver nitrate in 12.5 mM NaOH and 0.28% ammonium hydroxide at room temperature with gentle shake. After that gel was washed twice with ultrapure water. Protein pattern was obtained by soaking the gel in developing solution (0.2% citric acid and 0.02% formaldehyde for 2-4 minutes, or until the protein band began to develop. Remove the gel from the developer and stored in 7% acetic acid and 5% methanol (43).

CHAPTER III

RESULTS

3.1 Sucrose uptake in cassava leaf discs

Study of solute uptake in plant using leaf discs has been reported (44). In this study, cassava leaf disc was used to follow sucrose transport.

3.1.1 Preliminary screening of suitable preparation of leaf discs

Sucrose uptake of cassava leaf discs with different surface area (6 mm and 12 mm in diameter) was compared in order to select a suitable size for further study. The leaf discs with different diameter were separately incubated in the same concentration of ^{14}C- sucrose. The result showed that the uptake of sucrose in 12 mm diameter disc was greater than 6mm diameter disc. At 8 hrs incubation , the sucrose uptake by 12 mm diameter disc was 0.3% of total sucrose in the medium and only 0.07% by the 6 mm diameter leaf disc (Table 3.1). The radioactivity present in the washing medium was also determined. In the 3rd wash, the radioactivity was found at the same level as in the blank (43-60 dpm) indicating that there was no leakage of sucrose uptake during washing of the free space labeled.

Since the uptake of sucrose by normal leaf disc as rather low, the cuticle of the cassava leaves were peeled off and used to test whether the uptake of sucrose was enhanced. The cuticle peeled leaf discs were used in the sucrose uptake experiment in comparison with the un-peeled leaf discs. The peeled leaf discs were prepared as described in section 2.4.1.1 and sucrose uptake was

performed as described in sections 2.4.2 and 2.4.3. The result in Table 3.2 showed that the sucrose uptake in the peeled leaf discs were 3 times higher than the un-peeled leaf discs and the uptake by 12 mm diameter discs were higher than 6 mm diameter discs. Therefore, 12 mm peeled leaf discs were used in subsequent experiments.

3.1.2 The autoradiography of cassava leaf discs on sucrose uptake

In order to observe the path of C^{14} –sucrose uptake by cassava leaf disc, the autoradiographic technique was performed as described in section 2.4.2 and the result was shown in Figure 3.1. The radioactivity was accumulated in the small veins of the leaf disc and appeared on the autoradiograph picture as white track because the picture was recorded as negative print. The result indicated that sucrose was systematically transported into leaf cells not by random diffusion.

3.1.3 pH optimum of sucrose uptake

Sucrose uptake was performed at various pH as described in section 2.4.5. The results were shown in Figure 3.2. The uptake was highest at pH 5 and decreased to 90% at pH 6. Subsequent experiments were performed at pH 5.

Table 3.1 The time course of sucrose uptake into cassava leaf discs with different in surface area.

Diameter of leaf discs (mm)	Sucrose uptake in dpm/disc. Incubation time (hr.)						
	0	2	4	5	6	8	9
6 mm	40±1	121±18 (9)	ND	466±155 (7)	437±155 (6)	747±84 (6)	908±247 (7)
12 mm	67±2	233±82 (7)	1010±200 (7)	ND	2050±250 (6)	3213±1930 (9)	ND

No. in parenthesis indicated the number of leaf discs used.

ND. = Not determined.

Sucrose uptake is defined as the amount of ^{14}C-sucrose incorporated into each leaf discs.

1 mM (U-^{14}C) sucrose = 963,750. dpm

The values are expressed as dpm of mean mean ± SD of each disc. Data obtained from two experiments.

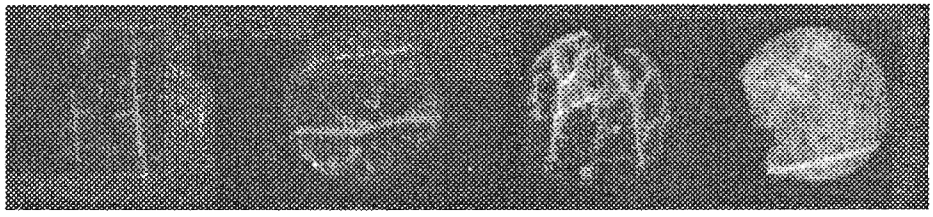
Table 3.2 The comparison of sucrose uptake in leaf disc of peeled and un-peeled leaf, (at 6 hrs. of incubation).

Leaf discs		Sucrose uptake in dpm/disc
Diameter (mm)	Type	
6 mm	Un-peeled leaf	167±124 (n=10)
6 mm	Peeled leaf	2197±705 (n=17)
12 mm	Peeled leaf	12,400±200 (n=6)

No. in parenthesis indicated the number of leaf discs used.

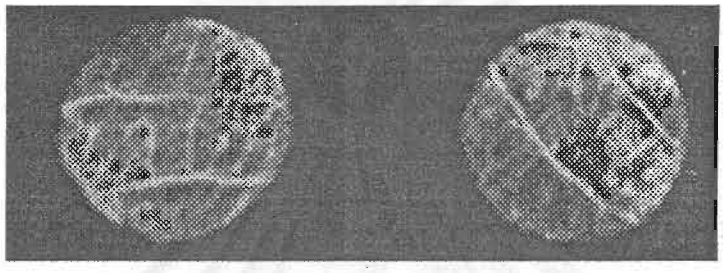
The values are expressed as dpm of mean ± SD of each disc.

Data obtained from two experiments.



2 hrs 4 hrs 6 hrs 8 hrs

A



B

Figure 3.1 Autoradiograph of ^{14}C- sucrose uptake into cassava leaf discs.

A = showed path of ^{14}C-sucrose uptake of cassava leaf discs at 2, 4, 6 and 8 hrs of incubation in 1 mM ^{14}C-sucrose.

B = showed path of ^{14}C-sucrose uptake of cassava leaf discs at 8 hrs of incubation in 1 mM ^{14}C-sucrose in different in image size.

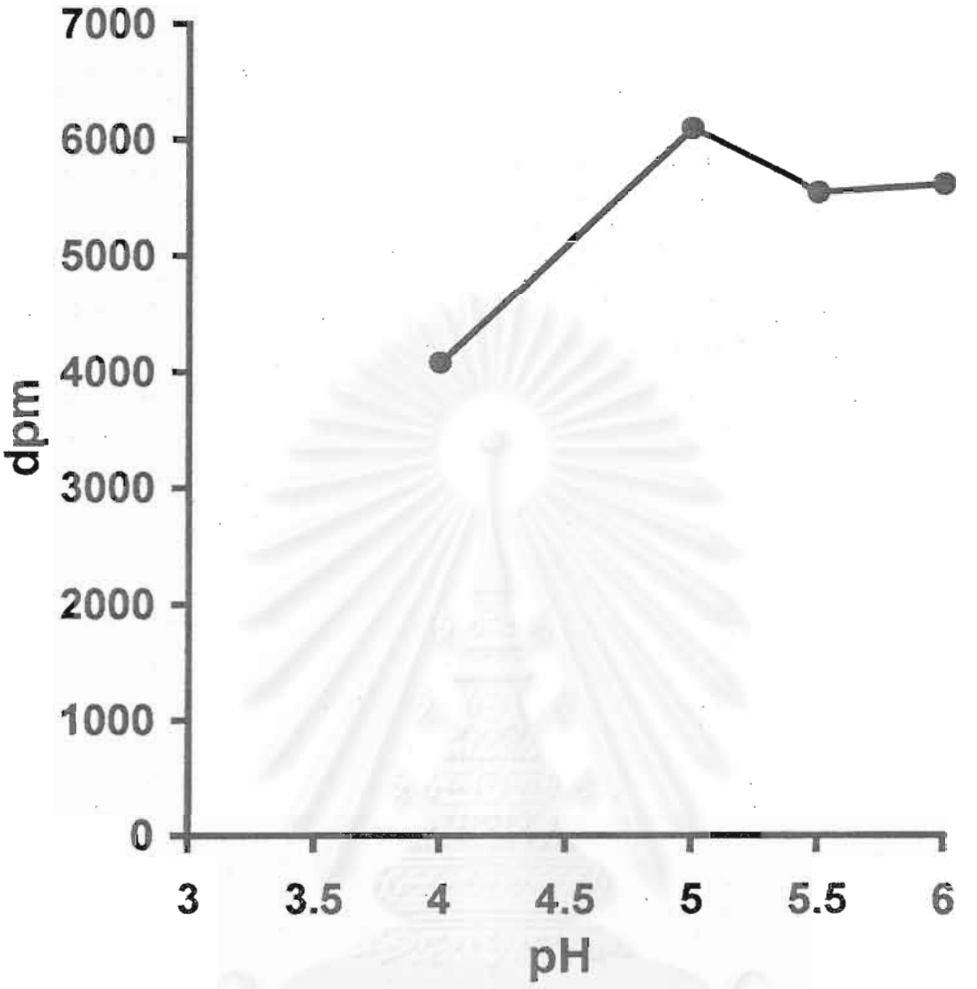


Figure 3.2 The pH optimum of sucrose uptake by cassava leaf discs (12mm in diameter).

The experiment was performed at various pH in MES/NaOH buffer.

The values are expressed as dpm and mean ± SD of 10 leaf discs and were calculated each experiments and an average from two experiments are shown.

3.1.4 The time course of sucrose uptake in cassava leaf discs

To determine the time course of sucrose uptake, sucrose uptake of the leaf discs were followed at various incubation times up to 8 hrs, under 2,000 luxs of light intensity. The result showed that the sucrose uptake of un-peeled leaf discs, both 6 mm and 12 mm sizes had a lag time of uptake during the first 2 hrs of incubation after which linearity was attained as shown in Figure 3.3.

For peeled leaf discs of both sizes, sucrose uptake was linear with time. However, in 12 mm diameter disc, the rate of uptake by the leaf discs appeared to accelerate after 4 hrs (Figure 3.4).

As the12 mm diameter leaf discs showed uptake of sucrose at about 3 time greater than the 6 mm diameter leaf discs, they were used in subsequent experiments. To keep the reaction in the linear range of initial velocity, the incubation time was fixed at 4 hrs.

3.1.5 The kinetics of sucrose uptake in cassava leaf discs

For determination of kinetic constants, experiments were performed as described in section 2.2.7. The results were shown in Figure 3.5A. Sucrose uptake by the leaf discs increased with increasing sucrose concentrations approaching maximal rate at the concentration of 5 mM to 10 mM sucrose. The sucrose saturation curve of the uptake appeared to follow the Michaelis-Menten kinetic (Figure 3.5A), indicating that the uptake of sucrose was carrier-mediated process. The double reciprocal plot of the saturation curve showed the apparent K_m of 1.31 mM and V_{max} of 4.18 nmole/hr/disc. The surface area of 12 mm diameter leaf disc was 1.13 cm^2 so the V_{max} is 3.699 nmole /hr/cm^2 (Figure 3.5B).

3.1.6 The effect of sugars on sucrose uptake of cassava leaf disc

Several sugars were tested for their effects on sucrose uptake in cassava leaf discs. The sugars used included glucose and fructose (monosaccharides), lactose (disaccharides), and some sucrose analogs. It was found that at the concentration of 1 mM glucose, fructose, lactose or palatinose, did not affect sucrose uptake. At higher concentration of 10 mM these sugars still showed little or no effect on sucrose uptake (Table 3.3). Other sucrose analogs such as IPTG, α- and β- PNPG significantly affecting the sucrose uptake. Inhibitory effects of α- or β- PNPG were observed at concentration as low as 0.1 mM and maximum inhibition of about 60% and 90% were achieved at 0.5 mM for α- and β- PNPG respectively (Figure 3.6). The test with IPTG was performed only up to 0.5 mM because the medium turned yellowish at high concentration. At 0.5 mM , IPTG can inhibit sucrose uptake by 30% (Figure 3.7), suggesting that IPTG had less effect compared to α-PNPG and β- PNPG. β- PNPG had the most effect inhibitory among the compounds tested (Figures 3.6 and 3.7).

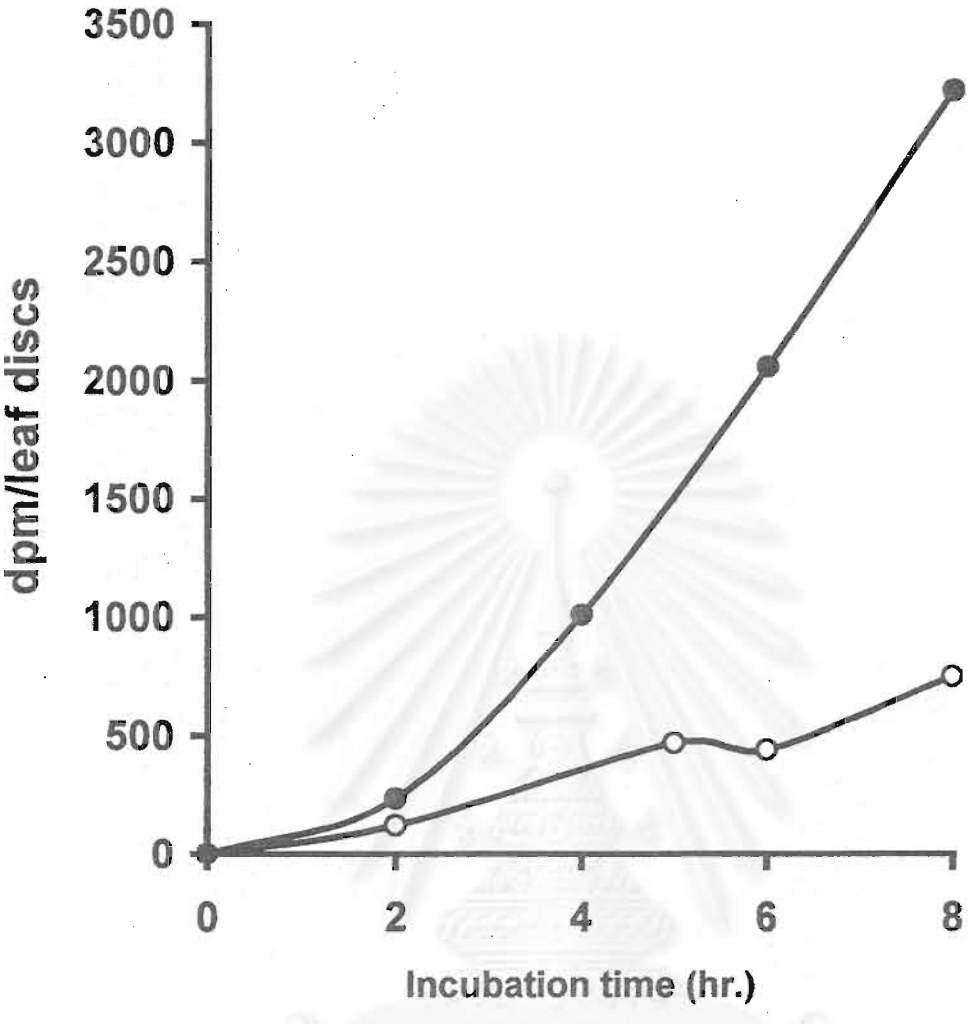


Figure 3.3 Time course of sucrose uptake in un-peeled cassava leaf discs.

o = uptake of 6 mm diameter leaf discs

● = uptake of 12 mm diameter leaf discs

The value are expressed as mean ± SD of 10 leaf discs.

Each value is an average of 2 experiments.

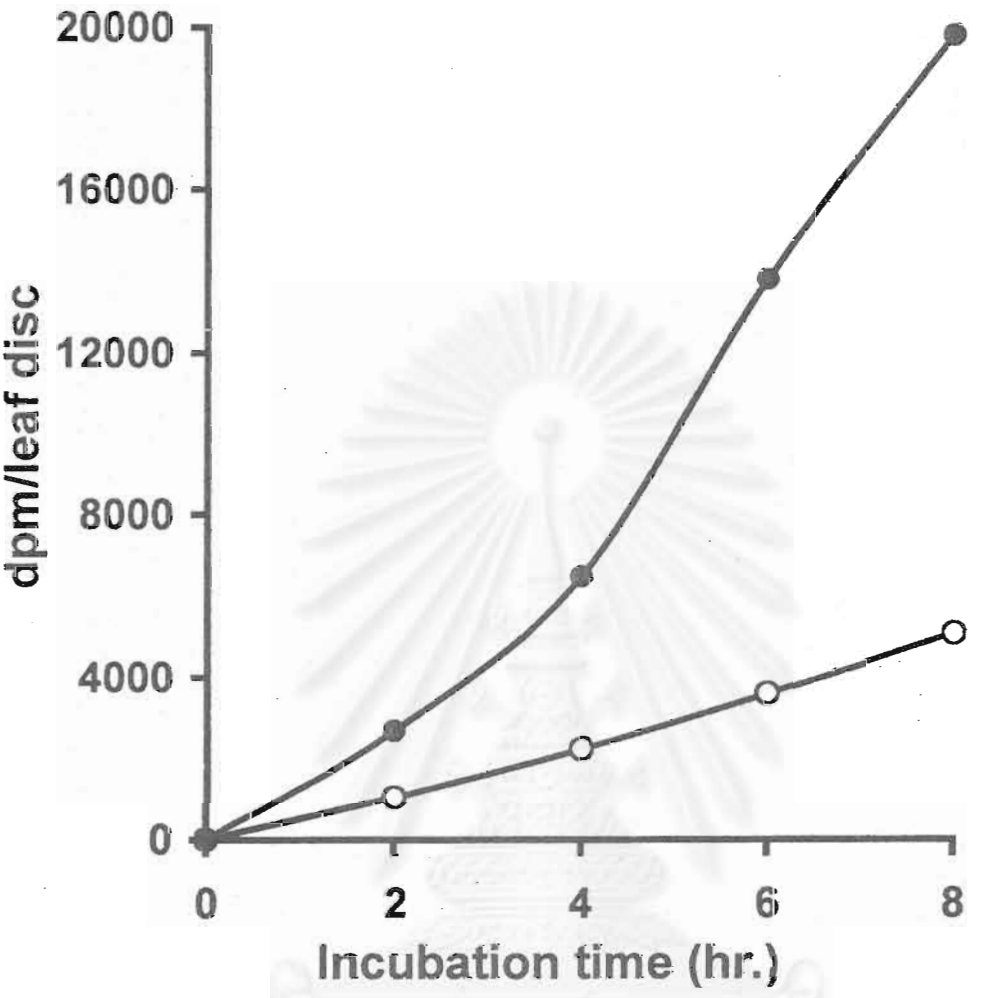


Figure 3.4 Time course of sucrose uptake of peeled cassava leaf discs.

o = sucrose uptake of 6 mm diameter leaf discs

● = sucrose uptake of 12 mm diameter leaf

The value expressed as mean ± SDof 7 leaf discs in each experiment.

Each value is an average of 2 experiments.

