

Enzyme-Substrate specificity of β -D-glucosidase from fungal, bacterial and cassava sources using p-nitrophenol β -D-glucoside and cassava linamarin as substrates.

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Abstract: The production of β -D-glucosidase from fungal, bacterial isolates and cassava tubers and the ability of the enzyme to hydrolyze cyanogenic glycoside were studied. Bacterial isolates fermented in mineral salt cassava medium failed to produce β -D-glucosidase while the aerobic fermentation of fungal extracts of *Aspergillus sydowi*, *A. terreus*, *A. Carneus* and *Fusarium equiseti* produced β -D-glucosidase with *A. Sydowl* and *F. equiseti* having the highest amount and activity. However, enzyme from these fungal sources failed to hydrolyze crude cyanogenic glucoside, linamarin, from cassava tuber but hydrolyzed p-nitrophenyl β -D-glucoside, a synthetic chemical analogue of linamarin. The linamarase also a β -D-glucosidase from cassava cortex hydrolyzed both p-nitrophenyl β -D-glucoside and cassava linamarin indicating that both partial and total hydrolysis of cassava linamarin could only be achieved by linamarase from cassava source showing enzyme-substrate specificity. Total cyanide hydrolysis in cassava tuber was achieved with 3-units of cassava linamarase in 10 minutes.

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1. Introduction

Cassava (*Manihot esculenta crantz*) contains an endogenous hydrolytic enzyme known as linamarase, a β -D-glucosidase (E.C 3.2.1.21), or β -D-glucoside glucohydrolase which hydrolyzes the cassava cyanogenic glucoside, linamarin, [2-(β -D-glucopyranosyloxy) Iso butyronitrile] and lotaustralin, [2-(β -D-glucopyranosyloxy) methyl butyronitrile] to hydrogen cyanide (HCN) on tissue damage (Conn 1969, Coursey 1973).

Cyanide in intact cassava exists largely as the aliphatic glucoside: linamarin and lotaustralin but only the free cyanide is available to direct assay because of the difficulty in achieving quantitative autolysis of the cyanogens in cassava extracts (Zitnak, 1973).

Acceleration of the rate of cassava pulp detoxification by the introduction of exogenous linamarase during fermentation of grated cassava for garri production and the precise role of this enzyme in cassava tissue during fermentation process have been indicated (Ikediobi and Onyike 1982a, 1982b). This development has created the need to have linamarase in sufficient quantity to satisfy the potential demand that would exist for its use in research and commerce.

Several fungi are known to produce amyloglucosidase (Wiserman, 1979) and some locally isolated fungi have been implicated in

linamarase production (Ikediobi and Ogundu 1985).

Experience of workers in industrial Biochemistry and Microbiology have shown that it is always cheaper, faster and more efficient in producing large quantities of enzymes from microorganisms than from other sources whenever such opportunity exists (Wang *et al* 1979b, Reese and Maguire 1969).

The objective of this work therefore was to produce linamarase from fungal, bacterial and cassava sources, test their ability to hydrolyze the cyanogenic

The objective of this work therefore was to produce linamarase from fungal, bacterial and cassava sources, test their ability to hydrolyze the cyanogenic glucoside, linamarin in cassava, ascertain for the first time which of the enzyme source that will bring about greater percentage detoxification of cassava cyanogenic glucoside, how effective and specific the enzymes from these sources will be in addition to the quantity of enzyme that will bring about a total detoxification when added exogenously and the time it will take to achieve such.

2. Materials and methods

Fungal and bacterial isolates

Five fungal and four bacterial cultures were obtained from the department of crop protection,

institute for Agricultural Research (IAR) and Microbiology Department respectively, Ahmadu Bello University, Zaria, Nigeria. The fungi and their identification numbers include *Aspergillus sydowi* (SA 1107); *A. ochraceus* (SA 1322); *A. Carneus* (SA 1326); *A. Terreus* (SA 562) and *A. equiseti* (SA 1109) while the Bacterial isolates are *Bacillus Licheniformis* (B 501); *B. Subtilis* (9.3) ; *B. Polymyxa* (WA 42) ; *B. Circulans* (Wa 113) and *B. Coagulans* (ST 21).

Preparation of Potatoe Dextrose Agar Streptomycin (PDAS) Medium For Culturing Fungal And Bacterial Isolates

Peeled Potatoes were cut into small pieces and boiled for one hour in distilled water (200g Potatoes per liter of water). The extract was filtered through glass wool. Dextrose and agar (20g each per liter of filtrate) were added to the filtrate. The volume was made up to the required volume and boiled till the agar dissolved. Streptomycin sulphate was added (0.05g Streptomycin sulphate per liter of solution).

