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Purification and detection of linamarin from cassava root cortex by high performance liquid chromatography

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Abstract

Linamarin, a cyanogenic glycoside in cassava (*Manihot esculenta* Crantz cv. KU-50) root cortex and parenchyma was extracted with different acids (HCl, H_2SO_4, H_3PO_4 or CH_3COOH) and detected by high performance liquid chromatography (HPLC). The highest linamarin was found in both tissues extracted with H_2SO_4. However, the concentration of linamarin in the extract of the root cortex was higher than that of the root parenchyma. Linamarin in crude extract of the root cortex using 0.25 M H_2SO_4 was purified by the second step HPLC with the yield of 91.54%. These extraction, detection and purification methods were useful to improve the purity of linamarin from cassava, especially the root cortex.

Keywords: Acid extraction; Cassava root cortex; HPLC; Linamarin detection; Linamarin purification

1. Introduction

Cassava or tapioca (*Manihot esculenta* Crantz) is widely cultivated in the tropics for its storage roots which are the staple food of about eight hundred million people in the world (Tatsuma, Tani, Oyama, & Yeoh, 1996). The bitter taste of fresh cassava roots is associated with high levels (>90% of total cyanogens) of cyanogenic glycosides, mainly linamarin (Sundaresan, Amma, & Nambisan, 1987). Linamarin is found in all parts of cassava except the seeds (McMahon, White, & Sayre, 1995). The cyanide liberated from linamarin as a result of hydrolysis is highly toxic. Generally, cyanide in cassava is not detected under physiological conditions. However, when cassava tissues are damaged, linamarase comes into contact with the linamarin, resulting in its hydrolysis and the subsequent release of cyanide. Then, it is a likely source of cyanide exposure in humans and presents a significant safety problem for humans. Many methods were developed for extraction and detection of the linamarin from cassava tissues (Bradbury, Bradbury, & Egan, 1994; Bradbury & Egan, 1992; Bradbury, Egan, & Lynch, 1991; Brimer, 1994; Cooke, 1978; Haque & Bradbury, 2004; O'Brien, Taylor, & Poulter, 1991). Linamarin is not only used as a substrate to detect the activity of linamarase but it also can be used in the preparation of standard linamarin filter paper discs that are needed to monitor the performance of picrate kits for determination of the total cyanide (Haque & Bradbury, 2004). Moreover, the purified linamarin can be used as an enzyme-prodrug system in cancer gene therapy (Cortes, de Felipe, Martin, Hughes, & Izquierdo, 1998; Vassaux & Lemoine, 2000). The objective of this work was to improve the extraction, detection and purification of linamarin from the highly bitter cassava cultivar KU-50 root for more applications in the future.

2. Materials and methods

2.1. Extraction of linamarin

The 12-month-old cassava roots of the high-cyanogen cultivar KU-50 were harvested from Rayong Field Crops

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Research Center in Thailand. All the roots were pooled, washed to remove grit and then the cortex tissue (root peel) was removed from the parenchyma tissue (peeled root). The root cortex and parenchyma were promptly diced (1 cm cubes) and randomised thoroughly before sampling.

Crude linamarin was extracted from cassava root cortex and parenchymal, using a modified version of the method of Cooke (1978). The 100 g of diced tissues were immediately homogenized with 160 ml of 0.1 M chilled acid (HCl, H_2SO_4, H_3PO_4 or CH_3COOH) for 15 s at low speed, followed by 1 min (2 times) at high speed in a blender. The homogenates were filtered through a filter cloth to remove insoluble materials. The homogenizer jar was rinsed with each acid (40 ml) which was filtered in the same way. The filtrates were centrifuged at 10,000 rpm for 10 min, at 4 °C. The clear supernatant fluids were collected and stored at −20 °C for further study.

