Bioscience Research Communications
Vol. 15, No. 3, June 30, 2003
Printed in Nigeria

0795-8072/2003 $12.00 + 0.00

http://www.klobexpublishers.com

BRC 2002057/15301

Partial purification of some properties of β-Glucosidase from the gut of the giant African snail, *Achatina achatina*.

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(Received August 15, 2002)

ABSTRACT: β-glucosidase was partially purified from animal source (*Achatina achatina*) by extraction with acetate buffer, separate fractional precipitation with ammonium sulphate and acetone followed by heat treatment. The activity and specific activity of the crude enzyme were 1.6U/ml and 2.35U/mg protein. The partially enzyme has activity of 4.70U/ml (for ammonium sulphate fraction) and 3.80U/ml (for acetone precipitate) with specific activity of 6.18U/mg protein and 6.03U/mg protein in the order above. Mathematical treatment of the data from the degradation of linamarin by the partially purified enzyme generated HCN values that were used to construct Lineweaver-Burk plot which gave apparent Km and Vmax values of 0.34mM and 0.25mmol HCN/min/ml of enzyme or 8.46mg HCN/min/mg protein respectively. The temperature and pH optima obtained for both the crude and partially purified enzyme were 55 – 60°C and 6.0 – 6.5. Both the crude and partially purified enzyme showed high degree of hydrolysis towards standard linamarin, the cynogenic glycosides of cassava and lima beans but did not significantly hydrolyse the cyanogenic glycoside of sorghum species.

Key words: β-glucosidase, *Achatina achatina*, partial purification, kinetic properties.

Introduction

Cassava (Manihot esculenta Crantz) has been considered to be the major human food crop with high content or cyanogenic glycosides (1) with the roots forming important food for more than 500 million people (2) mostly in the tropical countries of Africa, Asia and Latin America. An item of concern for public health is its potential toxicity (3,4,5,6) which is due to cyanogenic glucosides mainly limamarin (7).

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One of the optimum conditions for complete hydrolysis of cyanogenic glycoside to yield HCN, in routine and quantitative screening for cassava cyanogens and that of other cyanophoric plants (8,9) requires the input of exogenous β-glucosidases (EC.3.1.21.2). Quantitative hydrolysis (or autolysis) or by distillation method (acid hydrolysis) often result in gross underestimate of the the free HCN. Because complete hydrolysis or breakdown of cyanogenic glycosides is a pre-requisite for the removal of cyanogens during processing of cyanophoric plant materials and for monitoring safety of products, the need for the use of exogenous β-glucosidases cannot be over emphasized. Most of the exogenous β-glucosidases in use so far are from cyanophoric plant such as cassava, flax, sorghum and almond (10,7,11,12). The procedures involved in the purification of the β-glucosidase from those cyanophoric plants lead to low yields making commercial β-glucosidase hard to come by and highly expensive when available. Thus, there is need for a readily available and inexpensive source for this enzyme especially in the tropics where the presence of cyanogenic constituents in food and fodder is a severe problem.

In this context, Brimer *et al* (13) used β-glucosidase from *Helix pomatia* (Roman garden snail) for the hydrolysis of cyanogenic glycoside in foods and fodder samples. This strongly suggests that snails could be a good source of the aforementioned enzyme. African giant snail *Achatina achatina*, has been reported to be very rich in lytic enzymes (14, 15). However, there has not been any published report on the β-glucosidase activity of *A. achatina* which is the basis of this present investigation.

Material and Methods

Chemicals: Standard linamarin (from BDH Chemicals), chloramin T and isonicotinic acid (from Sigma), 1, 3 dimethyl barbituric acid (from Aldrich) and purified cassava linamarase of known activity were gifts from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. All other chemicals were from our laboratory and were of analytical grade.

Materials: Low cyanide cassava was obtained from National Root Crops Research Institute (N.R.C.R. 1) Umudike, Umuahia, Nigeria, while sorghum and lima beans seeds were purchased from Umuahia main market, Abia State, Nigeria.

The giant African snails (*Achatina achatina*) were purchased from Umuahia main market in Abia State, Nigeria. After removing the shells by cracking and peeling off, the gut juice was carefully drawn using syringes. The volume of the brown fluid collected was measured and filtered using Whatman No. 4 filter paper and stored in ice. Variation of the β-glucosidase activity of individual snails was therefore, not accounted for. The filtrate was then dissolved in 0.1M acetate buffer pH 5.5. This is to inactivate the β-glucosidase enzyme in the course of the following procedures.