10ml of the resulting solution was poured into universal MacCartney bottles with a 10ml dispensing syringe. They were tightly cocked and sterilized at 121°C in an autoclave for 20 minutes under a pressure of 15 psi. The bottles were allowed to cool to room temperature before use.

The pure fungal and bacterial isolates were transferred into the bottles of PDAS medium in an inoculating hood in a sterilized room. The inoculating needles used for the transfer were constantly sterilized with smokeless flame of a spirit lamp placed inside the inoculating hood. The bottles were left half-cocked and stored in an incubator for 4-7 days to ensure full growth of the fungi and 2-4 days of bacteria.

Preparation of sorghum – extract growth medium and fungal growth.

Sorghum (1.4kg) was boiled in 7 liters of distilled water (200g/litre) and left to cool for one hour. The cooked grain was upon cooling carefully filtered through a clean sheet of muslin cloth. The volume of the filtrate was made up to 7 liter. Two hundred milliliter of the filtrate was poured into each of the 250ml Erlenmeyer flasks and carefully plugged with cotton wads. The latter and their extracts were autoclaved for 15 minutes at 121°C under a pressure of 15psi. The flasks were removed and allowed to cool before use for fungal growth.

The fungus fully grown in potatoes-Dextrose- agar- streptomycin slant was inoculated into flasks inside an inoculating hood in a sterilized inoculating room and the wads of cotton wool were gently and carefully plugged firm after inoculation. The flasks were transferred to a rotary shaker

maintained at a speed of 100 revs/min. The fungus was allowed to grow for 18 days. Possible contamination by bacteria was eliminated by incorporating streptomycin sulphate in the growth medium at the concentration of 2% (w/v).

Extraction of crude fungal linamarase

At the end of the growth period, the contents of the growth flasks were pooled and centrifuged at 5,000 x g for 15 minutes. The Supernatant was saved for linamarase assay while the precipitated mycelia were discarded.

Preparation of Mineral-Salt-Cassava Medium (MSC) and Bacterial growth

One liter of mineral-salt-cassava medium contains 1g of KH₂PO₄; 0.2g of MgSO₄.7H₂O; 0.2g of CaCl₂; 0.2g of NH₄NO₃ and 22g of cassava flour. 200ml was dispensed into 250ml Erlenmeyer flasks. They were autoclaved at 121°C for 15 minutes under a pressure of 15 psi. After cooling, a loop full of bacteria was transferred into the flasks in duplicate and grown in orbital shaker. Ten milliliter was removed aseptically at three days intervals and assayed for linamarase.

Precipitation of enzyme extract by Ammonium Sulphate

The weight of ammonium sulphate required to give 90% saturation of a given volume of enzyme extract was determined from a table (Ferdinand 1978). This amount was dissolved in the enzyme extract with continuous stirring till a precipitate was observed. This was left in refrigerator at 4°C for 2 days for the precipitate to settle down and come together. The supernatant was carefully pipetted out using pipette filler. The precipitate was dialyzed against water for 24 hours at room temperature to remove remaining ammonium sulphate. The extract obtained after dialysis was assayed for enzyme activity and subsequently used for cyanide assay.

Preparation of Linamarase from Cassava tubers.

The thin brown outer skin of freshly harvested cassava tuber was carefully removed revealing the underlying cortex. The latter was removed and sliced into small pieces. 100g of the slices were homogenized in 300ml of pre-chilled 0.1M acetate buffer of pH 5.50. The homogenate was collected in a pre-chilled container and filtered by suction through a 1cm layer of kieselguhr. The filtrate was used to extract a second 100g batch of sliced cassava cortex as described above. The filtrate from the second extract was similarly used to extract third 100g batch. This final extract was kept overnight at 4°C.

To this extract was added 2.3 volumes of cold acetone and the resulting solution swirled several

times for 2 minutes within a 2 hour period. A pale-green precipitate was recovered upon decantation. This precipitate was subsequently extracted several times with 10ml portion of cold 0.1M acetate buffer of pH 5.50. The extract was centrifuged at 5000 x g for 5 minutes at room temperature and the resultant supernatant was dialyzed at room temperature (it can also be done in the cold room) against 0.1M acetate buffer of pH 5.50 to remove any residual linamarin. The extract obtained after dialysis was assayed for activity and subsequently used for cyanide assays.

Preparation of cassava extract for cyanide analysis

Fresh cassava parenchyma tissue (20g) was homogenized in 50ml of 0.1M HCl for 3 minutes. The homogenate was filtered by vacuum and the resultant filtrate adjusted to pH 6.8 with a base NaOH. This solution was centrifuged at 5000 x g for 3 minutes. The resultant supernatant was stored in a freezer until analysed.