2.2. Detection and purification of linamarin in crude extracts

HPLC (C-R1A Chromatopac, Shimadzu, Kyoto, Japan) was used for detection of the quantity and quality of linamarin from acid extracts of cassava roots. The column used for separation was the NH_2 Lichrospher 100 (size 4.00 × 250 mm, Hewlett Packard, USA). The HPLC was operated at 25 °C using 70% (v/v) acetonitrile at a flow rate of 1.0 ml/min and 1060 psi as the mobile phase. Refractomonitor-IV RI (LDC Analytical, USA) was used as a detector for analysis of the concentration of linamarin. Linamarin from A.G. Scientific, Biochemical Manufacturer, USA was used as a standard. Quantification of peaks was performed by integration of the peak areas. The high linamarin fractions were pooled for further experiments.

Linamarin separated by the first step HPLC was purified by second step HPLC with the same condition as described above but using 80% (v/v) acetonitrile at a flow rate of 0.5 ml/min as the mobile phase. The linamarin fractions were collected. Each fraction (100 μl) was dried by freeze dryer (FreeZone[6], Labconco, USA) and prechecked for purity of linamarin by thin-layer chromatography (TLC). The purified fractions were pooled and stored at −20 °C.

2.3. TLC

Each fraction of linamarin from second step HPLC was spotted on the TLC using silica gel 60, F_{254} plates (Merck) and chromatographed with a solvent system of *n*-butanol: acetic acid: distilled water (50:25:25) and the spots were detected by heating the plates over 100 °C after spraying them with a reagent of aniline: α-diphenylamine: acetone: 80% H_3PO_4 (1 ml:1 g:50 ml:7.5 ml).

2.4. Identification of purified linamarin

Purity of linamarin was confirmed by CHN elemental analysis (Perkin Elmer 2400 CHN/O Analyzer, USA) and NMR analysis. ^{1}H NMR spectra was recorded on a Fourier Transform Nuclear Magnetic Resonance Spectrometer (JNM-A500, Jeol, Japan), operating at 500.00 MHz. Samples for NMR analysis were dissolved in D_2O.

3. Results and discussion

3.1. Extraction and detection of linamarin from cassava root cortex and parenchyma

In previous works, acidic condition was selected for extraction of linamarin due to a convenient means of endogenous enzyme inactivation and the higher stability of acid extracts when compared with alkaline extracts (Cooke, 1978). In this study, the chilled H_2SO_4 and CH_3COOH were compared with HCl (Haque & Bradbury, 2004) and H_3PO_4 (Cooke, 1978) for extraction of linamarin from cassava root cortex and parenchyma. The concentrations of linamarin from crude extracts of the root cortex and parenchyma were determined by HPLC, and the results are shown in Table 1.

The highest concentration of linamarin in cassava root cortex and parenchyma extracts were obtained by using H_2SO_4, followed by HCl and H_3PO_4. In addition, the HPLC chromatograms showed that the concentration of linamarin in the extract of the root cortex was higher than that of the root parenchyma. Therefore, the root cortex extracted by $H_2SO_4$was selected for further steps. In the case of CH_3COOH extraction, we could not detect linamarin

Table 1
Concentrations of linamarin from crude extracts of cassava root cortex and parenchyma using various acids

Tissue	Extraction acid (0.1 M)	Linamarin (g)/kg fresh weight of tissue	Linamarin (g)/kg dry weight of tissue
Cortex	H_2SO_4	7.71 ± 0.97	28.40 ± 3.38
Cortex	HCl	3.91 ± 0.49	14.40 ± 1.52
Cortex	H_3PO_4	0.78 ± 0.10	2.86 ± 0.40
Cortex	CH_3COOH	ND	ND
Parenchyma	H_2SO_4	5.77 ± 0.74	14.71 ± 1.91
Parenchyma	HCl	0.73 ± 0.09	1.86 ± 0.22
Parenchyma	H_3PO_4	0.38 ± 0.05	0.97 ± 0.15
Parenchyma	CH_3COOH	ND	ND

ND = could not detect under the tested condition.
The values are means ± standard deviations from five independent experiments.