Purification Procedures

(i) Ammonium sulphate precipitation and dialysis.

Part of the enzyme solution prepared above was brought to 60% saturation of $(NH_4)_2SO_4$ and held at 4°C for 16 hours. The precipitate obtained by centrifugation at 10,000g for 1hour was dissolved in and dialysed against 0.1M phosphate buffer pH 5.5 with 3 changes of buffer every 8 hours. After dialysis the enzyme preparation was given heat treatment. Heat treatment consisted of 30 minutes at 55°C in a water bath. Heat treatment is to denature some of the other enzyme proteins such as arylsulphatases.

Arylsulphatases of the molluscs are known to be labile and were extensively denatured by slightly agitating their assay mixture (16). The enzyme preparation was then dispersed in small aliquots (5ml) and used for enzyme activity and kinetic studies. This method (except for heat treatment) is according to Cooke *et al* (12).

(ii) *Acetone precipitation and dialysis*

Part of the crude enzyme solution was gently added to analytical grade acetone solution (1: 2.3 v/v) with gentle shaking. This was allowed to stand at 4°C over-night and the precipitate formed collected by

centrifugation at 10,000g for 1 hour. The precipitate was dialysed and the sialysate collected as described above. This is according to the method of Bokanga (17) for the preparation of enzyme linamarase from cassava. This was then given heat treatment as above.

Protein determination

The protein content of both the crude and partially purified enzyme was determined using Biuret method as described by Layne (18).

Determination of β-glucosidase activity

The β-glucosidase activity of both the crude and partially purified enzyme was determined as follows:
The enzyme solutions were diluted 200 times with 0.1M phosphate buffer pH 6.0, o.1. 0.2……0.6ml of the enzyme preparation were pipetted into different stoppered test-tube and the volumes completed to 1ml in the 0.1M phosphate buffer pH 6.0. The test-tubes were placed in water-bath at 40°C and the reaction started by adding 0.1ml of standard linamarin stock solution (1mg/ml). Exactly 15 minutes after the addition of linamarin, the reaction was stopped by addition of 0.6ml of 0.2N NaOH. The cyaniade released was measured spectrophotometrically according to the method of Esser *et al*, (19).

Calculation of β-glucosidase activity

The volume of diluted enzyme was plotted on X-axis against the absorbance obtained (Y-axis). The slope b^1 gave absorbance per volume of diluted enzyme. The enzyme activity per ml is given as:

$$\frac{b^1 \times DF}{b \times 247.24 \times 15.} \quad \text{(EU)}$$

where b is the slope of the linamarin standard curve (Conc. in mg/ml).
b^1 is the clope of the assay curve (volume in ml)
Df is dilution factor
247.24 is the molecular weight of linamarin and 15 stands for 15 minutes.
EU ((enzyme unit) is defined as the amount of enzyme that can hydrolyse 1µmol of linamarin per minute under the experimental condition of the assay. This method is according to Bokega (17).

Prior to the determination of β-glucosidase activity described above, 0.1ml of the crude enzyme and 0.1ml of the substrate (linamarin) were incubated for 15 minutes at 40°C and HCN released measured. This was to determine whether the snails' gut enzyme has any β-glucosidase at all or not.

Linamarin standard curve

Linamarin standard curve was prepared from linamaria stock solution (4000ppm). 0.5ml of the stock solution was diluted 5 times with 0.1M phosphate buffer pH 6.0 to give a solution of concentration 40µg/ml. 0.1, 0.2, 0.3, 0.4 and 0.5ml corresponding to 4, 8, 12, 16 and 20ug were pipetted into stoppered test-tubes and each made up to 1ml. The solutions were then incubated with exogenous linamarase (3 units/ml) from cassava for 15 minutes in a water bath at 40°C. The reaction was stopped by addition of 0.2N NaOH. The cyanide released was then read spectrophotometrically according to (19). The concentration of cyanide relased (x-axis) was then plotted against absorbance (y-axis) and the slope calculated.

