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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. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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.

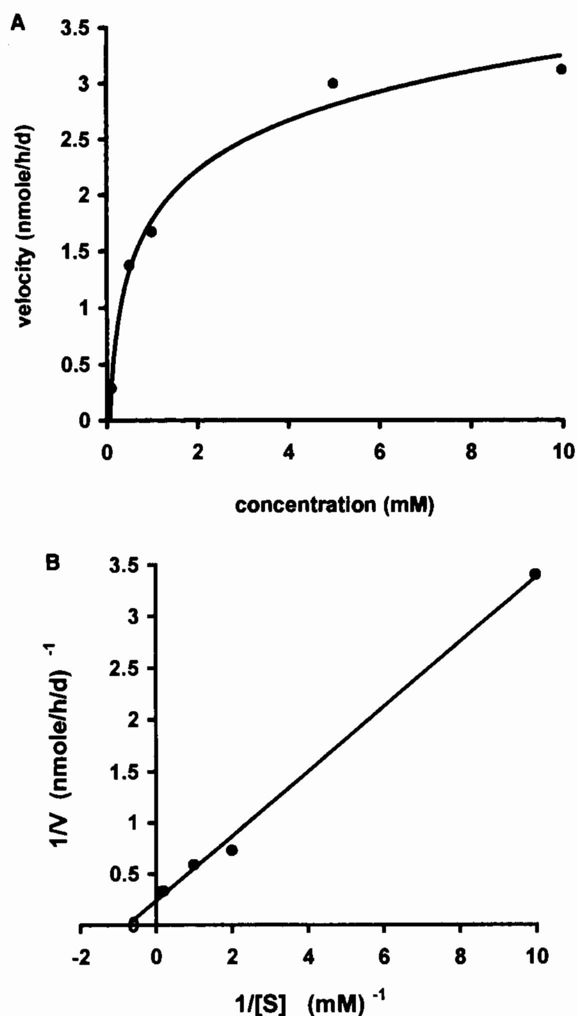

Fig. 1. (A) Saturation curve of the sucrose uptake by cassava leaf discs. The uptake rate is expressed in nmole per hour per disc. Three experiments were carried out. (B) Lineweaver–Burk plot of the saturation curve of the sucrose uptake by cassava leaf discs.

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-

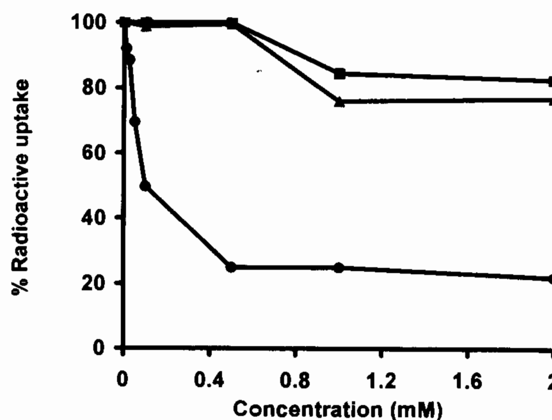

Fig. 2. The inhibition of the sucrose uptake of cassava leaf discs by linamarin (●; *n* = 4), prunasin (▲; *n* = 2) and amygdalin (■; *n* = 2).

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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