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KINETIC PROPERTIES OF β-GLUCOSIDASE FROM CASSAVA

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Key Word Index—*Manihot esculenta*; cassava; β-glucosidase; linamarase; K_m values; inhibition kinetics.

Abstract—β-Glucosidases from the leaf, peel and tuber cortex of cassava cv. Merah Jambu exhibited linamarase activity and had in common many kinetic properties. They were also capable of hydrolysing *p*-nitrophenyl-β-D-monoglycosides and cyanogenic β-monoglucosides but lacked activity towards the *p*-nitrophenyl-β-D-diglycosides, a cyanogenic diglucoside, and other β- or α-linked disaccharides. The K_m values for *p*-nitrophenyl-β-D-monoglycosides were generally lower than those for linamarin, prunasin and salicin. Ag^{2+} inhibited both β-glucosidase and linamarase activities. Glucono-1,5-lactone inhibited the enzyme competitively, irrespective of the substrates used, while imidazole showed competitive inhibition with linamarin but non-competitive (mixed) inhibition with *p*-nitrophenyl-β-D-glucoside. The enzyme was unaffected by glucose.

INTRODUCTION

Cassava is cultivated in many parts of the tropics primarily for the storage tubers but the young leaves are also consumed by humans or used as animal feed [1]. The leaves and tubers of these plants contain linamarase, a β-glucosidase (E.C. 3.2.1.21) [2–4] which catalyses the hydrolysis of linamarin, resulting in the release of hydrogen cyanide [5]. This enzyme, prepared from the peel of tubers, is used in the estimation of cyanide in cassava tubers [6]. The few reports on this enzyme have been mainly concerned with the peel linamarase [2–4]. There is hardly any published data on the kinetic properties of the leaf and tuber enzymes prepared from the same plant. It was felt, therefore, that a comparative study would yield valuable information on the enzymes found in different parts of the same plant and may help establish the nature of their origin. In addition, it might suggest an alternative source of linamarase (e.g. from the leaves) for the determination of cyanide. In this study, the kinetic properties and substrate specificity of β-glucosidase partially purified from the leaves, peel and tuber cortex of cassava cv. Merah Jambu were investigated. The linamarase activity was also characterized and comparisons made with those of other plant linamarases.

RESULTS AND DISCUSSION

Fractions eluted from a Sephacryl S-300 column contained both β-glucosidase and linamarase activity. The enzyme as prepared from the leaf, peel and tuber cortex showed maximal activity in the pH range 6–7.3 with both linamarin and *p*-nitrophenyl-β-D-glucoside (PNP-β-D-glucoside) as substrates. With both substrates, the enzyme exhibited some 85% of its maximum activity at pH 5 and 8, reflecting a broad pH range for activity. This property differs from those of linamarases from *Trifolium*, *Linum*, *Phaseolus* and *Hevea* which exhibit not only lower optimum pH values but also narrower pH ranges for maximal activity, irrespective of whether linamarin or PNP-β-D-glucoside is used as substrate [7–10].

Cassava β-glucosidase gave maximal linamarase activity at 55° in a 15 min assay. With PNP-β-D-glucoside, maximal activity was obtained at 60°. However, the enzyme was not stable at the optimal temperature for hydrolysis of the substrates. Both the leaf and peel β-glucosidase lost about 34 and 45% of their linamarase activity, respectively, after 30 min at 55° while the tuber cortex enzyme suffered a 10% loss. With PNP-β-D-glucoside, both the leaf and peel enzymes lost about 80% of their activity after 30 min at 60° but the tuber enzyme lost only 30% of its activity. At 50° for 1 hr, the leaf and peel enzymes lost 50 and 15% of their initial activity, respectively with either linamarin or PNP-β-D-glucoside. The tuber cortex β-glucosidase, however, retained its full activity with both substrates. Thus unlike the enzymes of the leaf and peel, the tuber cortex enzyme was more heat stable. However, for the kinetic studies, the incubations were carried out at 37°. Repeated freezing–thawing and storage at −10° for a year did not decrease the enzyme activity.