Substrate and substrate specificity

Pure linamarin, 2 (β-D-glucopyranosyl oxisobutyronitrile) was used as substrate to test the activity of the β-glucosidase. The cyanogenic compounds of cassava (linamarin and lotaustralin) and sorghum species (dhurin) extracted according to (20) were also used.

Physical and kinetic properties of the partially purified enzyme

The physical and kinetic parameters measured by conventional techniques were: temperature and pH optimum, apparent Km and Vmax, and the enzyme inhibition by some compounds.

Results and Discussion

The enzyme was extracted from gut contents (juice) of giant African snail; *Achatina achatina*. The β-glucosidase activity of the crude gut enzyme was 1.6 units/ml with specific activity of 2.35. Inclusion of 10mM EDTA or dithiothreitol; compounds that reduce enzyme inhibition by phenoliuc compounds did not alter the activity of crude enzyme.

Separate fractionation of the crude gut enzyme with 60% $(NH_4)_2SO_4$ and acetone (1: 2.3 v/v) followed by dialysis and heat treatment resulted in partially purified enzyme that has 4.7 and 3.8 Units/ml and specific activity of 6.18 and 6.03 respectively (see Table 1 and Fig. 2). Thus, the two partial purification procedures gave 2.63 and 2.57 fold increases in specific activity. Ammonium sulphate fractionation (12) and acetone precipitation (16) have been used in the purification of β-glucosidase of cassava with 60% $(NH_2)_2SO_4$ fractionation.

The substrate specificity of both the crude and partially purified enzyme was monitored by assaying with standard linamarin; the cyanogenic glucosides of cassava (linamarin and lotaustratlin), sorghum species (dhurin) and lima beans (linamarin) extracted from these plant materials according to O'Brien *et al*; (20). Both crude and partially ourified enzyme hydrolysed standard linamarin, the cyanogenic glucoside of cassava and lima beans, to near completion, but did not significantly hydrolyse the cyanogenic glucoside of sorghum (dhurin). The quantitative yield of HCN equivalent from fresh cassava pulp was 18.7 mgCN/100g (for crude enzyme), 28.42mgCN^-/100g (for acetate precipitate) and 31.16mgCN^-/100g (for NH_4SO_4 fraction), while the CN^- yield from lima beans were 6.25, 11.36 and 15.02mgCN^-/100gDM in the order above. The quantitative yield of HCN equivalent by purified cassava linamarase from fresh cassava pulp and lima beans were 31.76 and 14.84mgCN^-/100g respectively. The quantitative CN- yield from sorghum species by both crude and partially purified enzyme was < 1.0mg/100gDM. From our results, the β-glucosidase of *A. achatina* is highly specific for linamarin and lotaustralin, the cyanogenic glucosides of cassava and lima beans. In this connection, it has been suggested (21) that linamarase is specific for linamarin, however evidence is accumulating (22) that linamarase has broad specificity for compounds containing the β-glucosidic bound. The hydrolysis of linamarin to yield HCN depends on two enzymes (7); linamarase which produces acetone cyanohydrin and hydroxunitrile lyase which catalyse the dissociation of the cyanohydrin to yield HCN. Acetone cyanohydrin dissociates readily at alkaline pH used to stop the enzyme activity (0.6ml, of 0.2N NaOH) and thus assay does not require such a secondary enzyme activity.

Properties of the partially purified enzyme

Mathematical treatment of the data from the degradation of linamarin by the β-glucosidase, generated HCN values that were used to construct a Lineweaver-Burk plot which gave apparent Km and Vmax values of 0.34mM and 0.25mmol HCN/min/ml of enzyme or 8.46mg HCN/min/mg protein respectively (see Table 2 and Fig. 3). The Km of purified enzyme from cassava (12) was reported to be 1.45 x 10^{-3}M. The temperature optimum of both the crude and partially purified enzyme was 55-60°C while the pH optimum in 0.1M phosphate buffer was 6.0 – 6.5 (see Table 1). At 65°C, the enzyme lost about 14% of its activity and was totally destroyed at 75°C. It has been reported (23) that linamarase will act in cold and warm environment but is readily destroyed at 70 – 72°C. In cassava, the endogenous β-glucosidase cause optimum catalyisis of linamarin at pH 6.0 – 6.5 (7). The activity of the partially purified enzyme is unaffected by the inclusion of 10mM glucose, KCN, EDTA and dithiothreitol in the assay medium of linamarin as substrate. This demonstrates the non-inhibition or activation of the β-glucosidase enzyme by the compounds.