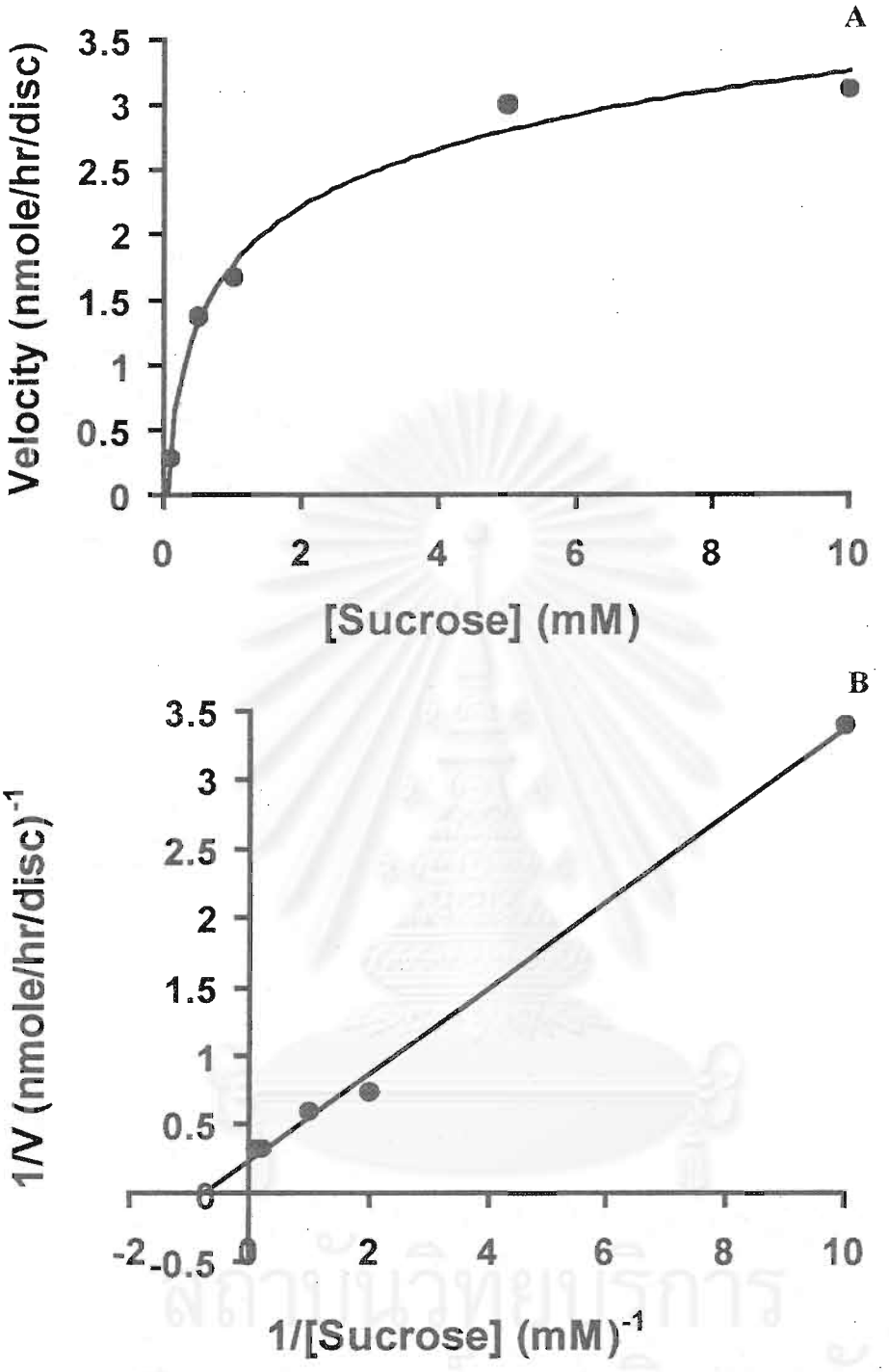


Figure 3.5 Kinetic study of ^{14}C-sucrose uptake by peeled cassava leaf discs

A = Substrate saturation curve

B = Lineweaver-Burk plot

Each experimental value is expressed as mean $\pm$ SD of 10 leaf discs.

Each value are an average of 4 experiments

Table 3.3 Effect of sugars on sucrose uptake by cassava leaf discs.

Sugar	^{14}C-sucrose uptake	
	Mean ± SD (dpm/disc)	% Relative
Sucrose 1 mM (control)	3620 ± 101	100
Glucose 1 mM	3885 ± 810	107
Glucose 10 mM	3338 ± 265	92
Fructose 1 mM	3005 ± 281	83
Fructose 10 mM	2583 ± 281	71
Lactose 1 mM	4021 ± 730	111
Lactose 10 mM	4321 ± 453	119
Palatinose 1 mM	4057 ± 442	162
Palatinose 10 mM	3926 ± 129	108

Each experimental value is expressed as mean ± SD of dpm of 7 leaf discs.

Each value is average of 2 experiments.

Control = sucrose uptake of leaf disc in 1 mM ^{14}C-sucrose without test sugar.

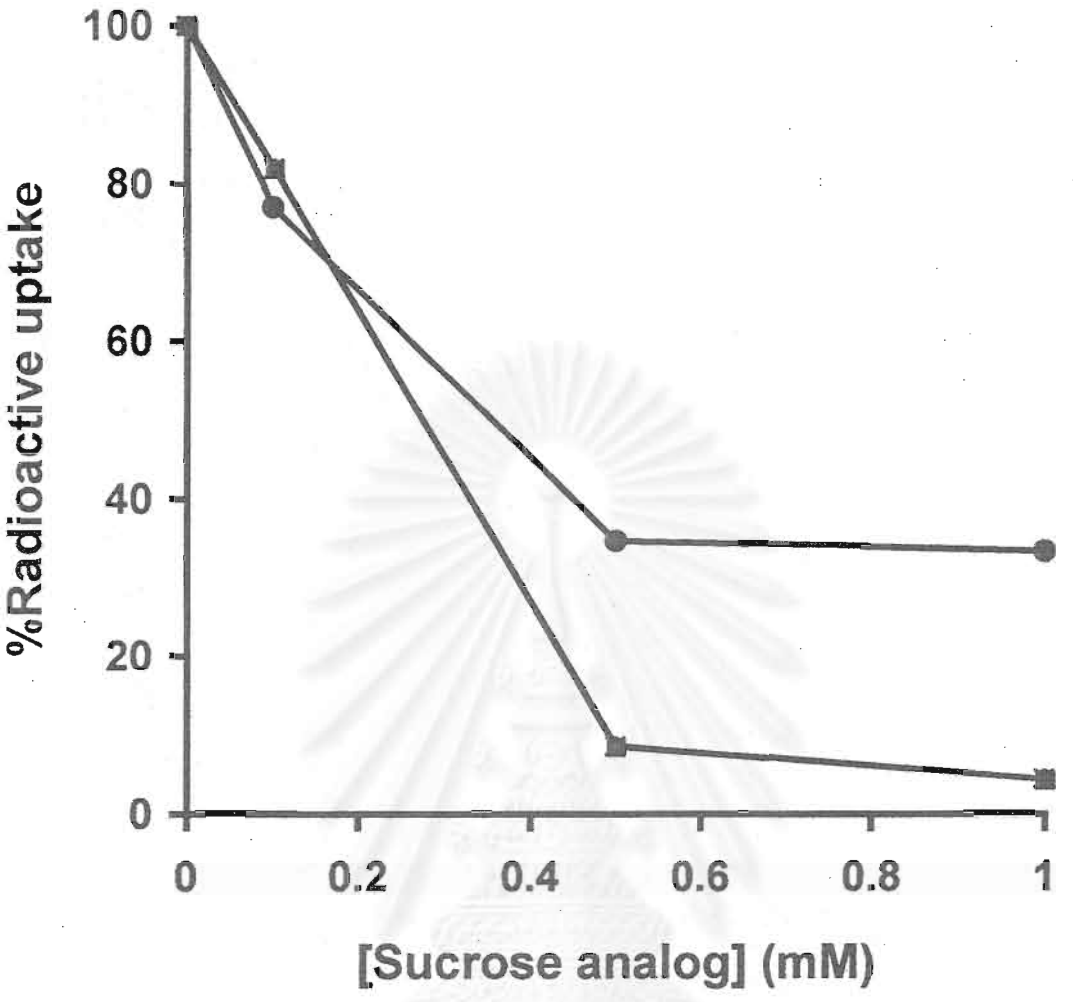


Figure 3.6 The inhibition curve of sucrose uptake of cassava leaf discs by

● = α-PNPG

■ = β-PNPG

Each experimental value is expressed as mean ± SD of dpm of 10 leaf discs.

Each value is average of 3 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 3240 dpm/disc

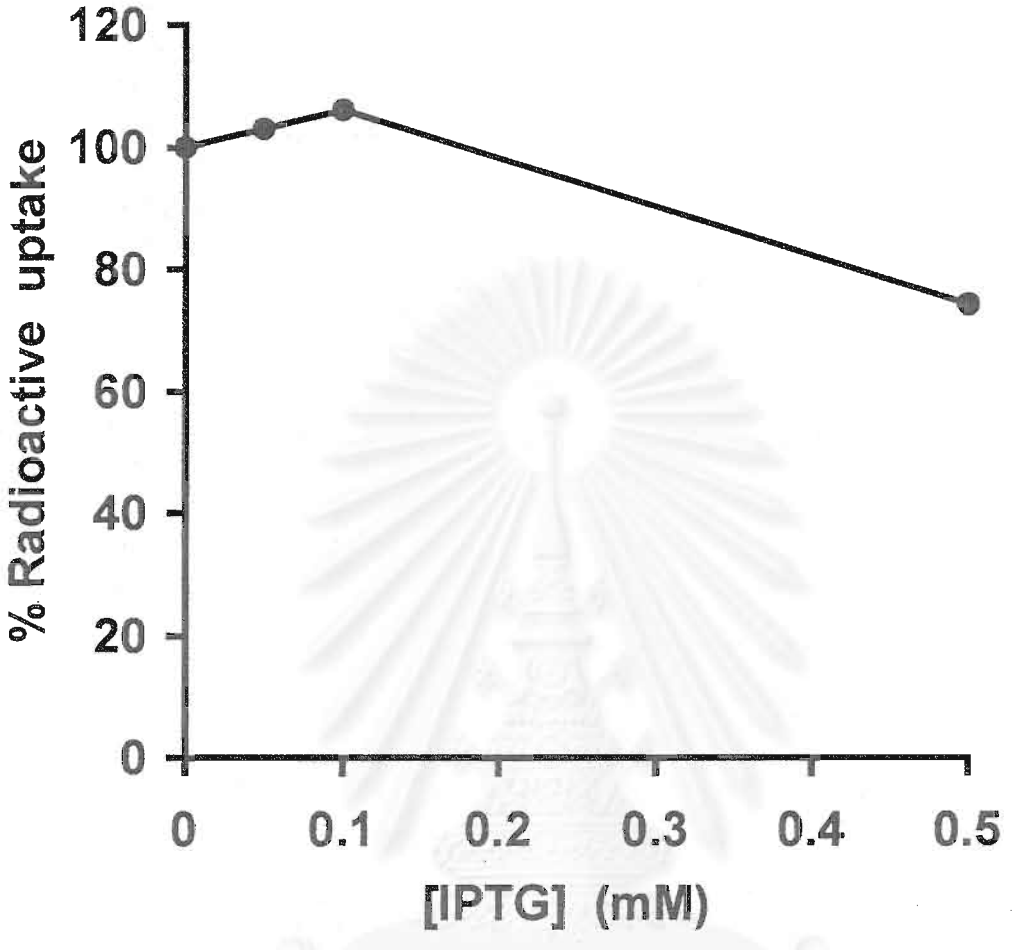


Figure 3.7 The inhibition curve of sucrose uptake of cassava leaf discs by IPTG (Isopropyl thio D-glucose).

Each experimental value is expressed as mean $\pm$SD of dpm of 10 leaf discs.

Each value is average of 3 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 3240 dpm/disc

3.1.7 The effect of sulfhydryl modifying reagents

Sucrose transport systems in many plants have been reported to involve sulfhydryl group and were sensitive to inhibition by sulfhydryl modifying reagents (44). Effect of such reagents on sucrose uptake of cassava leaf discs were also studied. NEM, PCMBS and IAA were each added to the sucrose uptake system of cassava leaf discs at the beginning of incubation. These reagents were found to inhibit the sucrose uptake (Figure 3.8). At the concentration of 0.5 mM, NEM gave nearly 100% inhibition while the same concentrations of PCMBS or IAA inhibited the uptake by about 70 % from the inhibition curves , PCMBS seemed to have a slightly stronger inhibitory effect than IAA.

3.1.8 The effect of metabolic inhibitors on the sucrose uptake cassava leaf discs

Some plant sucrose transporters were reported to function in association with ATPase or proton pump (45). To test whether sucrose uptake in cassava leaves also involved these systems, well known ATPase inhibitors were included in the test of sucrose uptake in cassava leaf. ATPase inhibitors such as vanadate and Erythrosin B and inhibitor of electron transport such as KCN were used in our study. The results in Figure 3.9 showed that sucrose uptake in leaf discs was inhibited by 60% at 0.05 mM vanadate while KCN and Erythrosin B gave only 20% inhibition at the concentration of 0.5 mM and 10 mM respectively.

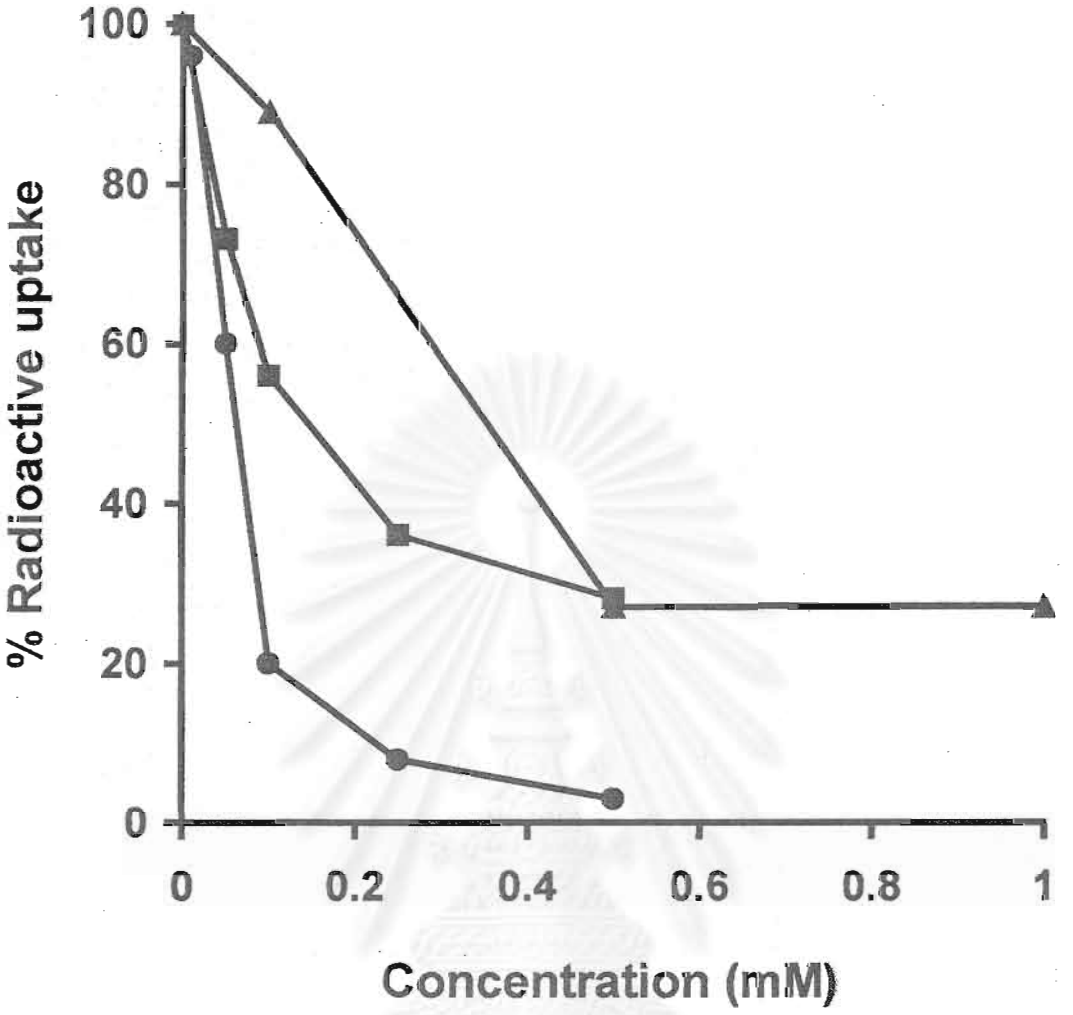


Figure 3.8 The inhibition curve of sucrose uptake of cassava leaf discs by NEM (●), PCMBS (■) and IAA (▲).

Each experimental value is expressed as mean ± SD of dpm of 7 leaf discs.

Each value is average of 3 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 4618 dpm/disc

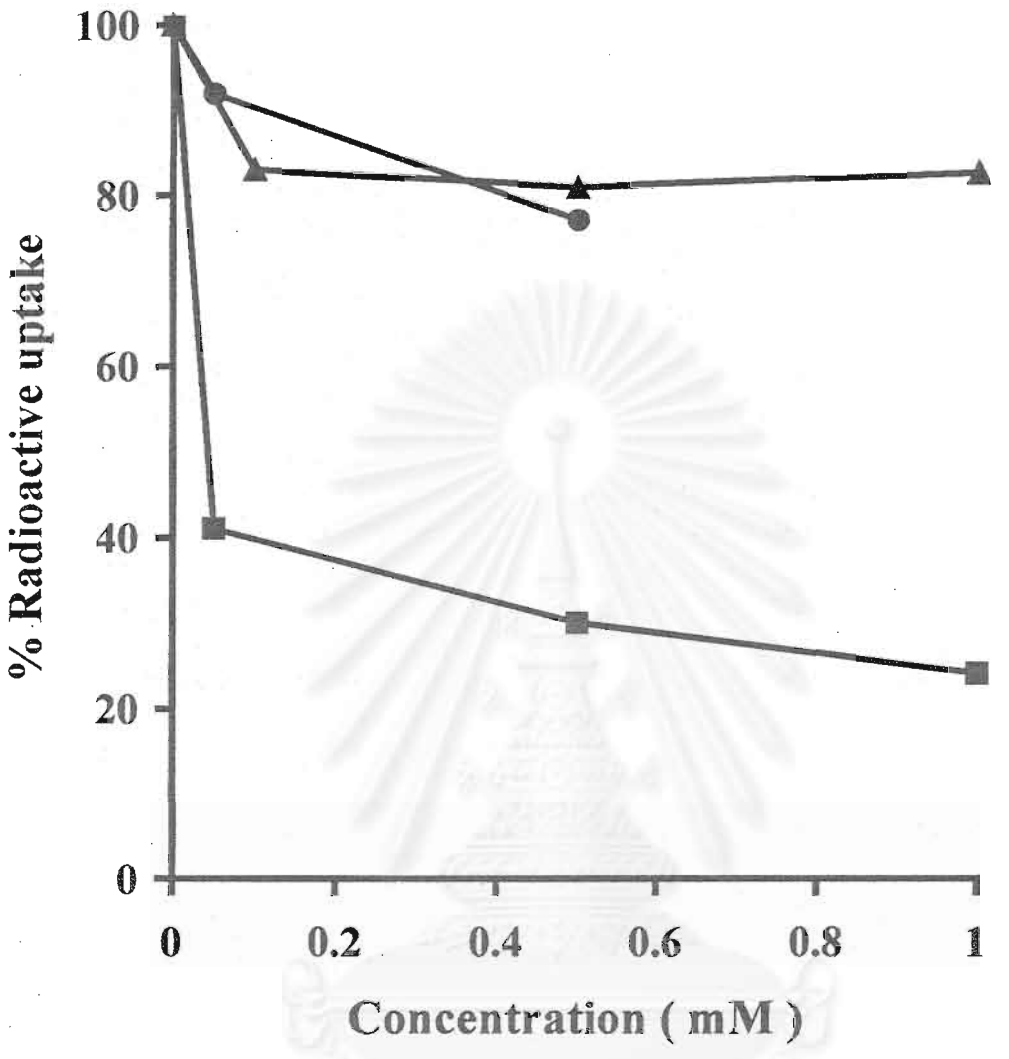


Figure 3.9 The inhibition curve of sucrose uptake of cassava leaf discs by KCN (●), V_2O_5 (■) and Erythrosin B (▲).

