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PURIFICATION AND CHARACTERISATION OF β-CYANOALANINE SYNTHASE FROM CASSAVA TISSUES

MERINA ELIAS, P. R. SUDHAKARAN* and BALA NAMBISAN†

Department of Biochemistry, University of Kerala, Kariavattom, Trivandrum. 695 581, India; † Central Tuber Crops Research Institute, Sreekaryam, Trivandrum 695 581, India

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Abstract—The occurrence of the β-cyanoalanine pathway for detoxication of cyanide in cassava was examined by studying the activity of β-cyanoalanine synthase (β-CAS) [EC.4.4.1.9], which is the major enzyme involved in the conversion of cyanide to β-cyanoalanine. β-CAS was partially purified from cassava leaf, rind and tuber. Its specific activity in the tuber was significantly greater than that in the leaf and rind. The purified enzyme appeared as a single band with M_r of 50 k in SDS-PAGE. On gel filtration over Sephadex G-150, the enzyme was eluted as a single peak with an average M_r of 51 k. β-CAS from all three tissues had an optimum pH of 9.0 and showed maximum activity at 30° and the K_m (cyanide) was similar. The presence of serine, an alternate substrate for the enzyme did not affect the utilisation of cysteine for the formation of β-cyanoalanine. © 1997 Elsevier Science Ltd

INTRODUCTION

Linamarin and lotaustralin are the cyanoglucosides present in cassava, linamarin being the major one [1]. The concentration of linamarin varies in different tissues of the cassava plant [2, 3] and is influenced by the rate of its biosynthesis, degradation and transport from and to the tissues. Although girdling experiments suggest that linamarin is transported from the leaves to the tubers [4–6], there is no accumulation of linamarin in tubers. The linamarin content of tubers is also lower than that of leaf and stem. The degradation of linamarin in cassava proceeds by the action of the enzymes linamarase, [EC 3.2.1.21] and hydroxynitrile lyase [EC 4.1.2.37], which hydrolyse linamarin to acetone cyanohydrin and then to cyanide. However, free cyanide is not detected in intact cassava tissues, indicating that it is rapidly detoxified. One of the pathways for the detoxication of cyanide is through conversion of cyanide to thiocyanate by the enzyme rhodanese. This pathway is apparently not significant in the cassava tuber because both rhodanese activity and thiocyanate are hardly detectable in the tuber [3]. Another pathway involves the conversion of cyanide to β-cyanoalanine (β-CA) which is hydrolysed to asparagine. The possibility of such a pathway was indicated in certain cyanophoric plants by the demonstration of β-cyanoalanine synthase (β-CAS) activity [7, 8]. Similar studies also showed high activity of β-CAS in cassava tissues, suggesting the importance of the latter pathway in the detoxication of cyanide in cassava [3]. Studies on the nature of β-CAS would help in providing an insight into the process of linamarin turnover in cassava. In this paper we report on the partial purification and characteristics of β-CAS in the leaf, rind and tuber tissues of cassava.

RESULTS AND DISCUSSION

Purification of β-cyanoalanine synthase from cassava tissues

The possibility of utilisation of cyanide formed by the degradation of cyanoglucosides in different tissues of cassava was examined by measuring the activity of β-CAS using cysteine and cyanide as substrates. Significant β-CAS activity was found in cassava leaf, rind and tuber tissues. A crude homogenate of tuber was found to have higher enzyme activity than that of the leaf and rind tissues. This is in contrast to the pattern of linamarase, which is higher in the leaf and rind as compared to tuber [3]. The tissue extract was subjected to fractionation for the purification of β-CAS. The results of purification of the enzyme from leaf, rind and tuber are given in Table 1. The extent of purification was 2–4-fold on ammonium sulphate

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* Author to whom correspondence should be addressed.

Table 1. Purification of β-cyanoalanine synthase from different tissues of cassava

Tissue	Sample	Volume (ml)	Protein (mg)	Total activity (units)*	Sp. activity (units mg^{-1})	Degree of purification	Yield
Leaf	1. Initial extract	28	83.2	108	1.3	1.0	100
	2.60% $(NH_4)_2SO_4$ Precipitate	4	5.3	30	5.6	4.3	25
	3. DEAE cellulose chromatography	4	0.93	13.0	13.8	10.6	12
Rind	1. Initial extract	38	69.8	105	1.5	1.0	100
	2. 60% $(NH_4)_2SO_4$ Precipitate	6	8.2	52	6.3	4.2	50
	3. DEAE cellulose chromatography	4	1.0	16	16.4	10.9	15
Tuber	1. Initial extract	54	85.3	150	1.8	1.0	100
	2. 60% $(NH_4)_2SO_4$ Precipitate	8	10.3	38	3.7	2.1	25
	3. DEAE cellulose chromatography	4	1.1	18	16.5	9.4	12