Assay of Enzyme activity using p-nitrophenyl β -D-glucosidase as the substrate.

The assay method was essentially that described by Ikediobi *et al* (1980). One milliliter of a solution containing 21mg of p-nitrophenyl β -D-glucoside (PNP-glucoside), an artificial analogue of linamarin in 0.01M sodium phosphate buffer of pH 6.8 was incubated for one hour at room temperature ($25 \pm 2^{\circ}\text{C}$) with 0.5ml of diluted enzyme extract (200 times dilution). At the end of the incubation period the reaction was stopped by the addition of 2ml of 0.2M borate buffer then brought up to 4ml with distilled water and the absorbance of the resulting solution read at 400nm in spectronic 20 spectrophotometer.

One unit of linamarase activity was defined as that amount of enzyme which produced one micromole of p-nitrophenolate ion at 400nm/min under the described assay conditions.

Quantitative determination of free and bound cyanide in cassava extracts

Cassava extract (0.1-0.5ml) was incubated with 1.0ml of β -D-glucosidase from Bacterial, fungal and cassava extracts containing 3 units of activity for 10-15 minutes at room temperatures in a tall stoppered test tube. The volume of the incubation mixture is made up to 2ml with 0.2M sodium phosphate buffer of pH 6.8. At the end of the incubation period, the reaction was stopped by the addition of 2ml of 0.2M borate buffer of pH 9.8. Acetic acid (0.2ml of 2M) was added and vortex mixed briefly. Sodium hypochlorite (0.1ml of 5mM) was added and mixed again. Within 1 minute, 0.5ml of barbituric acid – pyridine agent was added, mixed and the absorbance determined

at 580nm 5 to 15 minutes latter. The determination of free cyanide was performed as above method except that the exogenous linamarase preparation was not added to the aliquot of the extracts to be analyzed for free cyanide, instead 1ml of 0.2M sodium phosphate buffer was added. Cyanide concentration was determined from a standard cyanide curve using potassium cyanide as described by Lundquist *et al* (1985). Between the total and free cyanide gives the bound cyanide or unhydrolyzed cyanogenic glucoside. This determines the ability of the enzyme linamarase to hydrolyze the cyanogenic glucoside, linamarin. Protein content of the crude enzyme extracts from the bacterial, fungal and cassava sources were determined by Folin-Lowry method using bovine serum albumin as standard (Lowry *et al* 1951). This was used to calculate

the enzyme specific activity thus: Specific activity =

$$\frac{\text{Activity (Units/ml)}}{\text{Protein content (mg/ml)}}$$

3. Results

β -D-glucosidase production from fungi bacterial and cassava sources

Four fungal isolates, *Aspergillus terreus*, *Aspergillus sydowi*, *Aspergillus carneus*, *Fusarium equiseti* and five bacterial isolates, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus Circulans* and *Bacillus Coagulans* grown in sorghum extract medium for 18 days were tested for β -D-glucosidase production in the liquid medium. Extracts made from cassava cortex were tested for the same enzyme.

Results in tables 1 and 2 show that all the fungi isolates and cassava extract produced β -D-glucosidase while the bacterial isolates did not.

Table 1 β -D-glucosidase and linamarase production by fungal and bacterial isolates and cassava using PNP-glucoside and cassava linamarin as substrates.

Enzyme sources	PNP glucoside	PNP glucoside
<i>A terreus</i>	++	-
<i>A sydowi</i>	+	-
<i>A A carneus</i>	+	-
<i>F equiseti</i>	++	-
<i>B. licheniformis</i>	-	-
<i>B. subtilis</i>	-	-
<i>B-polymyxa</i>	-	-
<i>B. circulans</i>	-	-
<i>B. coagulans</i>	-	-
^a cassava	++++	++++

^a Denotes activity of enzyme from cassava cortex

Table 2. The activity and protein content of concentrated extracellular β -D-glucosidase produced by fungi grown in sorghum extract

Source of enzyme	Enzyme and crude enzyme (units/ml)	Protein content (mg/ml) x 10 ⁻¹	Specific activity (units/mg protein)
<i>A. terreus</i>	2.04	3.63	6.07
<i>A. sydowi</i>	0.67	6.88	0.97
<i>A. carneus</i>	0.44	0.84	5.24
<i>F. equiseti</i>	1.78	1.42	12.54
<i>cassava</i> ^a	2.69 ^a	0.72 ^a	41.11 ^a

a Denotes activity and protein content of linamarase from cassava cortex

Enzyme specificity using cassava linamarin and PNP-glucoside as substrate

Results in table 2 show that the specific activity of the enzyme is highest in cassava extract followed by that from *F. equiseti*, *A. terreus*, *A. carneus* and lastly *A. sudowi*.