Table 1: The Kinetic properties of A. achatina β-glucosidase under different purification procedures.

Purification procedure.	Protein content. (mg/ml)	Enzyme activity. Units/ml	Specific activity. Unit/mg protein	Temp. optima	PH. optima	$Km^{/}$. -	$Vmax^{/}$. -
Crude enzyme	0. 68	1. 60	2. 35	55 – 60°C	6.0 – 6.5	-	-
$(NH_4)_2SO_4$ fractionation product.	0. 76	4. 70	6. 18	55 – 60°C	6.0 – 6.5	0.34mM	8.46mgHCN/min/mg protein.
Acetone precipitation	0. 63	3. 80	6. 03	55 – 60°C	6.0 – 6.5	-	-

Note $Km^{/}$ = apparent Michalis-Menten constant.
$Vmax^{/}$ = apparent maximal velocity.

Table 2: Degradation of linamarin by the partially purified A. achatina β-glucosidase and cyanide production values.

Linamarin conc. [S] in incubation medium (mMol)	CN^- production (mMol) in 15min.	CN^-/min (V)	$\frac{1}{[S]}$	$\frac{1}{V}$
0. 016	0. 015	1.0×10^{-3}	62. 5	$1. 0 \times 10^{2}$
0. 032	0. 029	$1. 93 \times 10^{-3}$	31. 25	$5. 0 \times 10^{2}$
0. 048	0. 044	$2. 93 \times 10^{-3}$	20. 83	$3. 41 \times 10^{2}$
0. 064	0. 063	$4. 20 \times 10^{-3}$	15. 38	$2. 38 \times 10^{2}$
0. 080	0. 074	$4. 93 \times 10^{-3}$	12. 35	$2. 03 \times 10^{2}$
0. 096	0. 080	$5. 33 \times 10^{-3}$	10. 31	$1. 88 \times 10^{2}$

The values of linamarin concentration [S] and CN^- production are the mean of three determinations.

In conclusion, the crude and partially purified enzyme from *A. achatina* are both sufficiently active by hydrolyse quantitatively dilute linamarin, and the cyanogenic glucoside of cassava and lima beans and are being evaluated for use in the determination of cyanide in cassava and cyanogenic plant materials.

This finding should be of much interest especially to those in the tropical countries where cyanogenic plant materials are consumed frequently and in large amounts; considering the cheap source of this enzymes.

Acknowledgement

The authors wish to thank Dr.(Mrs.) B. Maziya-Dixon and Biochemistry Unit, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, for supplying us with standard linamarin, isonicotinic acid, 1.3-dimethyl barbituric acid and cassava β-glucosidase, used in this work.

Table 1: The kinetic properties of *A. achatina* β-glucopsidase under different purification procedures.

Purification Procedure	Protein content (mg/ml)	Enzyme activity (Units/ml)	Specific activity (Units/mg protein)	Temperature optima	pH optima	Km^1	$Vmax^1$
Crude enzyme	0.68	1.60	2.35	55-60°C	6.0-6.5	-	-
$(NH_4)_2SO_4$ fractionation precipitation	0.76	4.70	6.18	55-60°C	6.0-6.5	0.34mM	8.46mgHCN/min/mg protein.
Acetone precipitation	0.63	3.80	6.03	55-60°C	6.0-6.5	-	-

Note Km^1 = apparent Michalis-Menten constant.

$Vmax^1$ = apparent maximal velocity.

Table 2: Degradation of linamarin by the partially purified *A. achatina* β-glucosidase and cyanide production values.

Linamarin conc. [S] in incubation medium (mM)	CN production (mMol) in 15 min.	CN^- min (V)	1/[S]	1/V
0.016	0.015	1.0×10^{-3}	62.5	1.0×10^{2}
0.032	0.029	1.93×10^{-3}	31.25	5.0×10^{2}
0.048	0.044	2.93×10^{-3}	20.83	3.41×10^{2}
0.064	0.063	4.20×10^{-3}	25.38	2.38×10^{2}
0.080	0.074	4.93×10^{-3}	12.35	2.03×10^{2}
0.096	0.080	5.33×10^{-3}	10.31	1.88×10^{2}

The values of linamarin concentration [S] and CN^- production are the mean of three determinations.

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