* Microgram sulphide liberated/minute.

fractionation and about 10-fold after DEAE-cellulose chromatography. About 70–80% of the total activity was eluted from DEAE-cellulose with 0.2 M NaCl, and small amounts were eluted at 0.1 M and 0.3 M NaCl concentrations. The same pattern was obtained for the enzyme from the three different tissues tested. The fraction eluted with 0.2 M NaCl was dialysed, concentrated and further purified by gel filtration over Sephadex G-150. The enzyme was eluted as a sharp peak with M_r of 51 k. The purity of the fraction when checked by SDS-PAGE (Fig. 1) revealed the presence of a major polypeptide band with M_r of 50 k. The enzyme purified from blue lupine seedlings has a M_r of 52 k [9].

Kinetic analysis of β-CAS

K_m *(cyanide) of β-cyanoalanine synthase.* The effect of substrate concentration on the activity of β-CAS was examined by determining the activity at different concentrations of NaCN, and keeping the concentration of cysteine (10 mM) constant. It appeared to follow saturation kinetics. The k_m values of the β-CAS from leaf (7 mM), rind (8 mM) and tuber (5 mM), (cyanide) obtained from Lineweaver–Burk plots (Fig 2) were found to be similar. This is about 10-fold higher than that reported for β-CAS from blue lupine (550 μm) which is a non-cyanogenic plant [10]. But the k_m reported for the other substrate cysteine (2.5 mM) for the blue lupine β-CAS was high [10]. Although the k_m (cyanide) is high, the level of free cyanide in intact tissues of cassava is negligible, indicating the possible occurrence of a mechanism for activation of β-CAS *in vivo*.

Effect of temperature. The effect of temperature on β-CAS from different tissues was studied over the range from 20 to 45° using the assay conditions given in the Experimental section. Maximum activity was found at 30°; activity was significantly reduced at temperatures above 40°.

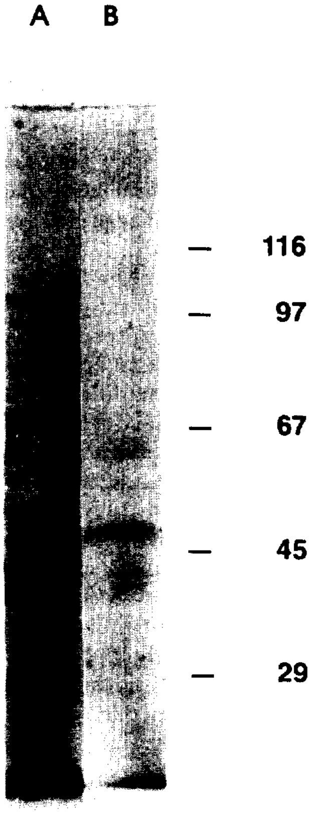

Fig. 1. SDS-PAGE analysis of purified β-cyanoalanine synthase. β-Cyanoalanine synthase purified from tuber tissue was subjected to 10% SDS-PAGE under reducing conditions and the bands were stained by Coomassie Blue. Lane A represents the initial extract and lane B represents the purified β-cyanoalanine synthase.

Effect of pH. β-CAS from different tissues was

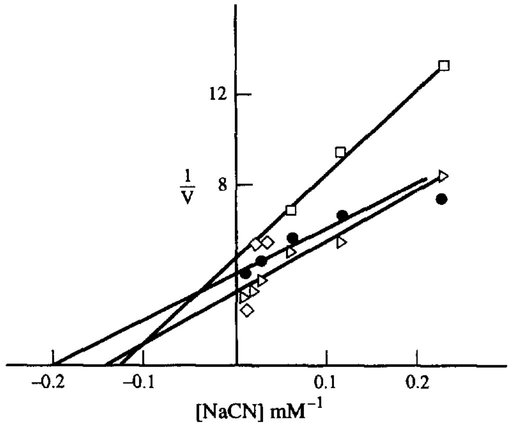

Fig. 2. Effect of concentration of substrate (cyanide) on β-cyanoalanine synthase activity. The activity of β-cyanoalanine synthase was determined using cysteine (10 mM) and different concentrations of sodium cyanide. Assay was carried out at 30° for 15 min. The amount of enzyme was equivalent to 0.6–1.0 mg protein. K_m (cyanide) of leaf (△), rind (●) and tuber (□) were calculated from Lineweaver–Burk plots.

assayed from pH 6.5 to 9.5 and maximum activity was found at pH 8.5–9.0. Unlike linamarase and hydroxynitrile-lyase, which have acidic pH optima (5.5 to 6.0), β-CAS of cassava tissues has an alkaline pH optimum (9.0) and shows low activity at lower pH.