The leaf, peel and tuber cortex enzyme exhibited a very similar energy of activation with respect to linamarin i. e. 3.0, 4.0 and 3.5 kcal/mol respectively. With PNP-β-D-glucoside, the leaf and peel β-glucosidase yielded an activation energy of 6.5 kcal/mol while the tuber cortex enzyme gave a value of 5.8 kcal/mol.

The optimum temperature recorded for PNP-β-D-glucoside activity is very similar to that of *Hevea* linamarase [9]. In addition, the poor thermal stability of the cassava leaf and peel enzyme is also seen in linamarases of *Phaseolus* and *Trifolium* [10, 11].

The catalytic specificity of the cassava β-glucosidase towards various glycosides was tested (Table 1). The leaf and peel β-glucosidase preparations gave higher specific activities than those of the tuber cortex enzyme. Nonetheless, they all showed a similar mode of action. They were better at hydrolysing the β-linkage of PNP from β-monoglycosides such as, PNP-β-D-glucoside, PNP-β-D-fucoside, PNP-β-D-galactoside, PNP-β-D-mannoside, PNP-β-D-xyloside, and showed little or no activity towards β-diglycosides such as PNP-β-D-cellobioside, PNP-β-D-maltoside, PNP-β-D-melibioside and PNP-β-D-gentiobioside. Among the PNP-β-monoglycosides, the enzyme was highly active towards PNP-β-D-glucoside and PNP-β-D-fucoside but showed little activity towards

Table 1. Activity of β-glucosidase towards various glycosides

Substrate (5 mM)	β-Glucosidase activity (nkat/mg protein)		
	Leaf	Peel	Tuber cortex
PNP-β-D-glucoside	917	400	3.3
PNP-β-D-fucoside	917	416	5.0
PNP-β-D-galactoside	33	13	0.2
PNP-β-D-mannoside	33	8	0.1
PNP-β-D-xyloside	12	5	0.1
PNP-β-D-cellobioside	0.2	0.1	0
PNP-β-D-maltoside	0.2	0.1	0
PNP-β-D-lactoside	0.1	0.1	0
PNP-β-D-melibioside	0.1	0.1	0
PNP-β-D-gentiobioside	<0.1	0.1	0
Linamarin	150	50	3.3
Prunasin	5	2	0
Salicin	5	2	0
Amygdalin	0	0	0
Cellobiose	0	0	0
Gentiobiose	0	0	0
Isomaltose	0	0	0
Maltose	0	0	0

the other monoglycosides. Of the cyanogenic β-monoglucosides used, linamarin, but not prunasin or salicin, was readily hydrolysed by the cassava β-glucosidase. It showed no activity towards amygdalin, a cyanogenic β-diglucoside, and disaccharides having α- or β-linkages, such as maltose, isomaltose, cellobiose and gentiobiose.

Despite the possibility that the enzyme preparations may contain other glycosidases as impurities, it is obvious that the cassava β-glucosidase is inactive with glycosides with a disaccharide structure, e. g. PNP-β-D-diglycosides and amygdalin. Their inability to hydrolyse the β-linkages of disaccharides further reflects the absence of β-diglucosidase activity. Although the cassava β-glucosidase behaves more as a β-monoglycosidase, the nature of the monosaccharide residue as well as the aglycone structure is important for enzyme activity. It is noteworthy that some of these observations are similar to those made for linamarases from *Linum*, *Hevea* and *Phaseolus* [8–10]; for example, the β-monoglycosides are better substrates than the β-diglycosides and among these, PNP-β-D-glucoside is often the preferred substrate.