The concentration of Erythrosin B was multiplied by 10^{-1}. (10 folds higher)

Each experimental value is expressed as mean $\pm$SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 2951 dpm/disc

Uncoupler or protonophore such as CCCP and DNP are known to dissipate H^+ gradient and consequently reduce ATP synthesis which is coupled to respiratory chain. In our experiment (Figure 3.10) both CCCP and DNP were found to be strong inhibitors of the sucrose uptake in leaf discs. From the inhibition curves, CCCP showed slightly stronger inhibition than DNP.

3.1.9 The effect of glycosides on sucrose uptake in cassava leaf discs

Phloridzin is a plant bioflavonoid glycoside and known to inhibit Na^+-glucose transport (46). When it was tested in sucrose transport system of cassava leaf discs, phloridzin was able to inhibit sucrose uptake in cassava leaves (Figure 3.11) with 50% inhibition achieved at the concentration 0.5 mM.

Cassava is a cyanophoric plant which produces cyanogenic glucosides such as linamarin and lotaustralin. When cyanogenic glycosides were included in the sucrose uptake experiment of cassava leaf discs, it was found that linamarin showed stronger inhibition on the sucrose uptake than other cyanogenic glycoside such as prunasin, amygdalin and lotaustralin (Figure 3.12). Linamarin gave 50% inhibition at the concentration about 0.1 mM.

Autoradiography of the leaf discs incubated with ^{14}C-sucrose in presence and absence of 0.1 mM linamarin were also performed. The autoradiography shown in Figure 3.13 showed that in presence of linamarin, appearance of ^{14}C-sucrose in the leaf veins was reduced, supporting the result in Figure 3.12.

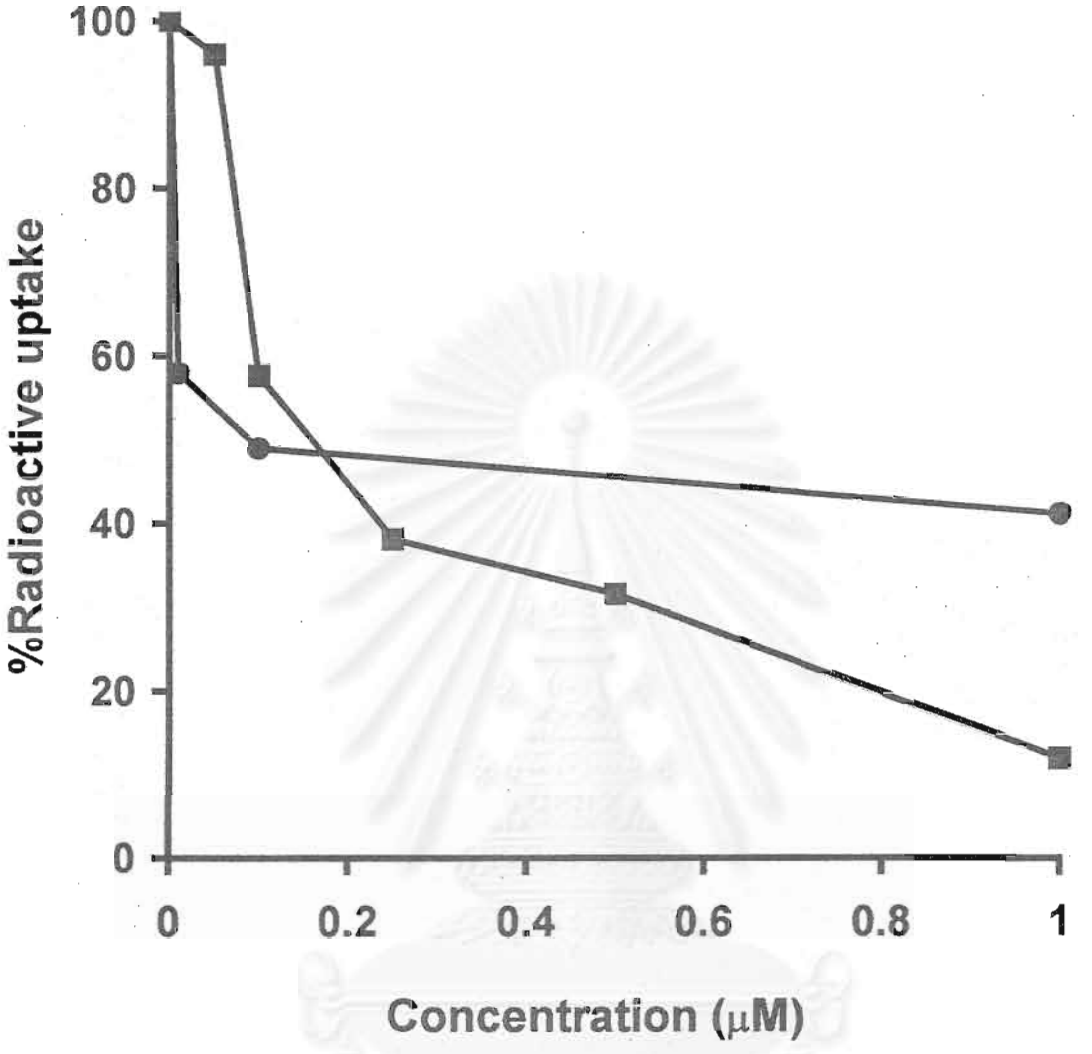


Figure 3.10 The inhibition curve of sucrose uptake of cassava leaf discs by DNP (●), CCCP (■).

Each experimental value is expressed as mean ± SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 3542 dpm/disc

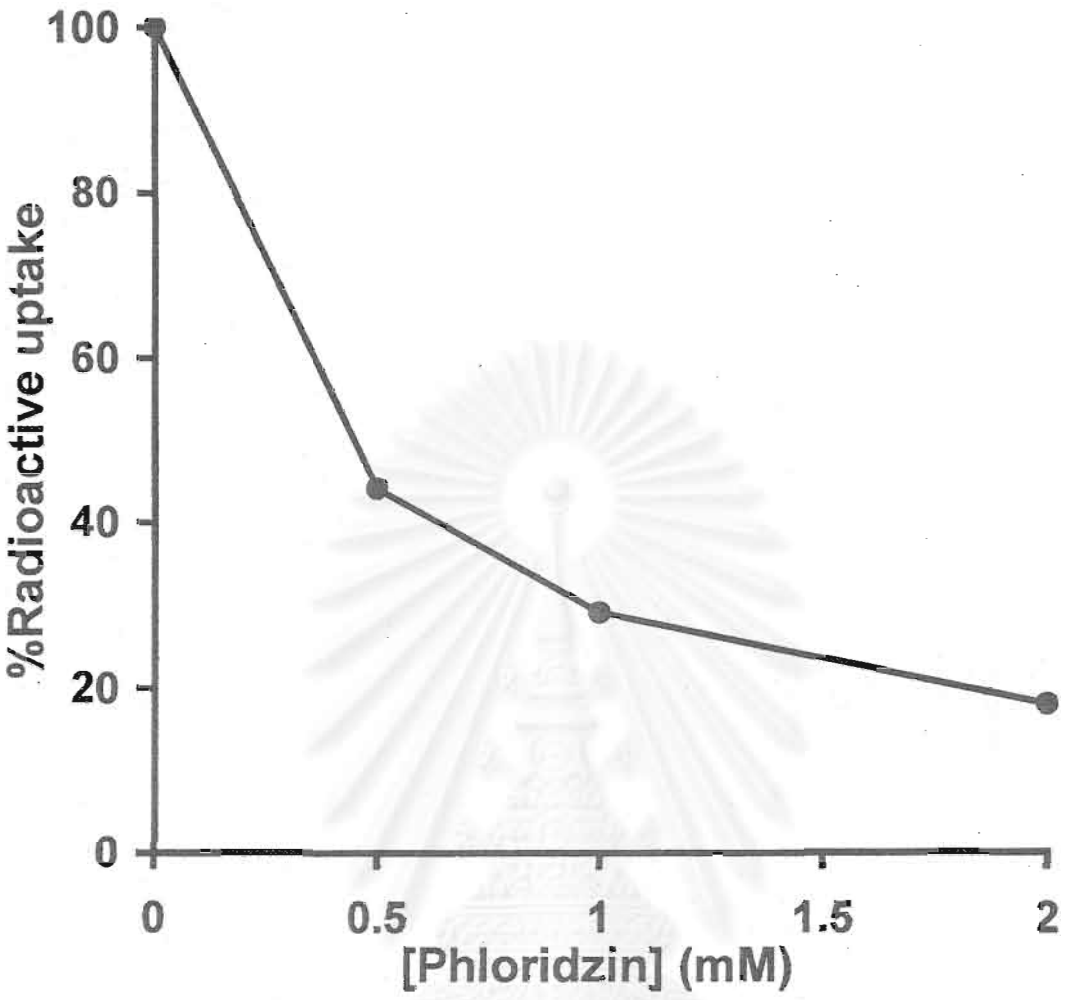


Figure 3.11 The inhibition curve of sucrose uptake of cassava leaf discs by Phloridzin.

Each experimental value is expressed as mean $\pm$SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 2450 dpm/disc

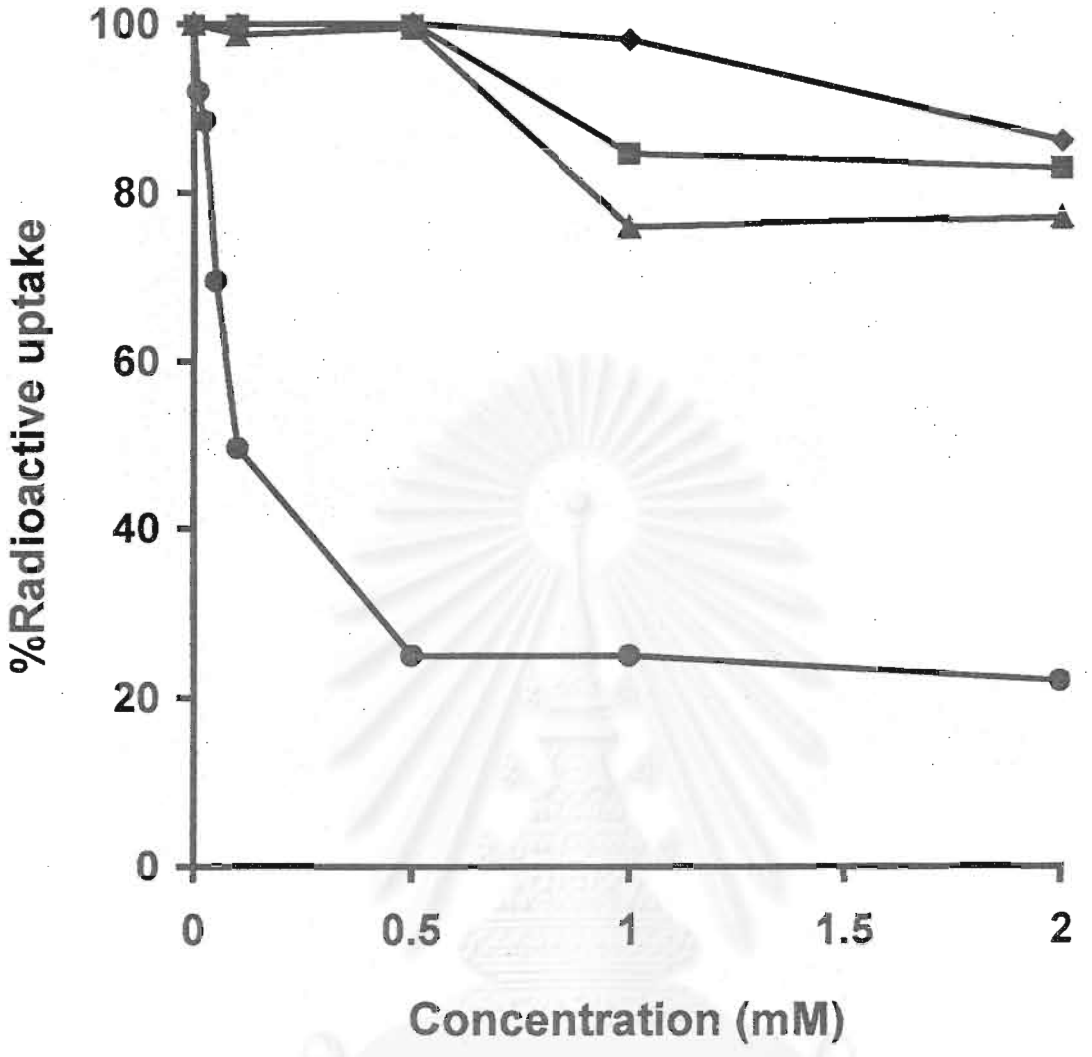


Figure 3.12 The inhibition curve of sucrose uptake of cassava leaf discs by Linamarin (●), Prunasin (■), Amygdalin (▲) and Lotaustralin (◆).

Each experimental value is expressed as mean ± SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 3500 dpm/disc

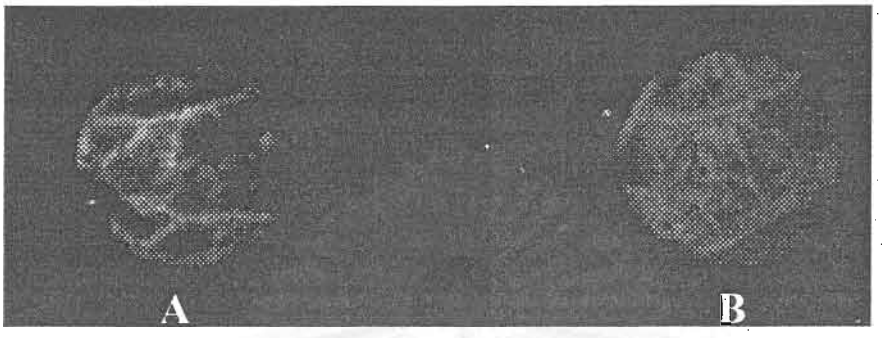


Figure 3.13 The autoradiograph of leaf discs in the absence and presence of linamarin after incubation for 4 hrs.

A = Leaf discs incubation in the absence of 0.1 mM linamarin

B = Leaf discs incubation in the presence of 0.1 mM linamarin

3.2 Sucrose uptake in root discs

With the results obtained in characterization of sucrose uptake in leaf discs, it was interesting to see if the same observations occurred in root which is a storage organ. Therefore, root discs were prepared and the interesting characteristics of sucrose uptake in cassava leaves and selected compounds which affected the leaf uptake were tested on root discs.

3.2.1 The time course of sucrose uptake in cassava root discs

The 12 mm. root discs were prepared as described in section 2.4.1.2. The root discs were incubated in the medium at optimum pH (pH 5) as described in section 2.4.5 and the time course of sucrose uptake was performed according to section 2.4.6. The results in (Figure 3.14) showed that the sucrose uptake in root was linear within 4 hr of incubation and gave a saturation characteristic. Therefore, the incubation time of subsequent experiments were fixed at 4 hrs. The washed root discs (section 2.4.1.2) was used in all experiment, since their uptake were 2 time higher than the unwashed root discs.

3.2.2 The kinetic of sucrose in root discs

The kinetic study of sucrose uptake in root discs was performed according to section 2.4.7, the concentrations of sucrose used in the experiments were 1,5,10,20,40 and 60 mM. The results in Figure 3.15A showed that sucrose uptake in root discs followed Michaelis-Menten kinetic in which the uptake increased with increasing sucrose concentration in the medium, approaching maximal rate

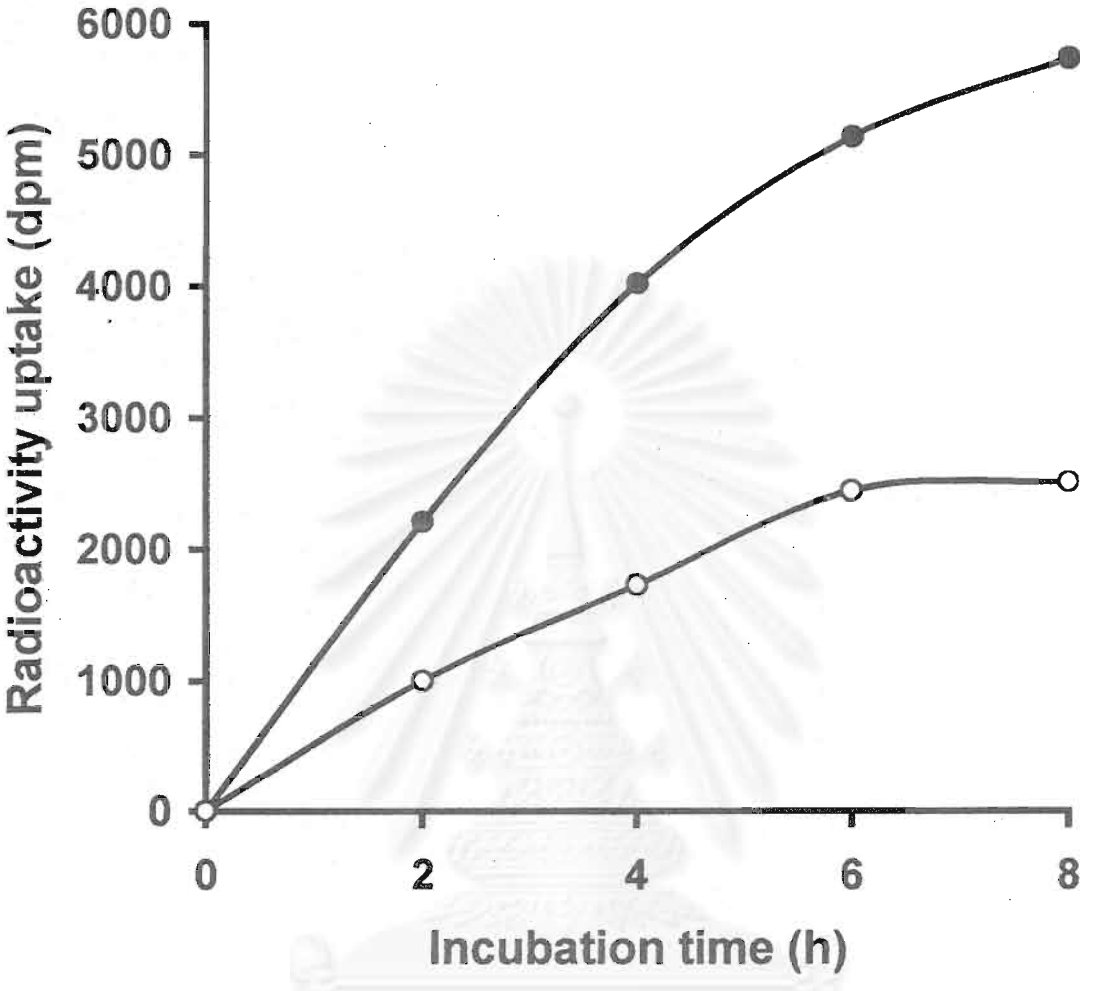


Figure 3.14 Time course of sucrose uptake by root discs.