The ability of the enzymes produced by the fungal isolates and that from cassava extract to hydrolyze cassava linamarin and PNP-glucoside are shown in tables 1 and 4. The results show that the enzyme from cassava was able to hydrolyze crude linamarin in cassava and PNP-glucoside while fungal enzyme was able to hydrolyze PNP-glucoside but not crude linamarin from cassava. Bacterial growth medium extract did not show any enzyme activity nor hydrolytic ability on the two substrates.

Table 3. Bound cyanide in fresh cassava tubers determined using enzyme from fungal and cassava sources

Source of enzyme	Enzyme activity (units/ml)	Bound cyanide (μ g/gm tuber x 10)
<i>A. terreus</i>	2.04	0.00
<i>A. sydowi</i>	0.67	0.00
<i>A. carneus</i>	0.44	0.00
<i>F. equiseti</i>	1.78	0.00
<i>cassava</i>	2.69	4.77

Enzyme activity shown in tables 2 and 3 were determined using p-nitrophenyl β -D-glucoside as earlier defined.

Cyanide determination and time of incubation for total cyanide hydrolysis in fresh cassava tubers using linamarase from cassava cortex

Different levels of linamarase from cassava cortex were used to determine the enzyme

concentration that will cause total cyanide hydrolysis and the time of incubation that will accomplish this results. Table 4 and fig 1 show that as the enzyme concentration increases, the hydrolysis of bound cyanide increases till a maximum of 3 units, above which there was a decline in cyanide hydrolysis

Table 4: Bound and total cyanide in fresh cassava tubers determined using different levels of cassava cortex linamarase.

Enzyme activity (units/ml)	Total cyanide (μ g/gm tuber x 10)	Bound cyanide (μ g/gm tuber x 10)
0.000	0.27*	0.00
0.592	2.91	2.64
1.480	4.73	4.46
2.960	4.95	4.68
5.920	4.45	4.18
14.800	3.68	3.41
29.600	3.68	3.41

*Free cyanide

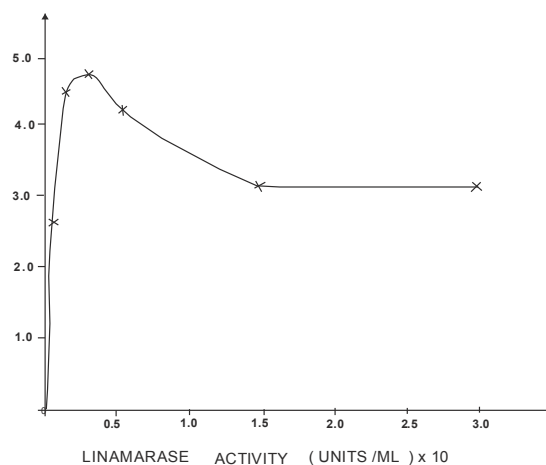


FIG. 1 MAXIMUM ENZYME ACTIVITY FOR TOTAL HYDROLYSIS OF BOUND CYANIDE

Table 5: Time of incubation for total hydrolysis of bound cyanide

Time (min)	Bound cyanide (μ g/gm tuber) x 10
5	3.82
10	4.68
20	4.68
30	4.68
40	4.41
50	4.41

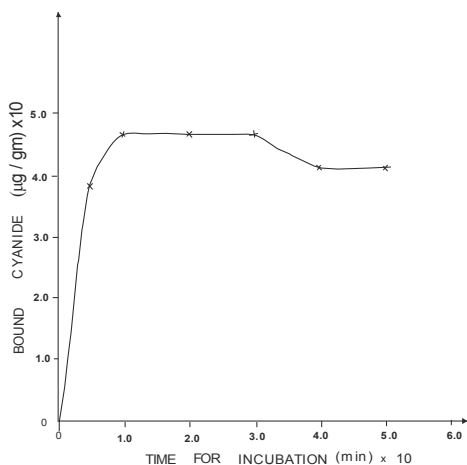


FIG. 2 THE CURVE FOR TOTAL HYDROLYSIS OF BOUND CYANIDE

The time of incubation in table 5 and fig 2 show that hydrolysis of Bound cyanide by linamarase increased with time till 10 minutes when it remained constant till the 30th minute. There was a decline after the 30th minute until the 40th minute and remained constant till the 50th minute.