β-Glucosidase from the leaf, peel and tuber cortex exhibited Michaelis–Menten kinetics with the different substrates tested (see Table 2). No substrate inhibition was observed at 20 mM. With respect to the leaf enzyme the K_m values for the various PNP-β-D-glycosides ranged from 0.26 ± 0.02 mM for PNP-β-D-galactoside to 0.42 ± 0.02 mM for PNP-β-D-fucoside except for PNP-β-D-mannoside ($K_m = 6.69 \pm 0.14$ mM). β-Glucosidase of the peel and tuber cortex gave K_m values within the same range as those for the leaf enzyme for the different substrates, with the exception of the tuber cortex K_m (PNP-β-D-mannoside). Cassava leaf β-glucosidase gave a lower K_m value for linamarin ($K_m = 2.08 \pm 0.15$ mM) as compared with those for salicin ($K_m = 3.56 \pm 0.38$ mM) and prunasin ($K_m = 12.22 \pm 1.40$ mM). The same kinetic pattern was observed with the peel enzyme. This reflects the higher affinity the β-glucosidase has for the natural substrate, linamarin. It is evident, however, that the enzyme has an even greater affinity for chromogenic substrates i.e. the β-monoglycosides. This is also true of other plant linamarases [9, 10].

There was no correlation between the substrate affinity (low K_m) and the catalytic ability (v_{max}) of the cassava β-glucosidase. Nevertheless, comparisons of the v_{max}/K_m values for the various substrates re-affirmed earlier observations on the substrate specificity of the cassava β-glucosidase (Table 2). Thus among the β-monoglycosides, the enzyme showed greater specificity for those having glucose or fucose as the monosaccharide residue, and among the cyanogenic monoglucosides, the aglycone moiety becomes important for catalysis.

Cassava β-glucosidase was inhibited by 1 mM Ag^{2+}, but not by Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} at the same concentration, irrespective of whether linamarin or PNP-β-D-glucoside was used as the substrate. The extent of inhibition was quite similar in all cases (65–77%) except for the leaf enzyme with PNP-β-D-glucoside as the substrate (93%) (Table 2). Glucono-1,5-lactone inhibited the cassava β-glucosidase competitively with respect to both linamarin and PNP-β-D-glucoside as substrates (Table 2). The K_i values for linamarase inhibition ranged from 0.36 mM for the leaf enzyme to 0.97 mM for the tuber cortex enzyme. With PNP-β-D-glucoside as substrate, the K_i values obtained for the enzymes were very similar, ranging from 0.54 to 0.56 mM (Table 2). Glucose, however, did not affect the β-glucosidase or its linamarase activity. Imidazole also inhibited the linamarase activity competitively, with K_i values ranging from 1.95 mM for the leaf enzyme to 5.71 mM for the tuber cortex enzyme. This inhibitor, on the other hand, inhibited the leaf, peel and tuber cortex β-glucosidase non-competitively (mixed). This was revealed by $K_{iES} > K_{iE}$ (Table 2). Comparisons of the K_i and K_{iE} values showed that glucono-1,5-lactone was a more potent inhibitor of the enzyme. *Phaseolus* linamarase is also competitively inhibited by glucono-1,5-lactone [8]. By contrast, the *Trifolium* enzyme is inhibited by both glucono-1,5-lactone and glucose [11]. Data on substrate specificity and inhibition by glucono-1,5-lactone and imidazole suggest a specific requirement of the enzyme active site, namely a planar half-chair conformation of the reactive intermediate [8].

Although differences in kinetic parameters have been observed among the three cassava β-glucosidase, overall, they share many common properties. In some respects, they are similar to other plant linamarases. More important, the close kinetic similarity among the cassava β-glucosidases reflects structural similarity in the enzyme molecules; this points to a common gene pool. However, variations in the kinetic parameters of the enzymes may reflect a certain degree of flexibility in the gene pool. This study has also shown that leaf β-glucosidase could be used a substitute for the peel enzyme in the determination of cyanide. In fact, the leaf represents a readily available source of enzyme.

EXPERIMENTAL

Plant material. Cassava (*Manihot esculenta* Crantz cv. Merah Jambu) cuttings were grown in the gardens of the Botany Department, National University of Singapore. Leaves and tubers were harvested from 8-month-old plants.