○ = sucrose uptake of unwashed leaf discs

● = sucrose uptake of washed leaf discs

Each experimental value is expressed as mean ±SD of dpm of 10 leaf discs.

Each value is average of 3 experiments.

at concentration of 20 mM to 60 mM sucrose. The result indicated that the sucrose uptake process in root discs was also carrier mediated process. The double reciprocal plot of the saturation curve showed the apparent K_m value of 20 mM and V_{max} value of 0.018 nmole/hr/cm^2 (Figure 3.15B).

3.2.3 The effect of sulfhydryl modifying reagents

The effects of NEM, PCMBS and IAA were tested on sucrose uptake of root discs. Ten root discs were used in each experiment as described in section 2.4 and the result showed in Figure 3.16. At the concentration of 0.5 mM, PCMBS and NEM inhibited the sucrose uptake by 60% and 35% respectively, while IAA did not significantly inhibit sucrose uptake in root discs. Inhibition by PCMBS seemed to be maximum at 0.2 mM with 60% inhibition. The effect of these compounds in ^{14}C-sucrose root discs were different from the effect observed in leave disc.

3.2.4 The effect of ATPase inhibitor on sucrose uptake of root discs

Since V_2O_5 is one of metabolic inhibitor which inhibited sucrose uptake in leaf disc by 60% at 0.05 mM (Figure 3.9), its inhibitory effect was also tested in root discs. It was found that V_2O_5 at the concentration of 0.05 mM had no inhibitory effect on sucrose uptake of root discs (Figure 3.17) but caused 30% inhibition at the concentration 1 mM.

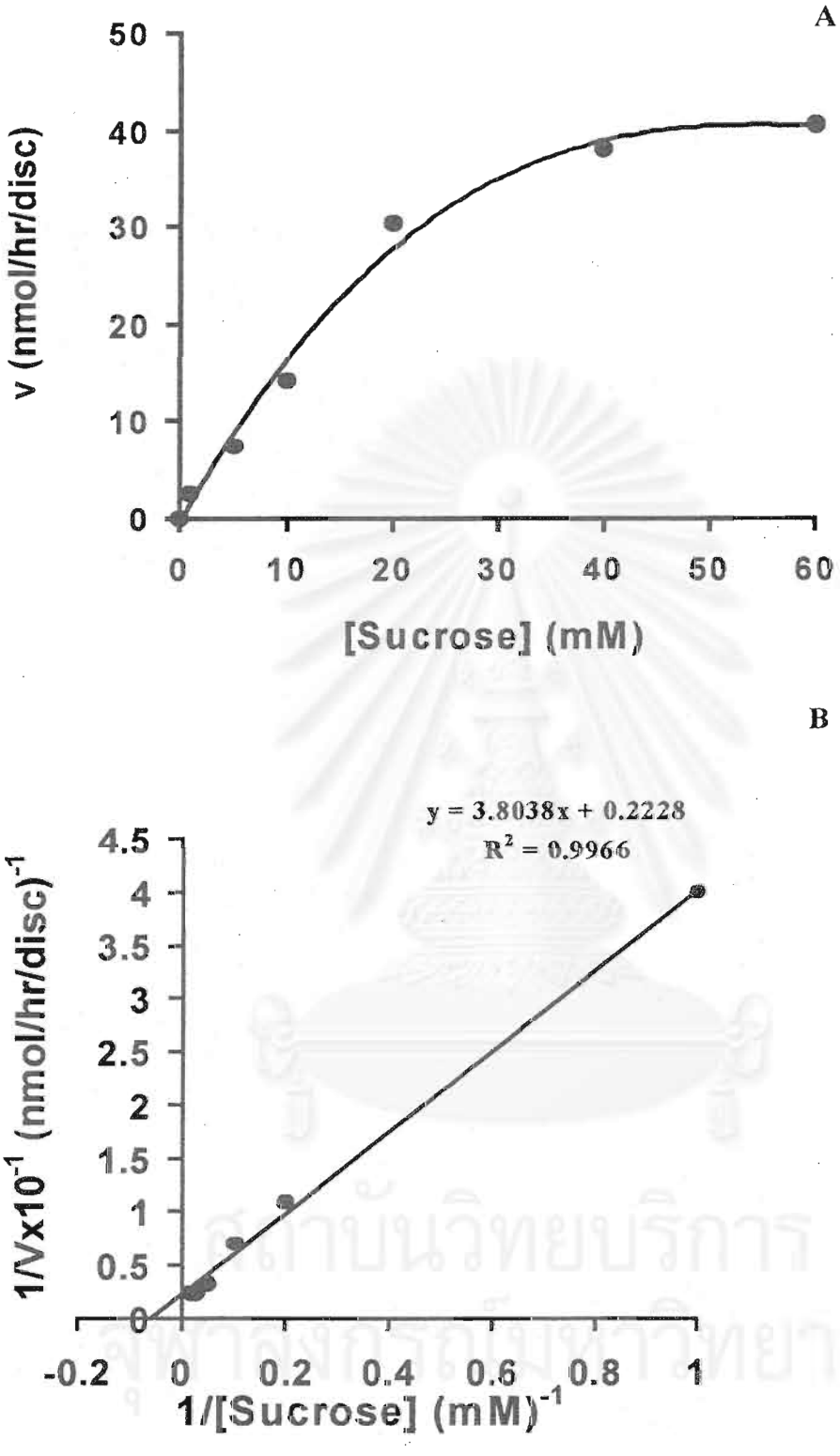


Figure 3.15 Kinetic study of ^{14}C-sucrose uptake by cassava root discs

A = Substrate saturation curve

B = Lineweaver-Burk plot

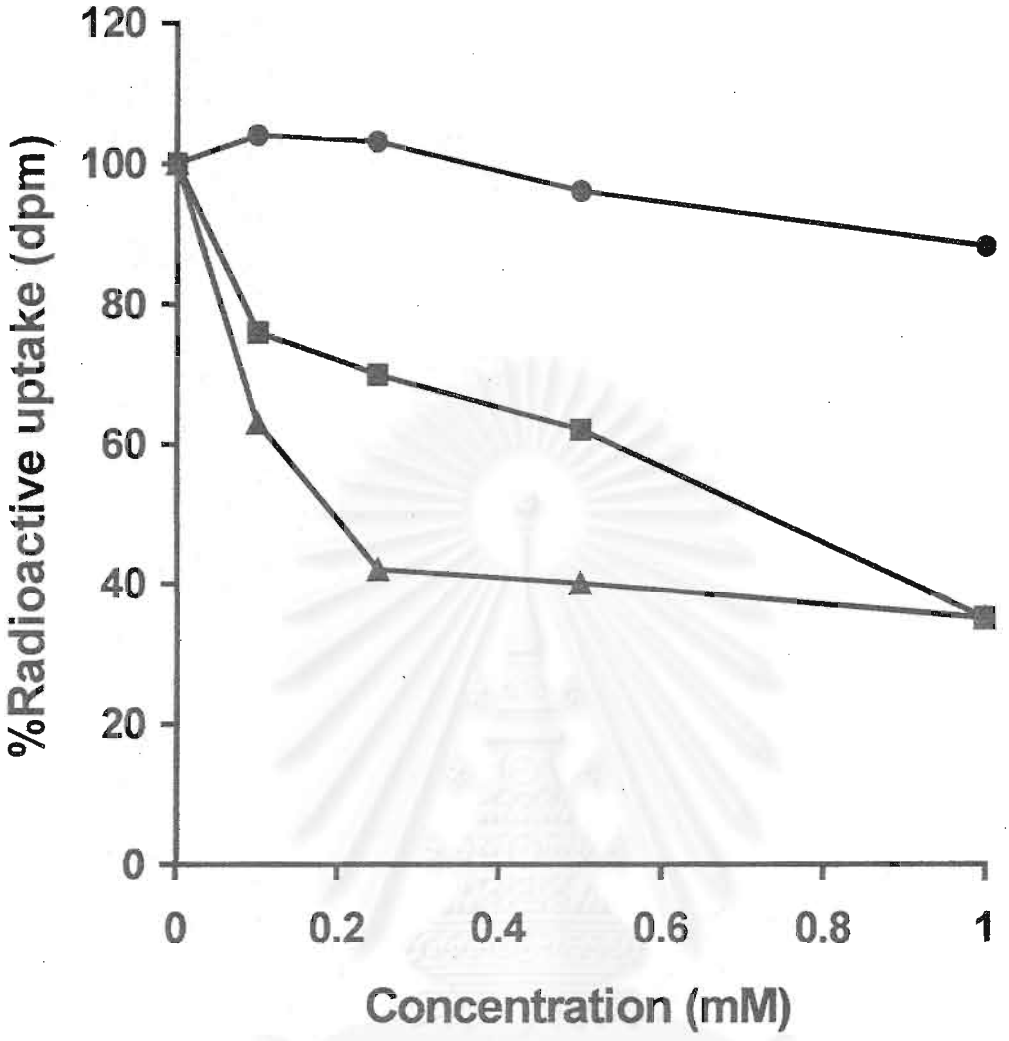


Figure 3.16 The inhibition curve of sucrose uptake of cassava root discs by NEM (■), PCMBS (▲) and IAA (●).

Each experimental value is expressed as mean ± SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 5550 dpm/disc

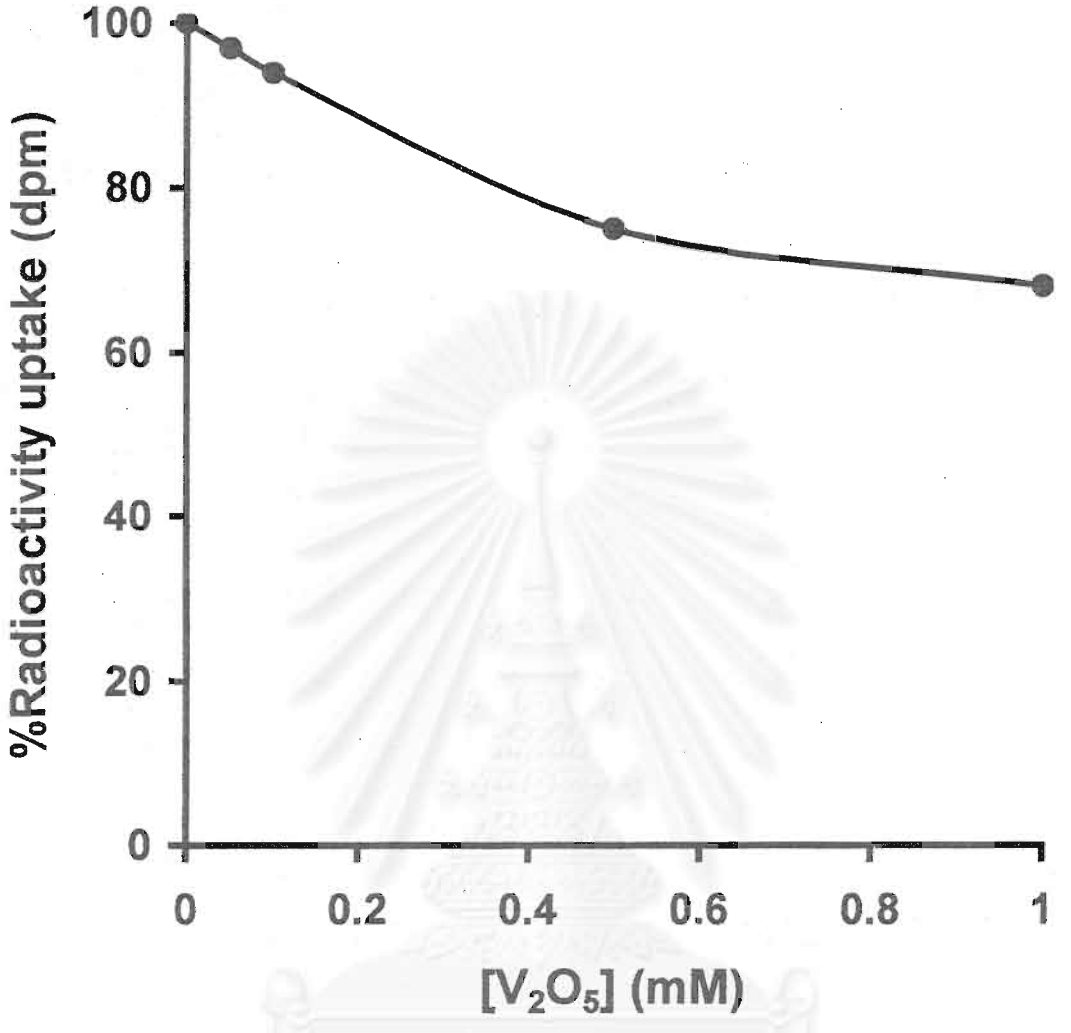


Figure 3.17 The inhibition curve of sucrose uptake of cassava root discs by V_2O_5

Each experimental value is expressed as mean $\pm$SD of dpm of 10 leaf discs.

Each value is average of 2 experiments.

100 % Radioactive uptake = Sucrose uptake of leaf discs in 1 mM ^{14}C-sucrose without test compounds = 2619 dpm/disc

3.2.5 The effect of linamarin on sucrose uptake in root discs

Linamarin is the major cyanogenic glycoside in cassava. It showed strong inhibitory effect on sucrose uptake in leaf discs (Figure 3.12). When was incubated with ^{14}C-sucrose in root discs, the result showed that linamarin activated the sucrose uptake in root discs (Table 3.4) which was contrary to the result observed the leaves.

3.3 Purification of Sucrose binding protein

Since the results in section 3.1.5 indicated that sucrose uptake in cassava leaf discs was carrier-mediated transport, experiment was performed to isolate and identify the existence of such protein on the cell membrane. Membrane fraction was prepared as described in section 2.5. isolate plasma membrane as described in section 2.5.2.

3.3.1 Preparation of microsomal fraction.

Homogenate of 250 g cassava leaves was centrifuged at 10,000xg for 10 min, the pellet obtained consisted of intact chloroplast, mitochondria and whole thylakoid. The supernatant was further centrifuged at 50,000xg for 30 min to obtain the pellet which was called the microsomal fraction. The microsomal fraction contained mixture of plasma membrane, tonoplast, internal membrane of the endoplasmic recticulum and membrane of cytoplasmic organelled (47). About 217 mg of micromal protein was obtained from 250 g of cassava leaves (Table 3.5). It was further used to isolate plasma membrane as describe in section 2.5.2.

Table 3.4 Comparison of linamarin effect on sucrose uptake in cassava tissue.

Compound & Tissue	Concentration test (mM)	% Relative uptake (n)	% Inhibition of sucrose uptake
Leaf discs			
Control	0	100	0
Linamarin	0.5	24.8 (4)	75
Linamarin	1.0	24.56 (4)	75
Linamarin	5.0	20.6 (4)	80
Root discs			
Control	0	100	0
Linamarin	1.0	161 (2)	-61
Linamarin	5.0	238 (2)	-138

No. in parenthesis indicated the number of experiment (7-10 discs were used in each experiment)

Each experimental value is expressed as percent relative uptake used in each

Control = Leaf discs or root discs incubated in medium containing ^{14}C-sucrose with 1 mM sucrose

100% Radioactive uptake of leaf discs = 3416 dpm/disc

100% Radioactive uptake of root discs = 4017 dpm/disc

3.3.2 Isolation of plasma membrane by aqueous two-phase system

Six identical tubes of 27 g phase mixture were prepared as describes in section 2.5.2 and Table 2.1. Figure 3.18 showed the membrane separation on the aqueous two phase system. Plasma membrane was fractionated in to the upper phase (PEG). About 40 mg of plasma membrane protein was obtained from 210 mg protein of microsomal fraction (Table 3.5).

3.3.2.1 The vanadate sensitive ATPase activity in plasma membrane preparation.

From the aqueous two-phase systems, the plasma membrane was obtained in the upper phase (PEG). To confirm the successful isolation of plasma membrane, the marker enzyme of plasma membrane, i.e. vanadate-sensitive ATPase, was assayed in the presence and absence 0.1 mM V_2O_5. ATPase activity was monitored by measuring ADP formation detected by HPLC. The column was equilibrated with standard ADP and ATP (appendix A). Table 3.6 showed that the membrane preparation contained ATP hydrolytic activity. Pi released cannot be used to monitor ATPase activity since the system of aqueous two-phase contained potassium phosphate.

The ATPase activity of the upper phase was about 1,750 nmole/mg protein /hr and was lower to about 968.5 nmole/mg protein/hr when V_2O_5 was included in the reaction (Table 3.6). ATPase in the lower phase was also assayed. It was found that ADP formation was undetectable similar to the experiment in which heated upper phase was used.

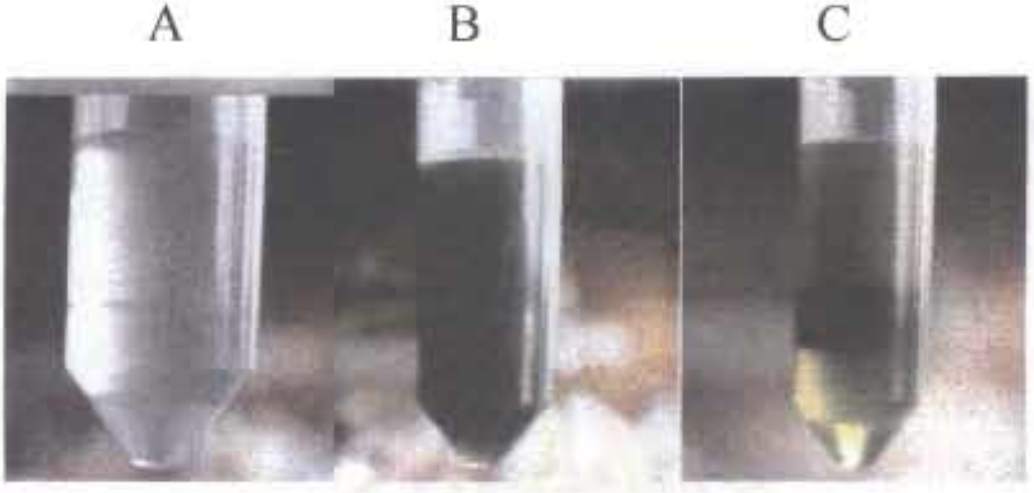


Figure 3.18 Aqueous two phase system in the isolation of cassava plasma membrane

A = Phases mixture

B = Mixing phases with sample

C = Phases separation

Table 3.5 Isolation of plasma membrane of cassava leaves.

Sample/fraction	Expt. I	Expt. II	Expt. III	Average
Wet wt of leave (g)	250	250	250	250
Microsomal fraction (mg protein)	177	289	187	217
Plasma membrane (mg protein)	40	45	43	42

Table 3.6 ADP formation in vanadate-sensitive ATPase assay in plasma membrane preparation.