Table 6 The result for standard curve for cyanide estimation

Cyanide concentration (µgCN/ reaction mixture)	Absorbance at 580nm
0.26	0.24
0.52	0.49
0.78	0.76
1.04	0.95
1.30	1.25

4. Discussion

The four fungal isolates grown in sorghum extract liquid medium produced β -D-glucosidase which had earlier been regarded by some workers as linamarase (Ikediobi and Ogundu, 1985; Ikediobi *et al* 1987).

Wiserman (1979) had earlier shown that these fungi among others were capable of amyloglucosidase production. The result shown in table 2 indicate that this enzyme was able to hydrolyze PNP-glucoside; a synthetic analogue of linamarin, with the enzyme from *Fubarium equiseti* showing the highest specific activity. This is in agreement with the word of Ikediobi and Ogundu (1985) who had shown that *F. equiseti* among other fungi, showed the highest soecific activity for the enzyme.

Although the fungal enzyme was ask to hydrolyze the PNP-glucoside, the results in table 1

and 3 show that it was unable to hydrolyze the crude natural linamarin in cassava. On the other hand, the enzyme from cassava cortex was able to hydrolyze both PNP-glucoside and crude linamarin in cassava while the bacterial enzyme did not produce the enzyme.

The work of Ejiofor and Okafor, 1983 had indicated that some bacterial isolates could produce linamarase when linamarin was incorporated in its growth medium and would have the ability to break down linamrin in fermenting cassava pulp. Ikediobi *et al* (1987) showed that the partially purified enzyme from *F.equiseti* had some ability on purified linamarin, However the observation in this work has revealed an enzyme – substrate specificity whereby the crude bacterial enzyme did not show any hydrolytic activity on both linamarin and its analogue PNP-glucoside. Fungi enzyme was able to show activity with the synthetic analogue of linamarin only, while the crude cassava enzyme showed activity with the natural linamarin and its synthetic analogue.

It thus seems reasonable to state that the fungal and bacterial enzymes are not the true linamarase and could therefore not be of much benefit in cassava processing if linamerin must be added in fermentation medium or the enzyme and linamarin to be in pure form to show any hydrolysis. This cassava enzyme could therefore be referred to as the true linamarase since it has high hydrolytic activity on the crude cassava linamarin. It would be more realistic to use cassava linamarase to achieve total or partial hydrolysis bound cyanide in cassava tubers though it has an economic disadvantage if it should be applied on industrial scale for cassava processing.

This work has shown as earlier indicated by Ikediobi and Onyike (1982a) that the cassava linamarase could cause cyanide reduction when added exogenously in fermenting cassava pulp. This shows the ability of cassava linamarase to hydrolyze crude linamarin.

To ascertain the concentration of the cassava linamarase that would cause a total hydrolysis of crude cassava linamarin and the duration of incubation, results in table 4 and fig 1 show that 3 units of enzyme activity caused total hydrolysis of bound cyanide per gramme of cassava tuber. This result agrees with that obtained by Rao and Hahn (1981) which was also 3 units but lower than that reported by Ikediobi *et al.* (1980) which was 30 units. The work of Cooke (1978) showed that 0.25-0.3 units of exogenous linamarase could cause 95±2% linamrin recovery from cassava parenchyma, peel or leaves. The apparent differences in these results could be due to the

sensitivity of the different assay methods used. Linamarin and other contaminants could affect cyanide availability (Cooke *et al.* 1978). Higher units of enzyme activity did not cause more hydrolysis rather there was a decline in the level of free cyanide in the medium available for assay (Table 4 and fig 1). This characteristic behaviour agrees with the reports of Cooke, (1978) and Ikediobi *et al.* (1980), which suggests that linamarin or a contaminant of the enzyme could sequester some of the free cyanide in the reaction medium.

The duration of incubation required for total hydrolysis was found to be between 10-30 minutes beyond which there was a decline (Table 5 and fig 2) This result agrees with the work of Cooke (1978) and Ikediobi *et al.* (1980), who used 15 and 10 minutes respectively. The decline is also attributable to cyanide sequestration by the enzyme.

5. Conclusion

The bacterial isolates failed to produce β -D-glucosidase while the fungal isolates did. The inability of the β -D-glucosidase from fungal source to hydrolyze linamarin in cassava tuber while similar enzyme from cassava cortex hydrolyzed the linamarin showed that the source of the enzyme is of great important in achieving partial or total hydrolysis of linamarin. This work has therefore revealed that cassava β -D-glucosidase is the true linamarase and is specific to its substrate linamarin showing enzyme-substrate specificity.

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