Effect of serine on the activity of β-CAS. The β-CAS enzyme can also utilize serine in place of cysteine *in vivo*, in which case the products formed would be β-CA and water. Since direct assay of the enzyme using serine as substrate, could not be done, the assay of the enzyme was carried out using cysteine as substrate, in the presence of varying amounts of serine. The activity of the enzyme in the presence of serine was not significantly different from the control. Since in cassava tissues the amount of sulphur containing amino acids is very low, it is possible that serine may be utilised *in vivo* by β-CAS. Since the presence of serine in the assay system did not affect the conversion of cysteine to β-CAL, this raises the possibility that cysteine and serine may be binding at two independent sites on the enzyme.

Comparison of the M_r and the kinetic properties indicates the similarity of cassava β-CAS and that of blue lupine, which is a non-cyanogenic plant. β-CAS purified from blue lupine also appeared to have an alkaline pH optimum [10]. β-CAS activity has been shown to be present in the mitochondrial fraction of etiolated blue lupine seedlings [10]. Although the occurrence of the enzyme for the detoxication of HCN formed during the degradation of cyanoglucoside in cyanogenic species can be explained, in higher plants β-CAS is suggested to be involved in the detoxication of HCN produced when 1-amino cyclo propane-1-carboxylic acid is converted to ethylene [11]. Detailed analysis of the subcellular distribution of the enzyme in cassava tissues is required to understand the segregation if any, of the cyanide detoxifying activity. Hydrolysis of β-CA by β-CA hydrolase produces asparagine which then enters the general metabolic pathway. Activity of β-CA hydrolase has been found in cassava tissues (M. Elias *et al.*, unpublished data). Like β-cyanoalanine synthase, β-cyanoalanine hydrolase also has an alkaline pH optimum. This suggests that in the cassava tissues, particularly in the tuber, the cyanoglucoside metabolism results in the conversion of the secondary metabolite to asparagine. It may therefore serve as a mechanism for the conversion of toxic nitrogenous components in the plant, which may have a protective role, into a useful primary metabolite.

EXPERIMENTAL

Plant material. Leaf, rind and tuber tissues from cassava cultivar H-165 were used for the studies.

Chemicals. L-Cysteine hydrochloride, *N,N*-dimethyl *p*-phenylene diamine sulphate (DPDS), DEAE-cellulose, Sephadex G–150 and bovine serum albumin, were purchased from Sigma. All chemicals used were of analytical grade.

β-Cyanoalanine synthase assay. The enzyme was assayed according to the method of [7]. The assay system contained 0.5 ml of 10 mM L-cysteine hydrochloride, 0.5 ml of 100 mM NaCN, aliquots of enzyme extract and Tris–HCl buffer 0.1 M pH 8.5 to make upto a total vol. of 2 ml. After incubation for 10 min at 30° in closed tubes, 0.5 ml of 0.03 M $FeCl_3$ in 1.2 M HCl was added, followed by 0.5 ml of 0.02 M DPDS in 7.2 M HCl. The samples were centrifuged after 20 min to remove pptd protein. *A* of samples were read at 650 nm. A unit of enzyme activity was defined as that amount of enzyme which releases 1 μg H_2S per min per mg protein.

Protein in the enzyme prepn was determined by the method of [12] using BSA as standard.

Extraction of enzyme. Dry powder was prepd by grinding the plant tissues (leaf, rind and tuber) with chilled Me_2CO in a Waring blender, filtered, dried *in vacuo* and stored at −20°. The dry powder was homogenised with (1:15) 0.01 M Tris–HCl buffer pH 8.9 at 4°C and then centrifuged at 20 000 *g* for 15 min. The supernatant was collected and used as crude extract.

$(NH_4)_2SO_4$ fractionation. The crude extract was pptd by addition of $(NH_4)_2SO_4$ to 60% saturation and kept overnight at 4°. The ppt obtained was dissolved in Tris–HCl buffer (0.1 M, pH 8.6) and dialysed overnight at 4° against the same buffer.

DEAE-cellulose chromatography. The dialysed samples were applied on a DEAE-cellulose column (1.5 × 10 cm) equilibrated with Tris–HCl buffer (0.1 M pH 8.6). The column was washed with the same buffer and eluted with an increasing amount of NaCl (0.1–0.5 M) in the same buffer. Frs were collected, assayed for β-CAS activity and those frs containing maximum activity were pooled and dialysed against

0.1 M Tris HCl pH 8.6 overnight in the cold. The purified enzyme was used for kinetic studies.

Gel filtration. The DEAE-cellulose purified enzyme prepn was dialysed, concd with dry powder of Sephadex G-25 and applied to a Sephadex G-150 column (1 × 90 cm) preequilibrated with 0.1 M Tris–HCl buffer pH 8.6. 2 ml frs were collected, assayed for β-CAS activity. Calibration for M_r was done using BSA, ovalbumin and lactoglobulin.

SDS-PAGE. The purified enzyme was subjected to SDS electrophoresis using 10% polyacrylamide [13].

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