Expt.	Upper phase assay	Peak area (mAu)	ADP formation (nmole/Rx)	ADP nmole/mg P/hr
I	- 0.1 M. V_2O_5	5316	78.5	1963
	+ 0.1 M. V_2O_5	2974	43.9	1098
II	- 0.1 M. V_2O_5	4242	62.7	1566
	+ 0.1 M. V_2O_5	2300	34.0	847
III	- 0.1 M. V_2O_5	4658	68.8	1720
	+ 0.1 M. V_2O_5	2569	38.3	959
Average	- 0.1 M. V_2O_5	4738	70.0	1750
	+ 0.1 M. V_2O_5	2623	38.7	968

Experiment I, II and III corresponded to the experiments in Table 3.5

From standard curve peak area 3250 was equaled to 200 μM ADP.

$$\text{Peak area 4738 equal to } \frac{4738 \times 200}{3250} \ \mu M$$

$$= 291.5 \ \mu M$$

The reaction volume 0. 24 ml containing 40 μg protein

$$= \frac{291.5 \times 1000 \text{ nmole} \times 0.24 \text{ ml}}{1000 \text{ ml}}$$

$$= 70 \text{ nmole/Reaction } 40 \ \mu gP$$

$$= \frac{70 \text{ nmole} \times 1000 \ \mu g}{40 \ \mu g}$$

$$= 1750 \text{ nmole/mg/hr}$$

3.3.2.2 Determination of membrane vesicle formation

Viewing the upper phase preparation under microscope showed vesicles formation of various sizes. The preparation was, thus, determined whether the vesicles observed were in the form of right-side-out vesicles by assaying the ATPase activity in the presence of 0.05% Triton X-100 (section 2.5.2.1). The results showed that the 40 μg of the protein in the upper phase there were 40% right side out vesicles (Table 3.7).

3.4 Sucrose uptake of native plasma membrane

The plasma membrane isolated by aqueous two-phase system were observed under the microscope. They appeared in the vesicle form and was designated as native plasma membrane vesicle. Experiment was set to test its ability of sucrose uptake. The vesicle was incubated in buffer pH 7.8 as described by Lemoine and Delrot (33) which was defined as non-energized condition and the result showed in Figure 3.19 that the sucrose uptake was linear with time up to 90 min at the rate of 0.16 nmole/min/mg protein.

3.5 Affinity chromatography of sucrose binding protein

The plasma membrane isolated from microsomal fraction by aqueous two phase system was solubilized with 1% CHAPS to obtain the membrane bound proteins. The solubilized proteins were obtained in the supernatant after centrifugation of the CHAPS treated membrane at 50,000 xg for 1 hr. The Solubilized proteins subjected to sucrose-affinity column (6 ml of affinity gel in 1.5 cm x 5 cm column) at described in section 2.5.4. The column was run at the

Table 3.7 Determination of the right-side t vesicle with ATPase activity of

Expt.	Upper phase	Peak area (mAu)	ADP nmole/mg P/hr
I	- 0.05% triton X-100	2399	
	+ 0.05% triton X-100	4095	41.4
II	- 0.05% triton X-100	2352	
	+ 0.05% triton X-100	3791	38.0
III	- 0.05% triton X-100	2460	
	+ 0.05% triton X-100	4198	41.4
Average			40.3

%Right side-out (% latent activity) as :

$$\frac{\text{(activity in presence of detergent) – (activity in absence of detergent)}}{\text{activity in presence of detergent}} \times 100$$

From : (36).

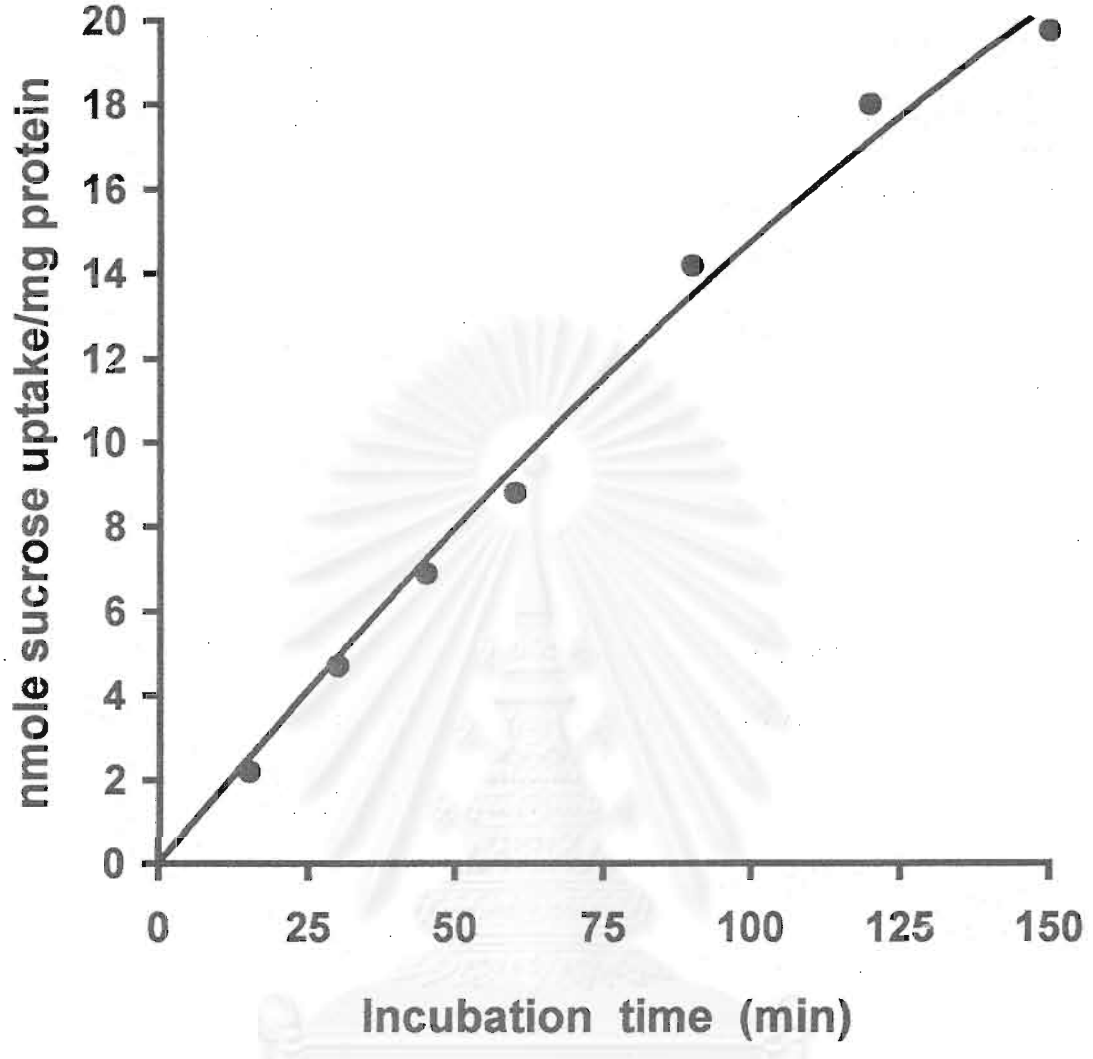


Figure 3.19 Time course of sucrose uptake of isolated plasma membrane Vesicles.

flow rate 0.3 ml/min and fractions collected at 5 min/fraction. The unbound protein was eluted with starting buffer (section 2.5.4) until the OD_{280} of the collected fractions were undetectable. The bound protein or sucrose binding protein was then eluted with 0.1 M sucrose. The chromatogram was shown in Figure 3.20. The sucrose binding protein obtained was about 0.09 % of protein loaded with 0.06 mg sucrose binding protein purified from 64 mg solubilized membrane protein.

3.6 SDS-polyacrylamide gel electrophoresis

The purified sucrose binding protein was subjected to electrophoresis on 10 % SDS-PAGE as described in section 2.8. The result was shown in Figure 3.21. The sucrose binding protein showed one single band with estimated MW. of 62 kDa when the gel was stained with silver stain using the standard curve in Appendix C. Large amount of protein was removed from the membrane preparation (lane A, B) using the sucrose-affinity column, indicating high specificity of the method.

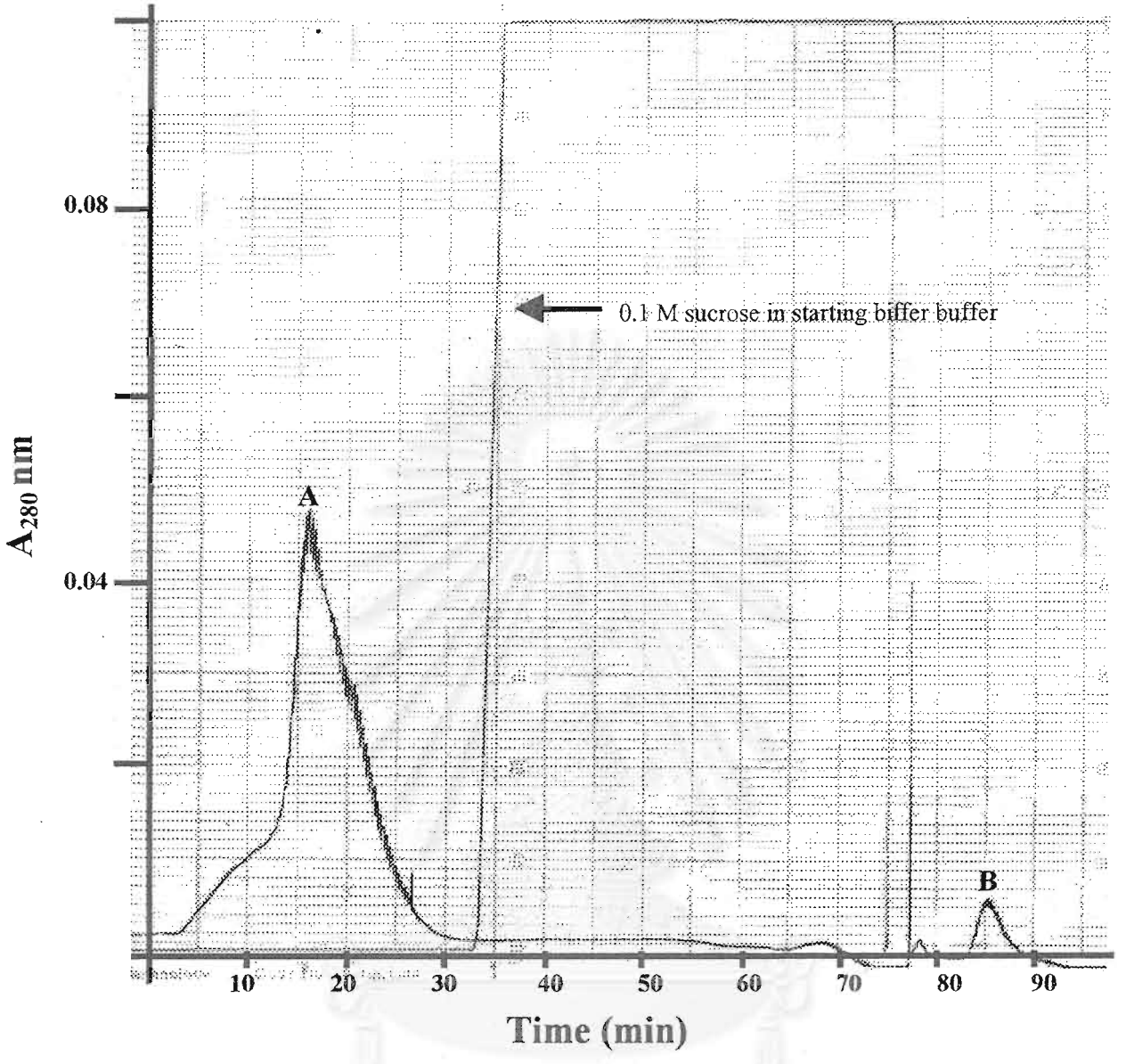


Figure 3.20 The profile of sucrose binding protein from sucrose–affinity column. The bound peak or sucrose binding protein was eluted with 0.1 M sucrose in 50 mM sodium phosphate buffer pH 5.2 containing 0.1% CHAPS, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$.

A = unbound protein , B = bound or sucrose binding protein

Starting buffer = 50 mM Sodium phosphate pH 5.2 with 0.1 % CHAPS , 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$

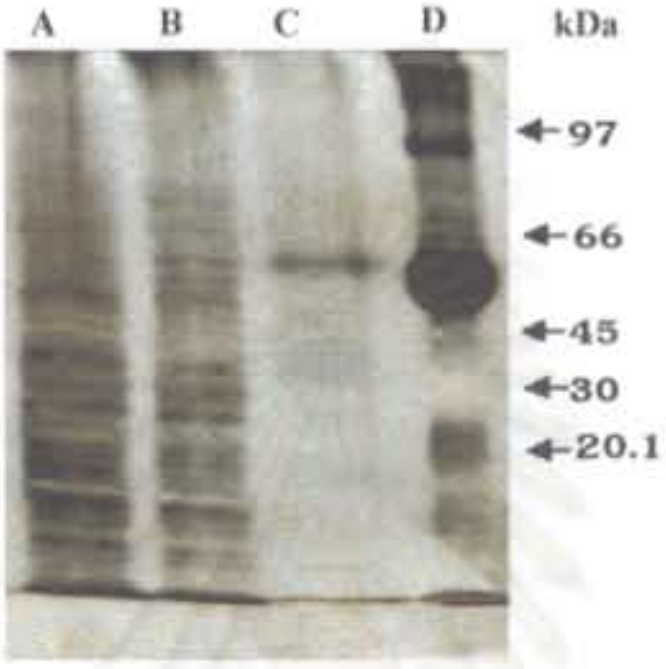


Figure 3.21 SDS - PAGE of sucrose binding protein from cassava leaves

A = Upper phase from aqueous two phase system. (5 µg)

B = CHAPS solubilizing fraction. (1 µg)

C = Sucrose binding protein from sucrose affinity. (10 ng)

D = Standard protein marker

CHAPTER IV

DISCUSSION

Sucrose is a principal form of carbohydrate in many higher plant species and is an important intermediate for may biological processes(Figures 1.1and 1.3). Sucrose also plays a role as a storage carbohydrate in the storage organ such as sugar cane and sugar beet or converted into starch in the storage roots such as cassava tubers. Moreover, it is the major transport form of photosynthetic product for delivery to other non-photosynthetic organs of most plants so sucrose transport is an important process for crop productivity (44).

Cassava is a tropical root crop which stores a large amount of carbohydrate in the form of starch. In Thailand, cassava is one of the most economically important crops and cassava is produced in large scale for export. Sucrose uptake has been studied in many plants such as sugar beet, broad bean, potato and spinach (45, 46, 48) but not in cassava. Understanding sucrose transport in cassava may be a useful fundamental knowledge leading to the explanation of accumulation of other sugars especially cyanogenic glycosides which are synthesized and accumulated in cassava.

4.1 Characterization of sucrose uptake in cassava

There have been reports that absorption of exogeneous sucrose by source leaf discs closely resembles sucrose uptake in intact leaves (45, 48, 49). Examples of studies using leaf discs system in study of sucrose uptake were sugar beet (49), broad bean (50) and potato (47).

The sucrose uptake in broad bean was studied in the leaf (51). However, the presence of hydrophobic cuticle resulted in less permeability due to less contact between reaction medium and plasma membrane of leaf cells. Technique of removing lower epidermis of the leaf was applied in the study of sucrose and hexose carrier in broad bean leaves (46). The sucrose uptake in cassava leaf discs were performed under light condition to keep them close to natural condition.

The preliminary screening of suitable preparation of cassava leaf discs in this study showed an observable sucrose uptake. However, the level of uptake was rather low which would make it difficult to study on effects of substances on the uptake system especially inhibitory effects (Table 3.2). As mentioned above, the peeled leaves which were used in the study of sugar uptake in broad bean were prepared by abrasion with a carborundum and soft cotton. In our study, the modified technique of Beier and Bruenin (29) was used for protoplast preparation. Briefly, the leaf was immersed into 70% ethanol to reduce the surface tension of cuticle and the excess ethanol was removed by washing in distilled water. The leaves were slightly brushed and any damaged to the parenchyma cells was observed by the amount of green colour of chloroplast released. Like other plants, cassava is expected to possess a sucrose transport system. The distribution of sucrose in the small veins of leaf disc after incubation with ^{14}C-sucrose, and the autoradiograph (Figure 3.1) sucrose was systematically transported into the leaf cells, not just a random diffusion. Moreover, the uptake of sucrose was not due to the diffusion of sugar in the medium into plant cell via the injury of phloem cells from leaf disc preparation. The phloem cells contains P-protein which has the gelling properties. It appears to function as protective agent by sealing off sieve

plates in areas of injury of the phloem. Another component in the phloem has the same function as P-protein is callose. Callose will accumulate in the sieve area.

The characteristics of sucrose uptake by cassava leaf discs, which followed the Michaelis-Menten kinetics as known in Figure 3.5A implies that the system is carrier-mediated. The binding affinity or K_m of the carrier for sucrose was calculated by the Lineweaver-Burk plot to be 1.31 mM and V_{max} or rate of transport was 3.699 nmole/hr/cm^2 (Figure 3.5B). The K_m value is lower than in other plants studied as shown in Table 4.1. The lower K_m value suggests that the carrier protein in cassava leaves has a high affinity for sucrose than other sugars.

From Table 4.1 the K_m for sucrose in cassava leaves is similar to that of potato.

Of several sugars tested on the effect of sucrose uptake in leaves as showed in Table 3.3, most of them showed no effect to the sucrose uptake system except PNPG. The β-analogs of PNPG, exhibited strong inhibitory effect at the concentration 0.5 mM while α-PNPG showed only 60% inhibition (Figure 3.6). The different effect of the two analogs may be due to the different orientation of phenol group.

Glucose and fructose which were the hydrolytic products of sucrose did not have effect on the sucrose uptake. This should imply that sucrose was taken up as sucrose not by the breakdown of sucrose into smaller units by hydrolytic activity. The uniformly labeled sucrose would yield labeled glucose and fructose. If sucrose was broken down before transported, the presence of added unlabeled glucose or fructose would complete with the radioactive uptake and effectively lower the amount radioactive uptake monitored.

Table 4.1 Comparison of kinetic parameter of sucrose transport in plants.

Plants	Km	Vmax	Ref.
Beta Vulgoris L	25 mM pH 5	30 μmole/hr/dm^2	Plant Physiol (1974) 54:880-891
Red-beet	12 mM	-	Plant Physiol (1985) 78: 871-875
Broad Bean	3 mM	-	Plant Physiol (1981) 67: 560-564
Potato	1 mM	-	Plant Cell (1993) 5: 1591-1598
Cassava*			This study
Leaf	1.13 mM	3.69 nmole/h/cm^2	
Root	20 mM	0.018 nmole/h/cm^2	

Study on sucrose uptake in cassava tuber also showed the characteristics of a carrier mediated system (Figure 3.1 A and Figure 3.15B). The root disc showed the K_m value of 20 mM and V_{max} value of 0.018 nmole/hr/cm^2. The K_m for sucrose of root disc was still in the lower range but higher than in the leaf disc. The result indicating the nature of carrier mediated sucrose uptake system in leaf and root implicated that sucrose in leaf and root cassava used the apoplastic pathway in sucrose transporting.