Enzyme preparation. Unless otherwise stated, all preparations were carried out at 4°. Freshly harvested and deveined leaves (50 g fr. wt) were homogenized in a blender with 100 mM Na citrate buffer (pH 6) containing 1% (w/v) PVP-10 in a ratio of

Table 2. Kinetic properties of cassava β-glucosidases

Property	β-Glucosidase of		
	Leaf	Peel	Tuber cortex
K_m values (mM)			
PNP-β-D-glucoside	0.31±0.03	0.31±0.03	0.28±0.03
PNP-β-D-fucoside	0.42±0.02	0.32±0.07	0.49±0.07
PNP-β-D-galactoside	0.26±0.02	0.27±0.05	0.41±0.10
PNP-β-D-mannoside	6.69±0.14	6.68±0.16	3.61±0.22
PNP-β-D-xyloside	0.39±0.15	0.27±0.07	0.31±0.14
Linamarin	2.08±0.15	2.34±0.33	3.93±0.33
Prunasin	12.22±1.40	12.96±1.30	nd
Salicin	3.56±0.38	3.83±0.56	nd
V_{max}/K_m (nkat mg protein/mM)			
PNP-β-D-glucoside	3280	1397	12
PNP-β-D-fucoside	2023	1300	10
PNP-β-D-galactoside	147	49	1.2
PNP-β-D-mannoside	9	3	0.1
PNP-β-D-xyloside	51	24	0.5
Linamarin	101	38	2
Prunasin	0.2	0.2	nd
Salicin	4	2	nd
Inhibitors			
1 mM Ag^{2+} (% inhibition)			
with linamarin	67	75	65
with PNP-β-D-glucoside	93	77	70
Glucono-1,5-lactone			
with linamarin (K_i, mM)	0.36	0.78	0.97
with PNP-β-D-glucoside (K_i, mM)	0.56	0.55	0.54
Imidazole			
with linamarin (K_i, mM)	1.95	2.69	5.71
with PNP-β-D-glucoside			
K_{iES} (mM)	21.14	22.32	13.80
K_{iE} (mM)	0.98	1.85	1.05

nd = not determined due to absence of activity.

1 g fr. wt leaf to 5 ml buffer. For the peel (42 g) and cortex (200 g) of the tuber, the ratio was 1 g fr. wt material to 2 ml buffer. The homogenate was centrifuged and the supernatant pptd with $(NH_4)_2SO_4$ to 60% satn. The ppt. was recovered by centrifugation and resuspended in a minimum vol. of 100 mM Na citrate buffer (pH 6). 5 ml of the $(NH_4)_2SO_4$ fraction was then eluted through a Sephacryl S-300 column (33 × 2.5 cm) and fractions (3.8 ml each) with high β-glucosidase activity were collected for kinetic studies. Fractions were also monitored for linamarase activity. Protein content was determined according to the modified Lowry's method [12].

β-Glucosidase assay. The enzyme activity was assayed using 10 mM PNP-β-D-glucoside in 50 mM Na citrate, pH 6, at 37°. The total reaction vol. was maintained at 600 μl. The reaction was stopped after 15 min by the addition of 3 ml 0.1 M Na_2CO_3, and the absorbance was measured at 420 nm [13].

Linamarase assay. This was carried out using 10 mM linamarin in 50 mM Na citrate, pH 6, at 37°. The total reaction vol was 600 μl. After 15 min, the glucose liberated was determined by the glucose oxidase method [14].

Kinetic studies. Kinetic studies were carried out using both linamarin and PNP-β-D-glucoside as substrates. The effect of pH on enzyme activity was studied using citrate-phosphate buffer (pH range 3–7) and phosphate buffer (pH range 6–8). The temp. optimum was determined over the temp range 30–70°. The energy of activation was calculated using the Arrhenius equation. Thermal stability of the enzyme was studied at temperatures ranging from 30 to 80° over a period of 60 min. The ability of the enzyme to hydrolyse various PNP-β-D-glycosides, cyanogenic glycosides and disaccharides was measured at a substrate concentration of 5 mM. For cyanogenic glycosides and disaccharides, the glucose released was determined by the glucose oxidase method [14]. K_m values were determined using substrate concentrations ranging from 0.2 to 20 mM and were calculated using the method of Wilkinson [15]. The K_i for glucono-1,5-lactone or imidazole was determined using the above substrate concentrations and in the presence of 1, 5 and 10 mM inhibitor.

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