The difference in K_m values of leaf and root of cassava suggests that the sucrose carrier proteins in leaves and root were different. The sucrose transport system in leaves usually functions to move sucrose in the leaves to supply other non-photosynthetic tissue including roots. The carrier proteins involved should have a high affinity for sucrose so that sucrose is efficiently transferred from the leaves. On the other hand, transport in the tuber should serve only to accumulate sucrose which would be stored in the tuber as starch. Therefore, sucrose transport or uptake in the two tissues should be different. This was further supported by the results on the effects of sulfhydryl reagents, metabolite inhibitors especially linamarin.

Sucrose transport in many plants have been reported to involve sulfhydryl group and is sensitive to sulfhydryl modifying reagents (49). PCMBS is a non-penetrating, reversible sulfhydryl modifying reagent while NEM penetrates and binds irreversibly to sulfhydryl group. In cassava leaf, NEM gave nearly 100% inhibition at 0.5 mM while PCMBS or IAA showed less effect (Figure 3.8). The results suggest that the sucrose carrier proteins in leaf involve sulfhydryl groups.

When the compounds which showed effect on the leaf sucrose uptake was

tested in the root discs, PCMBS inhibited the sucrose uptake by 60% at 0.5 mM, more effective than NEM while IAA has no inhibitory effect (Figure 3.16). The different effect of sulfhydryl modifying reagents in roots and leaves of cassava suggests that carrier protein in root and leaf discs are different. Root disc may have the sulfhydryl group exposed outside of the plasma membrane more than cytoplasmic side since it was more affected by non-penetrating PCMBS This result confirmed the earlier suggestion from the study of kinetic data that sucrose uptake system in root and leaves differed.

Several plant sucrose transports were reported to function in association with ATPase and proton pump (43). CCCP and DNP are uncouplers that dissipate H^+ gradient and reduce the production of ATP. Both of them inhibited the sucrose uptake to 50% at the concentration in μM level with CCCP showed slightly stronger inhibition than DNP. Their inhibitions were considered strong which implicated the involvement of proton transport in sucrose uptake in cassava leaves. However erythrosin B which was H^+-ATPase inhibitor gave only 20 % inhibitory effect at the concentration up to 1 mM and also like KCN which was well known to inhibit the electron flow in respiratory chain and ATP production (Figure 3.9).

The ATPase present in organelles such as mitochondria and chloroplasts, has long been known as “F-type” ATPase. Another two types of ATPase found in plants are P-type and v-type ATPases which differ in their reaction with vanadate, P-type ATPase is inhibited by vanadate while V-type is not. The P-type ATPase or vanadate-sensitive ATPase is characteristics of plant plasma membrane while v-type ATPase is the characteristics of tonoplast or vacuole membrane (47). In this study, vanadate was found to inhibit sucrose uptake in leaf disc by 60% at the concentration 0.05 mM

(Figure 3.9) while its inhibitory effect on sucrose uptake in root disc was less (Figure 3.17), with 30% inhibition at concentration 1 mM. The observation suggested the sucrose uptake in cassava tissue was energy–dependent process requiring ATP hydrolysis. The small effect of V_2O_5 on sucrose transport in root discs may imply that the sucrose transport observed in root disc may not involved only plasma membrane but also included the transport in the storage organelles such as vacuoles which was not V_2O_5 sensitive. Since, cassava root is a storage organ, the sucrose transport on vacuole membrane may be more prominent than plasma membrane. However, the effect of inhibitors on other types of ATPase was not investigated.

Cassava is known to produce cyanogenic glucosides, with linamarin as major form and lotaustralin as minor form. The cyanogenic glucosides are synthesized in leave and transport to all tissues except seeds. It is interesting to see if the transport of these two compounds is interelated. When linamarin was included in the sucrose uptake experiment it showed inhibitory effect on the sucrose uptake in leaves while other naturally occurred cyanogenic glycosides did not (Figure 3.12). At 0.1 mM linamarin caused 50% inhibition of sucrose uptake in cassava leave but at 1 mM and 5 mM linamarin activated sucrose uptake in root disc by 61% and 138% respectively (Table 3.5). The linamarin inhibition was unlikely to be due to its break down products, KCN and glucose, since neither showed inhibitory effect on the sucrose uptake system (Table 3.3 and Figure 3.9). Linamarin is known to be synthesized and accumulated in cassava leaves more than in root tissues and is translocated from leaves to other organs. (52 and 53).

Phloridzin, which is a plant flavonoid glycoside known to inhibit sucrose uptake and Na^+- glucose transport in plants without being transported (46) also showed the

inhibitory effect on sucrose uptake in cassava leaves (Figure 3.11). However, its effect was less than linamarin. Further investigation on the inhibitory effect of linamarin should be carried out to verify whether it compete for the same carrier as sucrose or its inhibitory nature was the same as phloridzin.

4.2 Isolation of sucrose binding protein from plasma membrane

Since the characterization of the sucrose uptake in leaf discs implicates a carrier-mediated system, an attempt was made to isolate such a carrier protein. Purification of the sucrose carrier protein of cassava leaves, started with the isolation of the freshly prepared plasma membrane. The aqueous two-phase method was selected. Two phase system required two water soluble polymers, which would co-dissolve in water without phase separation at moderate condition. This system have been successful used in the isolation of plasma membrane with H^+-glutamine symporter, H^+-sucrose symporter in *Ricinus communis* L. (54 and 55), sucrose transporter of tonoplast of sugarcane (37) and sucrose carrier of plasma membrane of sugar beet leaves (35).

In this study, Dextran T500 and PEG 3350 were used to form an aqueous two phase system which would give mild, highly discriminating separation with minimum sheering force. The separation achieved by two phase partition depends primarily on surface properties of the sample such as inside-out and right-side-out membrane vesicles. The two phase system can be affected by content of phase compositions, pH, salt concentration and temperature.

The condition, used in this study, followed the condition reported for effective isolation of pure plasma membrane up to 90 % by Larsson *et al* (36). The cassava leaf

plasma membrane preparation obtained appeared as vesicle forms under microscope (data no shown) similar to the result reported by Lemoine *et al.* as shown in Figure 4.1(56). About 40-45 mg protein of plasma membrane was obtained from 250 g of mature leaves used. In previously report on sugar beet leaves, 30-36 mg protein of plasma membrane was obtained from 250 g of leaves.

To confirm that the preparation contained plasma membrane, vanadate-sensitive ATPase which is the marker of plasma membrane was monitored. The membrane preparation was shown to produce ADP when incubated with ATP, indicating hydrolysis of ATP was assayed. The system was proved to be vanadate-sensitive ATPase by the result that ATP hydrolytic activity was inhibited to 50% at 0.1 mM V_2O_5 (Table 3.6). This suggested that the preparation obtained from the aqueous two phase system contained cassava leaf plasma membrane. Although the technique of aqueous two-phase was reported to yield highly purified plasma membrane, it was some what tedious, tricky and requires large amount of starting material. It is also important to characterize the plasma membrane preparation in term of purity and membrane orientation. This characterization provides basic information to interpret uptake data. To check for purity, the marker enzymes of other organelles such as cytochrome C oxidase, a marker of mitochondrial membrane, chlorophyll for chloroplast should be monitored. These organelles should be absent in the upper phase or PEG phase. Plasma membrane was monitored by the presence of vanadate-sensitive ATPase. In our study, only vanadate-sensitive ATPase was assayed, the other markers for membrane of cell organelles were not determined due to the limitation of yields. For orientation of membrane vesicle, right-side-out mean the vesicle was formed with membrane orientation similar to intact cell with ATPase located inside.

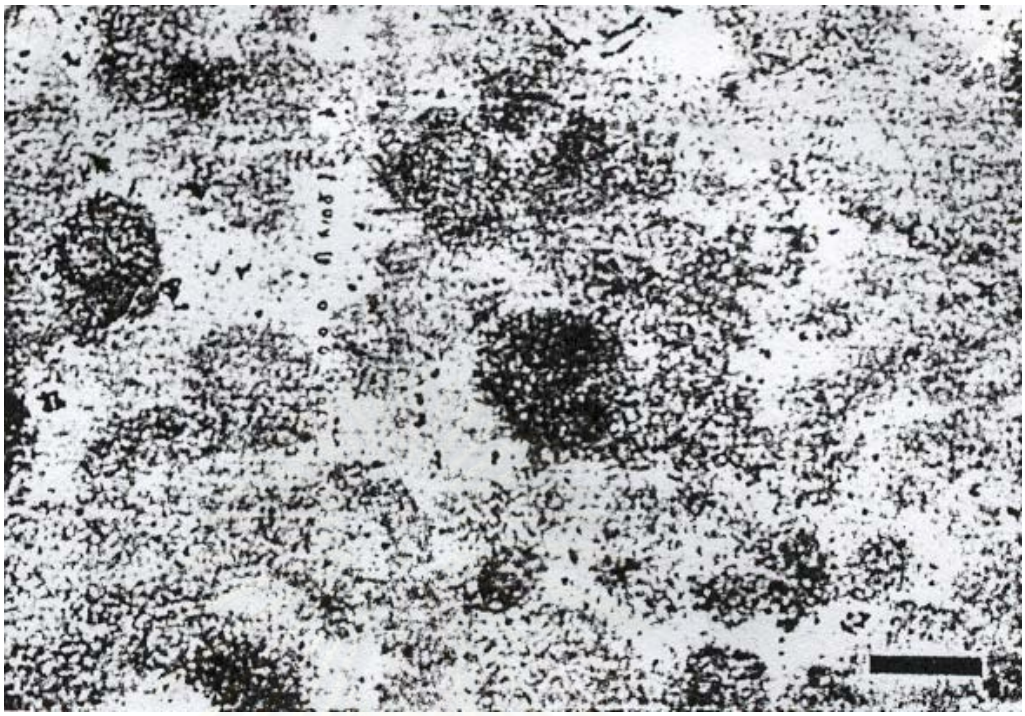


Figure 4.1 Electron micrographs of sugar beet plasma membrane vesicle.

From: (56).

The technique used to determine the amount of right-side-out vesicle employed Triton X-100 which will solubilize the membrane resulting more access of ATP to the active site of the ATPase. Right-side-out vesicles treated with Triton X-100, therefore, exhibited increase sucrose uptake activity. The activity was calculated as % latent activity by :

$$\frac{100\text{x(activity in the presence of detergent - activity in the absence of detergent)}}{\text{activity in the presence of detergent}}$$

The higher % latent activity suggested most the vesicle were in the right side-out. When the % latent activity or % Right-side-out of plasma membrane was monitored in our preparation, only 41% was achieved. This may be due to the concentration of Triton X-100 used was not, the maximal effective concentration to expose all the active sites in plasma membrane. Similar experiment on sugar beet by Lemoine *et al.*(56) obtained 58.5% latency. On the other hand, the percentage of the right side-out vesicle could be estimated by staining the vesicle with peroxidase labeled Concanavalin A (Con-A). Con-A will bind the glycoprotein of plasma membrane was mainly restricted to the outer surface of the membrane. Lamoine *et al.*(56) showed both the peroxidase activity staining and Triton X-100 treatment of inside-out and right side-out vesicles to give comparable results.

The isolated plasma membrane was tested for sucrose uptake. The result in Figure 3.18 showed the plasma membrane was still active. The sucrose uptake was 18-20 nmole/mg protein/hr higher than in sugar beet (5 nmole/mg protein/hr) under the same condition (39).

Since the amount of protein in the membrane preparation was low, an one step purification should be more effective. Affinity column with sucrose as binding ligand was used because it is highly specific and more suitable for use with small amount of protein. Sucrose affinity column was, therefore, prepared by linking sucrose to epoxy-activated Sepharose 6B resin. Capacity of sucrose linkage was not determined because it required the digestion of sucrose from portion of the prepared affinity resin (39). To purify the carrier membrane protein fraction was treated with 1% CHAPS to solubilize the protein in plasma membrane. The solubilized proteins were subjected to sucrose-affinity column. From 64 mg protein obtained in the solubilized membrane, only 60 μg protein was eluted at 0.1 mM sucrose. This protein showed as single major protein band on SDS-PAGE stained with silver nitrate. Its molecular weight was estimated at 62 kDa. It has been reported that the molecular weight of sucrose carrier protein in sugar beet leaves was about 42 kDa and most gene product of sucrose transporter were around 55 kDa (18).

Li Z-G *et al*.(39) reported the purification of sucrose binding protein on affinity column which yielded only 0.22% of sucrose binding protein from sugar beet plasma membrane. Our preparation obtained only 0.09% of sucrose binding protein from the plasma membrane of 250 g cassava leaves. The low yield obtained may be the low efficiency of the affinity column prepared. The eluting concentration of 0.1 M sucrose was sufficient since further washing of the column did not yield additional protein.

A few enzymes utilizing sucrose as substrates were reported in cassava leaves (26), these enzymes can also bind sucrose. Kham-ai reported that a membrane-bound sucrose synthase, a soluble sucrose synthase and a soluble invertase were purified

from cassava leaves. The membrane-bound sucrose synthase was a polymeric enzyme with a molecular mass of 669 kDa, consisting of 9-10 subunits molecular weight of 68 kDa. Its K_m for sucrose was 52.68 mM. The soluble sucrose synthase was a polymeric enzyme with a molecular mass of 470 kDa, consisting of 9 units molecular weight of 53 kDa. Its K_m for sucrose was 53 mM. The soluble invertase was trimeric with a molecular mass of 158 kDa and a subunit molecular weight of 57 kDa. Its K_m for sucrose was 32 mM. From the report on the K_m for sucrose of these enzymes, the cytosolic localization of most enzymes except membrane-bound sucrose synthase together with our finding on the non effect of glucose and fructose on sucrose uptake, it can be concluded that the sucrose carrier protein was unlikely of these enzymes.

From all the experiments performed on sucrose uptake in cassava leaves, we can conclude that there existed a sucrose transport system in the leaves which was carrier mediated and was apoplastic transport. It was located on the plasma membrane and its activity was associated with ATPase and proton pump. Linamarin, a cyanogenic glucoside which was also synthesized in the leaves and exported to other organs, can inhibit the sucrose transport system in cassava leaves. The nature of linamarin inhibition remained to be determined and may provide evidence whether they share the same transporter protein or associated in any way. The sucrose carrier protein was purified, further experiments such as reconstitution of the protein on liposome would provide more useful information on the mechanism of the sucrose transport in cassava leaves. Although the study on sucrose transport root discs was only a preliminary results but yielded interesting features. The results indicated a different sucrose transport system in the root which exhibited several opposite

properties. The difference may implicate the different function of the sucrose transport system in these organs. Characterizations of root sucrose transport system would lead to more understanding of their mechanism.

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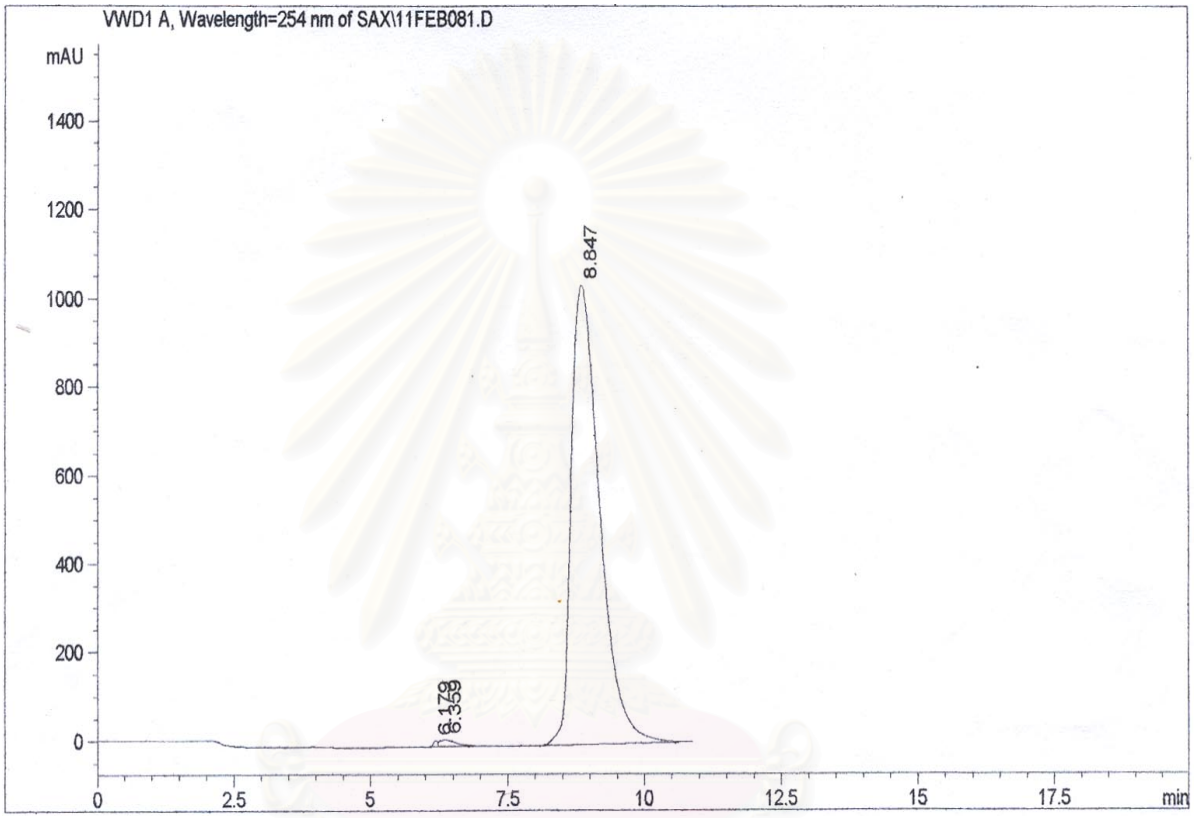
APPENDICES

APPENDIX A

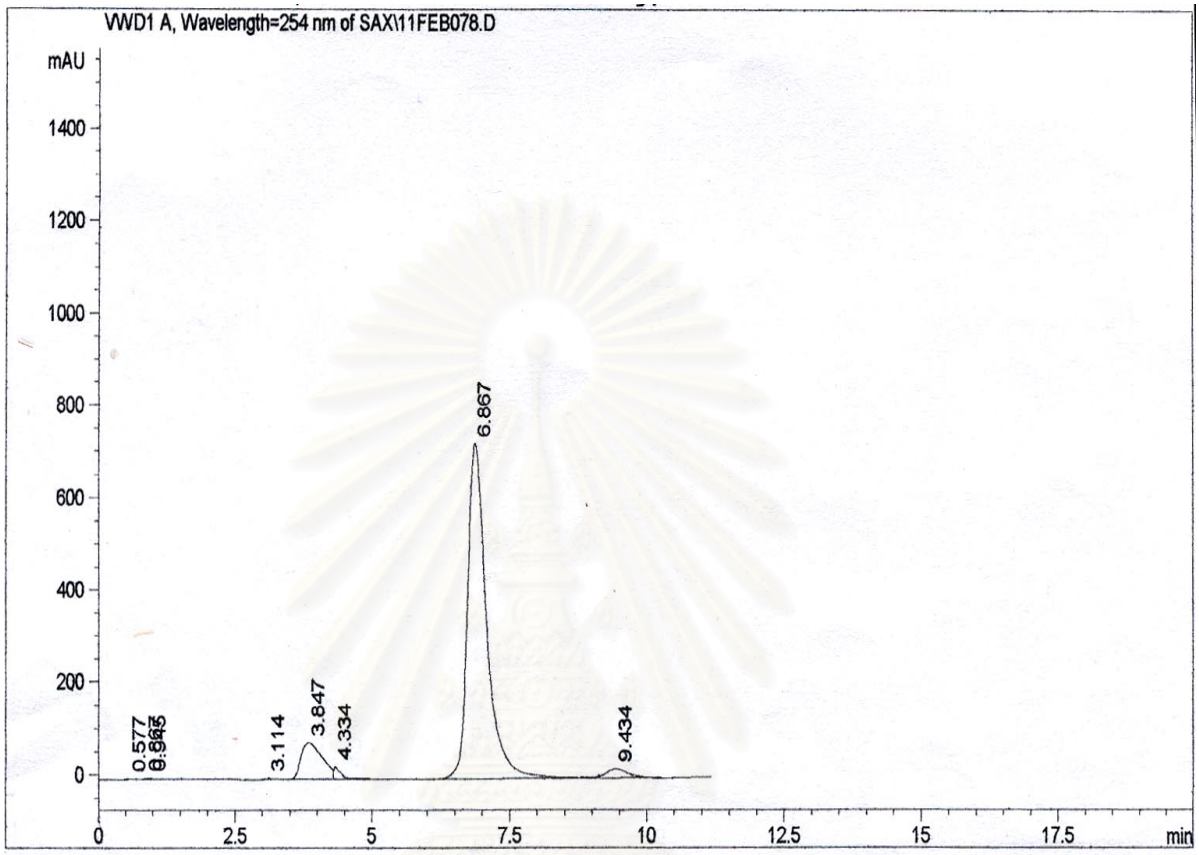
1. HPLC profile of ATP and ADP

1.1 Standard of 3 mM ATP



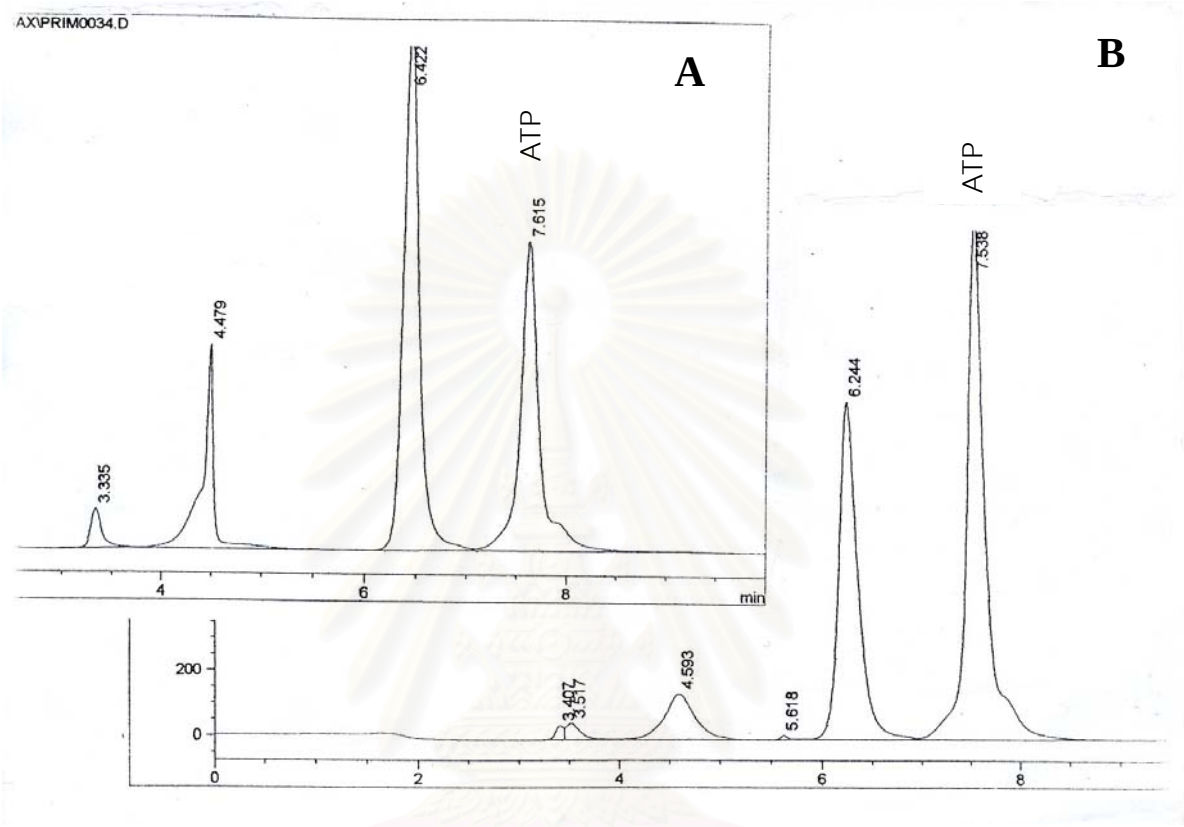
ATP : RT = 8.847

1.2 Standard of 1.5 mM ADP

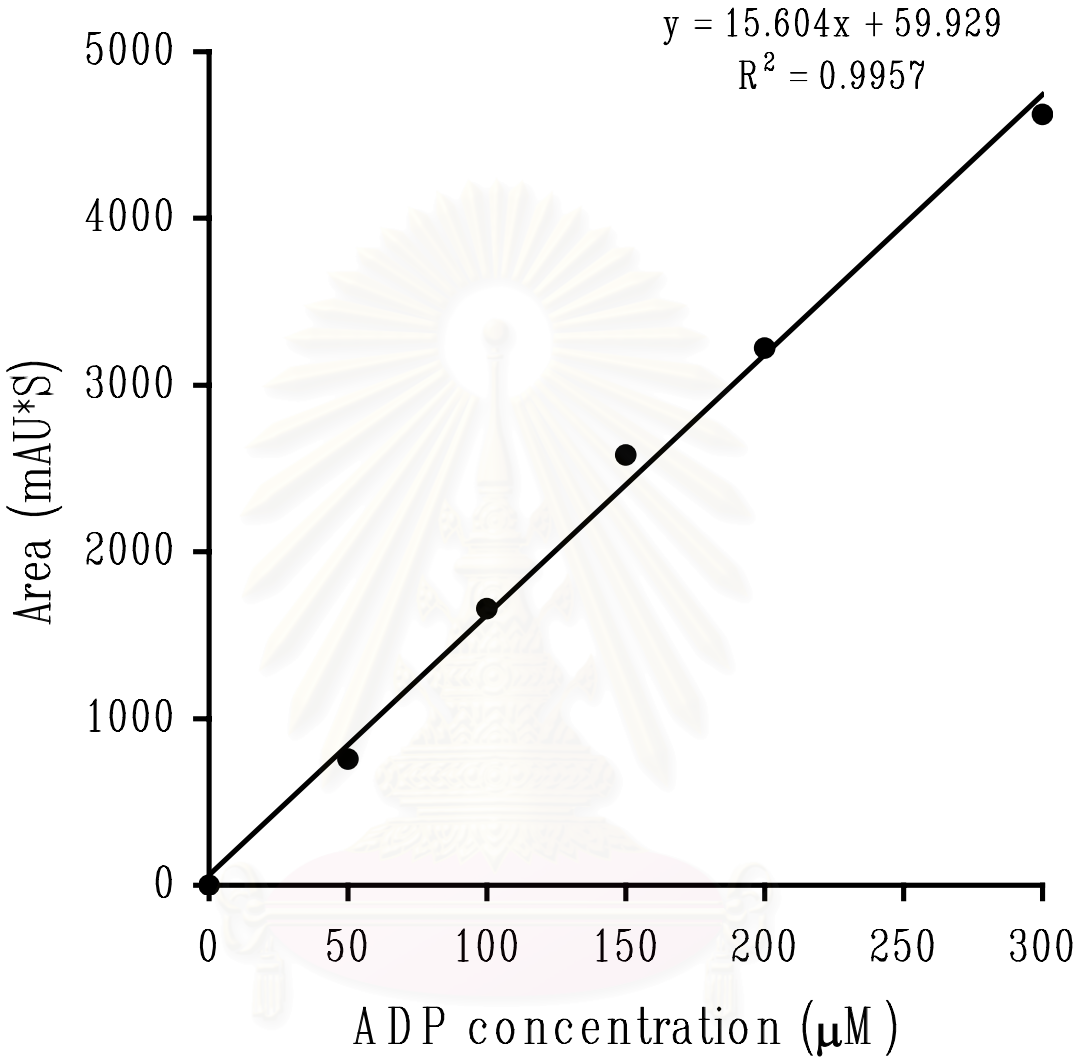


ADP : RT = 6.867

1.3 HPLC profile of reaction mixture with ATPase activity (A) and ATPase activity + 0.1 mM V_2O_5 (B)

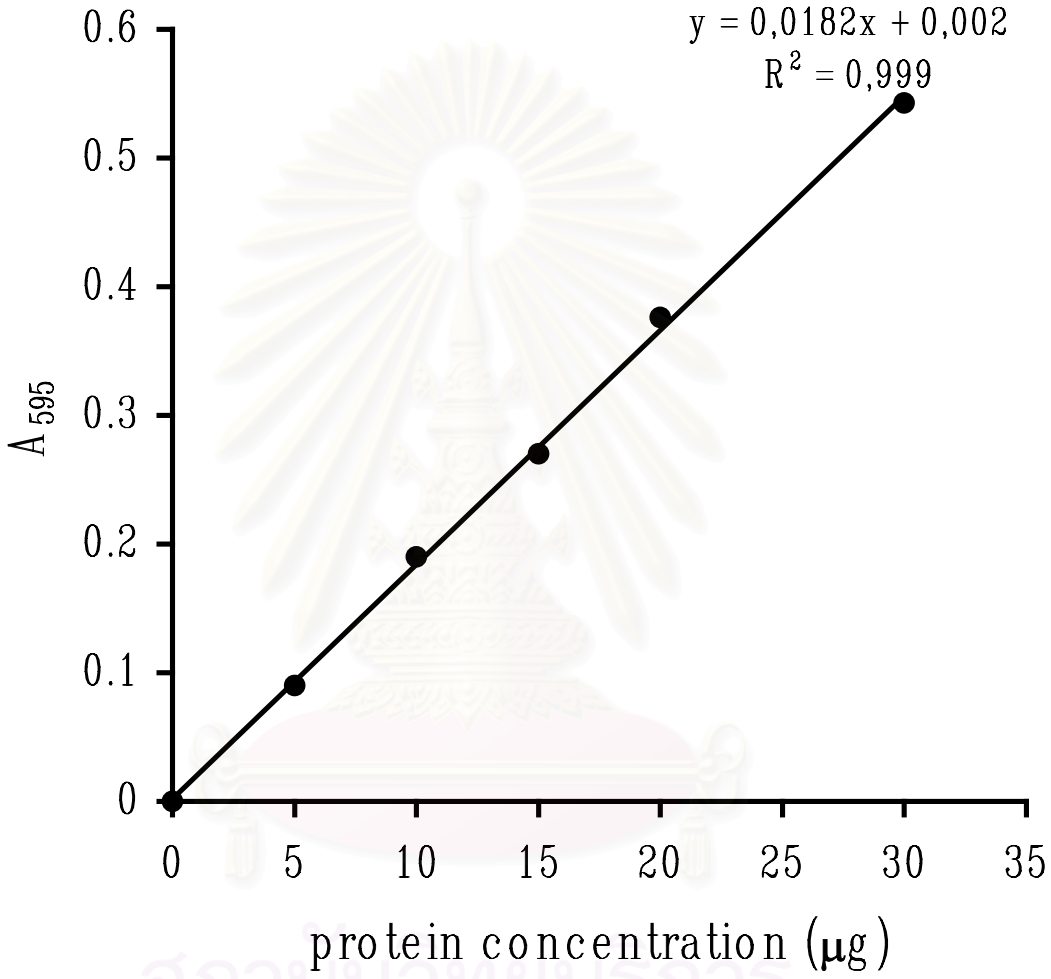


2. Calibration curve of ADP concentration



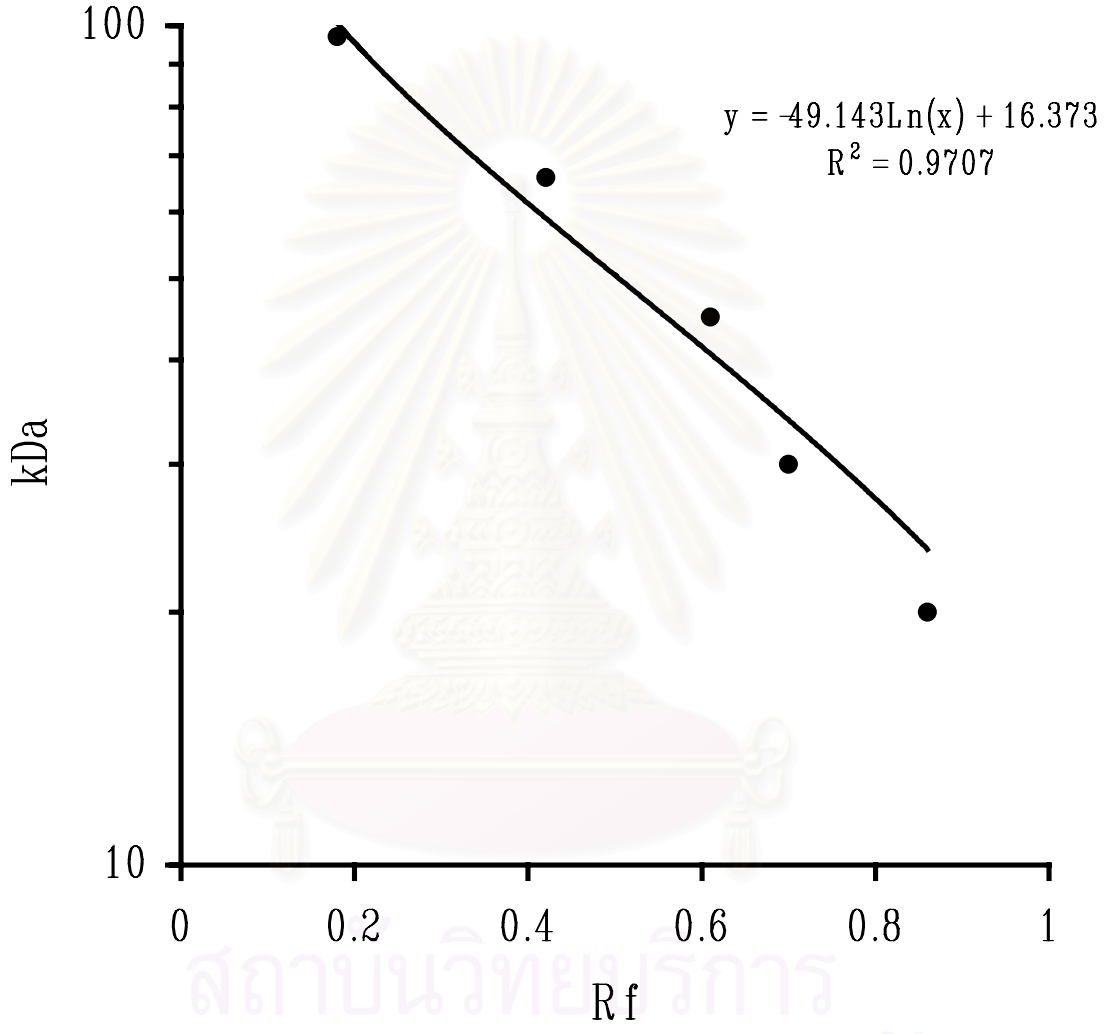
APPENDIX B

Calibration curve of protein concentration



APPENDIX C

Calibration curve for molecular weight on SDS-PAGE



APPENDIX D

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock reagents

30% Acrylamide, 0.8% bis-acrylamide, 100ml

acrylamide	29.2 g
N.N -methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water

1.5 M Tris-HCI pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with 1 M HCI and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCI pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06 g

Adjusted pH to 6.8 with 1 M HCI and adjusted volume to 100 ml with distilled water

Solution B -SDS (1.5M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCI pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21ml

Solution C –SDS (0.5M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCI pH 6.8	50 ml
Distilled water	50 ml

5x Sample buffer

1M Tris-HCl pH 6.8	0.6 ml
50% Glycerol	5.0 ml
10% SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% Bromphenol blue	0.5 ml
Distilled water	1.4 ml

SDS electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine, SDS 0.1%)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g
SDS	1 g

Dissolved in distilled water to 1 litre without pH adjustment.

(Final pH should be 8.3).

3. Preparation of SDS-PAGE

10 % separating gel

Prepare as described for non-denaturing gel but using with solution B containing SDS instead solution B.

3% stacking gel

Prepare as described for non-denaturing gel but using with solution C containing SDS instead solution C.

1400 Sucrose Uptake in Leaves of Cassava *Manihot esculenta*Crantz

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Sucrose is a major translocated form of carbon assimilates in most plant species.It has been shown to be actively transported across membrane by transport protein to heterotrophic organs for its growth development and storage.In cassava,sucrose is transported from leaves and stored as starch in the roots.In this study,cassava leaf disc,12 mm.in diameter,were incubated in the medium containing ^{14}C-sucrose.It was found that the sucrose uptake was linear with time and exhibited Michaelis-Menten characteristics with K_m 1.31 mM and V_{max} of 3.7nmole/hour/cm^2 indicating that sucrose transport in cassava leaves was carrier mediated.The sucrose uptake was inhibited by N-ethylmaleimide,p-chloromercuribenzenesulfonic acid and iodoacetic acid,suggesting involvement of thiol groups in the process.It was strongly inhibited by dinitrophenol,carbonyl cyanide m-chlorophenylhydrazone, vanadate,erythrosin B and KCN,suggesting that the uptake involved proton transport and was energy dependent.
Cassava is a cyanophoric plant.All cassava tissues,with the exception of seeds,contain the cyanogenic glycosides linamarin(>93% total cyanogen) and lotaustralin (< 10%total cyanogen). They were synthesized in leaves.When effect of cyanogenic glycosides on sucrose uptake was studied,it was found that linamarin showed a prominent inhibition on the process.In presence of linamarin,sucrose transport was significantly reduced.Other mono and disaccharides tested showed little effect.It appears that cyanogenic glycosides may regulate the sucrose transport in cassava.
Supported by National Science and Technology Development Agency

1401 Leucine uptake into membrane vesicles from midge larvae.

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Membrane vescicles obtained from the brush border membranes of the midge larvae *Chironomus riparius* (Insecta, Diptera) were used to study the patways for neutral amino acid transport. Larvae were collected in the wild and then either maintained in a cold room or stored in liquid nitrogen without significant changing their physiological properties. Larval size ranged between 4 to 10 mm. Vesicles were purified by homogenisation in hypotonic Hepes-Tris buffer, two precipitations with 12 mM $MgCl_2$ and differential centrifugation as described by Biber et al. (*Biochim. Biophys. Acta*1981, v. 647, p.169). The resulting preparation was 16-fold enriched of leucine aminopeptidase and alkaline phosphatase, not enriched of enzime markers from basolateral membranes and free of contaminant of mitochondrial membranes. For the high activities of typical brush border marker enzymes our preparation was judged consisting mainly of midgut luminal membranes. Membranes exhibited pH-dependent, Na^+/gradient coupled transport phenomenon for L-leucine. Initial rate or leucine uptake at varying leucine concentration was a function of a saturable component plus a linear one. Fitting the former with the Michaelis-Menten equation gave a high affinity, low capacity transport system (Km = 0.01 mM and Vmax = 400 pmoli/ 10s/ mg protein). Leucine uptake was strongly inhibited by all neutral amino acid, including the 2-amino-2-norbornanecarboxylic acid (substrate for the mammalian leucine transport system) and by some polar amino acid, such as serine and histidine. For this inhibition pattern the system much resembles the broad-scope high affinity, high capacity, K^+-dependent neutral amino acid transport system from lepidopteran larvae. This represents the first report on amino acid uptake in dipteran larvae.

1402 Calcium Binding and Translocation by VDAC: a possible regulatory mechanism in mitochondrial function.

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Mitochondria play a central role in energy production, Ca^{2+} signaling, aging and cell death. To control cytosolic or mitochondrial calcium concentration, mitochondria possess several influx and efflux Ca^{2+} transport systems in the inner membrane. However, the pathway for Ca^{2+} crossing the outer mitochondrial membrane has yet not been identified. Our results indicate that the Voltage-Dependent Anion Channel (VDAC), an outer mitochondrial membrane protein, provides that pathway. VDAC is a large channel that transports anions, cations, ATP and other metabolites. In this study we show that: (i) Purified VDAC reconstituted into a planar lipid bilayer or liposomes is highly permeable to Ca^{2+}; (ii) VDAC contains Ca^{2+} binding sites; (iii) La^{3+} and the polycationic dye ruthenium red completely close the VDAC channel and inhibit Ca^{2+} transport in isolated mitochondria. VDAC permeability to Ca^{2+} and binding of Ca^{2+} provide the answers to how the inner membrane located Ca^{2+} transport systems sense cytosolic Ca^{2+}. VDAC is a component of the mitochondrial Permeabilite Transition Pore (PTP), a large, high conductence, non-specific channel spanning both the inner and outer mitochondrial membranes. We suggest that VDAC as a Ca^{2+} binding protein has a role in the regulation of the PTP activity, and in intracellular Ca^{2+} signaling.

1404 Metabotropic Glutamate receptors expressed in bone cells

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Glutamate is an important neurotransmitter in the CNS. On the Basis of pharmacological, electrophysiological and biochemical studies, glutamate receptors can be categorized into two groups termed ionotropic and metabotropic. RT-PCR, antibody labeling and electrophysiological investigations have demonstrated expression of NMDA receptors in rat osbeoblasts, MG63 cells and rat osteoclasts. Recent evidence suggests that bones may receive glutamatergic innervation.
We have found discrepancies between the electrophysiological effects, on osteoblasts, of glutamate and NMDA, suggesting that other (possibly metabotropic) glutamate receptors may be present in these cells. Using RT-PCR we have detected expression of metabotropic glutamate receptor subunits in rat femoral cells in primary culture. Osteoblasts express mRNA for mGluR1b (but not mGluR1a), but no mGluR2, mGluR4 and mGluR6, whereas in marrow stromal cells we detected mGluR6 and (apparently) low levels of mGluR1a and b, but no mGluR2 or mGluR4 . Further more, we observed glutamate induced Nitric Oxide reduction in marrow cells which expressed mGluR6. This effect would be blocked by PKC inhibitor. These finding support a signaling role for glutamate in bone, possibly associated with cell differentiation.

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Characterization of sucrose uptake system in cassava (*Manihot esculenta* Crantz)

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Abstract

A leaf disc system was developed to study sucrose uptake in cassava (*Manihot esculenta* Crantz). The uptake of (U-^{14}C) sucrose by cassava leaf discs followed Michaelis–Menten kinetics with a K_m value for sucrose of 1.3 mM. It was found to be strongly inhibited by sulfhydryl reagents, *N*-ethylmaleimide, *p*-chloromercuribenzene sulfonate and iodoacetate. Several metabolic inhibitors were also tested. Among these, dinitrophenol, chlorocarbonyl cyanide phenylhydrazone, phloridzin and vanadate inhibited the sucrose uptake by the leaf discs. Linamarin, the main cassava cyanogenic glucoside, strongly inhibited the sucrose uptake by the leaf discs, while other cyanogenic glycosides tested (prunasin and amygdalin) showed a much weaker inhibition. A linamarin analog, isopropylthioglucoside, was a weaker inhibitor than *p*-nitrophenyl glucosides (both α and β forms). Cassava root discs were also capable of taking up sucrose, but linamarin activated its uptake. The observations suggested that linamarin may regulate the sucrose transport in cassava.

Keywords: Cassava; *Manihot esculenta* Crantz; Sucrose uptake; Linamarin

1. Introduction

Because of its high starch content, tubers of cassava (*Manihot esculenta* Crantz) have long formed a part of the staple diet for millions of poor people in the tropics [1]. For some countries, cassava is also an economic crop because it is sold as animal feed and cassava starch has a wide range of industrial applications. However, long-term cassava consumption has been associated with certain health consequences due to chronic cyanide toxicity [2–4]. Much research attention is, therefore, devoted to controlling the cassava cyanide that exists in two forms of cyanogenic glucosides, linamarin and lotaustalin [5]. It remains unknown if the cyanogenic glucosides will have any role in the starch synthesis or accumulation. Since sucrose metabolism and transport are closely linked to starch formation, this study has been undertaken to characterize the cassava sucrose transport or uptake system and to explore the interaction between cyanogenic glucosides and the sucrose uptake system. As sucrose uptake in cassava has never been studied before, this study began with the development of a leaf disc system to characterize the sucrose uptake in cassava. Furthermore, the effects of linamarin, the main cyanogenic glucoside in cassava, on the sucrose uptake system were studied.

Abbreviations: CCCP, chlorocarbonyl cyanide phenylhydrazone; DNP, *p*-nitrophenol; IA, iodoacetate; IPTG, isopropylthioglucoside; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzene sulphonate; PNPG, *p*-nitrophenylglucoside.

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2. Materials and methods

2.1. Chemicals

Radioactive sucrose (U-^{14}C) was purchased from Amersham Life Science. All fine chemicals were from Sigma and Merck.

2.2. Plant material

Fresh and fully expanded cassava leaves, usually the 4th and 5th leaf, were collected from plants grown in the experimental field of Mahidol University, Bangkok.

2.3. Preparation of cassava leaf discs

The thin cuticle at lower epidermis of cassava leaves that cannot be peeled off was partially removed by repeated light brushing using a fine plastic toothbrush. The brushing technique was adapted from that used in preparing protoplasts [6]. A leaf disc of 12 mm in diameter was then punched out using a cockborer [7]. The fresh leaf discs were immediately used in the sucrose uptake experiment.

2.4. Preparation of cassava root discs

Slices of 1.5 mm thick were prepared from fresh cassava root parenchyma using a Super slicer (UK Patent No. 2256579). A root disc of 12 mm in diameter was then punched out using a cockborer [7]. The root discs were washed three times in 0.3 M mannitol before use in the experiments.

2.5. Sucrose uptake measurement

The freshly prepared leaf discs (seven to ten discs) were floated with the brushed side down in 5 ml of standard medium in a petri dish (47 mm diameter). The standard medium consisted of 20 mM MES/NaOH, pH 5.0, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.3 M mannitol, 1 mM sucrose and (U-^{14}C) sucrose (7.4 M Bq/ml or 22.8 M Bq/mmole) [8]. The incubation dish was left at room temperature under fluorescent light. After 4 h, or the time specified otherwise, the leaf discs were removed from the incubation medium and washed three times in 20 ml of the medium without sucrose and radioactive sucrose. Each washing was done with gentle agitation for 3 min. Then, each washed disc was put into a scintillation vial containing 0.1 ml of 65% $HClO_4$ (v/v), 0.1 ml of 0.2% Triton X-100 and 0.3 ml of 35% H_2O_2. The vial was then tightly capped and incubated at room temperature for 2 h, then at 75°C for 8–12 h with occasional agitation to complete the digestion and decolorization [8]. The radioactivity (dpm) in the vial was measured using a Beckman LS 6000 liquid scintillation counter after adding 5 ml of PPO-POPOP-Triton-toluene scintillation fluid.

When the standard medium contained >1 mM sucrose or an additional sugar, the concentration of mannitol was correspondingly lowered in order to keep the osmolarity of the solution constant. When a test compound was dissolved in ethanol, a control experiment containing the same amount of ethanol was included to correct for the solvent effect.

The uptake of sucrose by cassava root discs was measured by the same method using the root discs instead of the leaf discs.

3. Results

3.1. Sucrose uptake by the cassava leaf discs

At 1 or 10 mM sucrose, the uptake of radioactivity by the leaf discs increased linearly with time up to 4 h. After that, the uptake proceeded with a slight acceleration. So, a fixed time of 4 h was used in further experiments. When the concentration of sucrose increased, the rate of the sucrose uptake also increased, approaching a maximal rate as the sucrose concentration was reaching saturation. The saturation curve of the sucrose uptake appeared to follow the Michaelis–Menten kinetics (Fig. 1). The double reciprocal plot of the saturation curve yielded a K_m value for sucrose of 1.3 mM.

To establish the characteristics of the sucrose uptake system of the cassava leaf disc, several agents were tested for their effects on the rate of uptake. These agents were: (a) sulfhydryl reagents; (b) metabolic inhibitors; and (c) linamarin and other glycosides. In order to detect the effect on the sucrose uptake, the tests were performed using 1 mM sucrose, which was near its K_m value.

3.2. Effects of sulfhydryl reagents and metabolic inhibitors on the sucrose uptake by cassava leaf discs

NEM, PCMBS and IA were found to be inhibitory to the cassava sucrose uptake system (Table 1). Among these reagents, NEM was the strongest inhibitor, giving a nearly complete inhibition at 0.5 mM.

The metabolic inhibitors selected for the test were those acting on the oxidative phosphorylation (DNP, CCCP and KCN) and those acting on the ATPases (vanadate and erythrosin B) [9]. Both DNP and CCCP were found to be strong inhibitors of the sucrose uptake, but KCN was only slightly inhibitory (Table 1). For the ATPase inhibitors, vanadate was a much better inhibitor than erythrosin B (Table 1).

Table 1
Effects of various compounds on ^{14}C-sucrose uptake by cassava leaf discs[a]

Compounds	Maximal conc. tested (mM)	Relative uptake rate % (n)
Control	–	100 (5)
NEM	0.5	3 (3)
PCMB	0.5	28 (3)
IA	1	27 (2)
Phloridzin	2	18 (2)
DNP	0.001	41 (3)
CCCP	0.001	12 (3)
KCN	0.5	77 (2)
V_2O_5	1	24 (2)
Erythrosin B	10	82 (2)
D-Glucose	10	92 (2)
D-Fructose	10	71 (2)
Lactose	10	119 (2)
Palatinose	10	108 (2)
IPTG	0.5	74 (2)
β-PNPG	1	4 (2)
α-PNPG	1	33 (2)

[a] The compounds were added at the beginning of incubation. n, number of experiments.

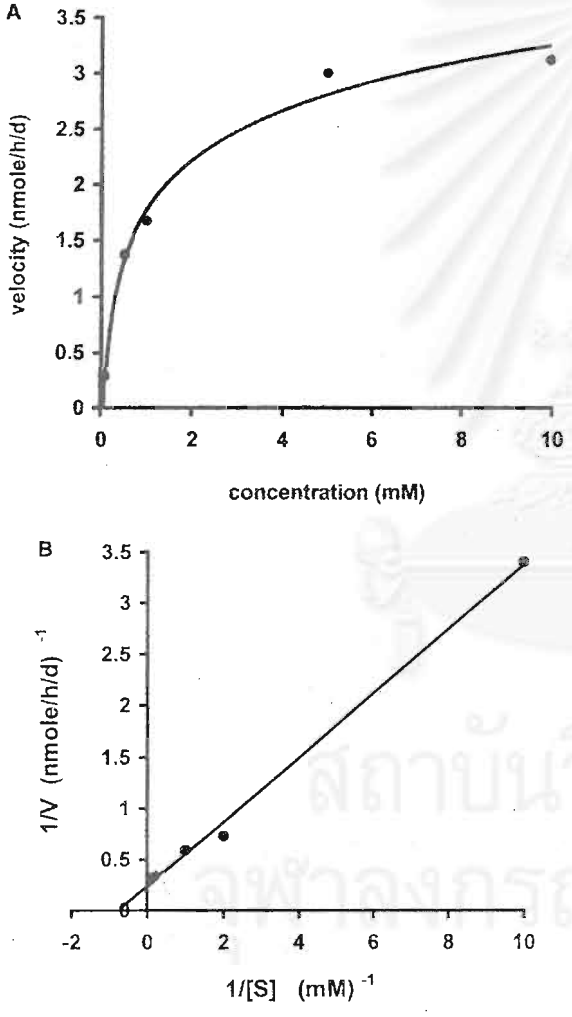

Fig. 1. (A) Saturation curve of the sucrose uptake by cassava leaf discs. The uptake rate is expressed in nmole per hour per disc. Three experiments were carried out. (B) Lineweaver–Burk plot of the saturation curve of the sucrose uptake by cassava leaf discs.

3.3. Inhibition of the cassava sucrose uptake by linamarin and other glycosides

Linamarin strongly inhibited the cassava sucrose uptake by the leaf discs, giving ≈80% inhibition at 0.4 mM (Fig. 2). Two other cyanogenic glycosides, prunasin and amygdalin, were much less inhibitory. Among the selected synthetic glu-

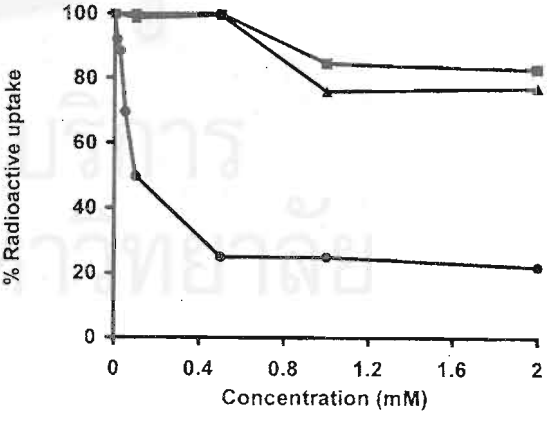

Fig. 2. The inhibition of the sucrose uptake of cassava leaf discs by linamarin (●; $n=4$), prunasin (▲; $n=2$) and amygdalin (■; $n=2$).

Table 2
Effects of various compounds on ^{14}C-sucrose uptake by cassava root discs[a]

Compounds	Maximal conc. tested (mM)	Relative uptake rate % (*n*)
Control	–	100 (3)
NEM	1	34 (4)
PCMB	0.5	37 (1)
IA	1	89 (1)
V_2O_5	1	62 (1)
Linamarin	1	161 (2)
Linamarin	5	238 (2)

[a] The compounds were added at the beginning of incubation. *n*, number of experiments.

cosides tested, both α and β forms of PNPG were better inhibitors than IPTG (Table 1). PNPG was a known inhibitor of sucrose uptake [8]. Phloridzin, which was a plant bioflavonoid glycoside and known to inhibit sucrose and Na^+-glucose transport in plants [10], was able to inhibit the cassava sucrose uptake (Table 1).

In contrast, other simple sugars, namely glucose, fructose, lactose and palatinose, only weakly inhibited or stimulated the sucrose uptake by the cassava leaf discs (Table 1).

3.4. The sucrose uptake by cassava root discs

Preliminary experiments showed that cassava root discs were also found to take up sucrose and its rate at 1 mM sucrose was ≈1.2–1.4 times that of the leaf discs. The uptake by the root discs was also inhibited by NEM, PCMB, IA and V_2O_5 (Table 2). In contrast to the cassava leaf discs, the uptake of sucrose by the cassava root discs was stimulated by linamarin (Table 2).

4. Discussion

Like other plants, cassava is expected to possess a sucrose transport system. However, this is the first experimental evidence for its existence in cassava leaf because the system shows a simple substrate saturation curve (Fig. 1). In other plants, the sucrose transport proteins are sensitive to sulfhydryl agents and its function is dependent on ATP and/or proton [11,12]. The sucrose uptake or transport system of cassava leaf appears to share some of these common characteristics, as implied by the following lines of evidence. Firstly, it is sensitive to sulfhydryl reagents (Table 1). Secondly, it is inhibited by protonophores DNP, CCCP and phloridzin (Table 1). Thirdly, it is also inhibited strongly by vanadate that is a *P*-type ATPase inhibitor (Table 1). However, the observations of the weak inhibition of the system by KCN, that is a respiratory chain inhibitor and by erythrosin B (Table 1), which is a H^+-ATPase inhibitor [13] appear inconsistent with the above suggestion. To explain these conflicting observations, one may speculate that the respiratory chain in cassava is CN-insensitive and H^+-ATPase does not associate itself with the sucrose uptake system of cassava leaf. Alternatively the weak effects by KCN and erythrosin B may be simply due to poor penetration of the compounds.

An interesting characteristic of the sucrose uptake system of cassava leaf is its sensitivity to the inhibition by its major cyanogenic glucoside, linamarin (Fig. 2). This is rather specific, since two other tested cyanogenic glycosides are much less effective (Fig. 2). The linamarin inhibition is unlikely to be due to its breakdown products, KCN and glucose, since neither shows a strong inhibitory effect on the sucrose uptake system. The inhibition of the cassava sucrose uptake by PNPG, but not IPTG, further supports the notion that there is a specificity of the inhibitory site. The inhibition mechanism of linamarin is yet unknown. Linamarin may act by competing for the sucrose-binding site of the sucrose transport protein. Further purification of the cassava sucrose transport protein will have to be carried out before the mechanism of the linamarin inhibition can be investigated.

This study also provides the first evidence on the existence of the sucrose transport system in cassava root parenchyma. The preliminary evidence suggests that, like the sucrose uptake by the cassava leaf discs, the sucrose uptake by the root discs is sensitive to inhibition by sulfhydryl reagents and ATPase inhibitors (Table 2). The lower sensitivity to the inhibitory agents may simply be due to poorer permeabilities of the test compounds in the root discs. However, it is interesting to note the different effects of linamarin that inhibits the sucrose uptake in the leaf (Fig. 2) but activates the uptake in the root parenchyma (Table 2). Linamarin is known to accumulate in cassava leaves and roots to concentrations as high

as 500 mg/kg fresh weight [5,14] or ≈2 mM. Therefore, the observed effects of linamarin in the millimolar range (Fig. 2, Table 2) should have certain physiological relevance. Although the observed differences in the leaf and the root need further investigation using purified membranes, the results imply that the cyanogenic glucoside may play a role in regulating the sucrose transport that may alter the subsequent distribution of sucrose and its metabolism in cassava. If this is the case, it will be a new function of the biological cyanide in cassava. To explore this possibility further, study will be made on the fate of the absorbed sucrose in the cassava leaf and root discs.

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