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ScienceDirect
Food Chemistry 104 (2007) 1750–1754
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

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

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Received 1 May 2006; received in revised form 28 July 2006; accepted 27 October 2006

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.
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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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doi:10.1016/j.foodchem.2006.10.071

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.

under this HPLC condition. CH_3COOH might be interfere with the linamarin HPLC profile. Then, it could not present the peak at the retention time of linamarin. The chromatogram of crude linamarin extracted from the root cortex by H_2SO_4 is shown in Fig. 1. The peak at retention time of 3.13 min is linamarin, and the high contents of linamarin in this peak were pooled.

It was found that the content of cyanogenic glycosides in cassava roots is dependent on the cultivar (Santana, Vásquez, Matehus, & Aldao, 2002). Most cassava cultivars are correctly called low-cyanogenic because the cyanoglycoside content (less than 0.1 kg^{-1} fresh roots) but there are high-cyanogenic cultivars called bitter cassavas, which contain cyanoglycosides of up to 0.5 g kg^{-1} fresh roots (Wheatley, Orrego, Sanchez, & Granados, 1993). However, the highest linamarin content from cassava root of KU-50, extracted by 0.10 M H_2SO_4 was 7.71 ± 0.97 and 5.77 ± 0.74 g linamarin kg^{-1} fresh weight for the cortex and parenchyma tissues, respectively. Linamarin content in this work was higher than those of previous studies (Bradbury et al., 1994, 1991; Bradbury & Egan, 1992; Brimer, 1994; Brimer, Abrahamsson, Mlingi, & Rosling, 1998; Cooke, 1978; Tatsuma et al., 1996; Yeoh, 1993; Yeoh, Lim, & Woo, 1996; Yeoh & Tan, 1994; Yeoh & Truong, 1993). However, the determinations of linamarin by those methods are based on the enzymatic hydrolysis of linamarin, followed by spectrophotometric, potentiometric or amperometric measurement. But in this study, we used a HPLC method for detecting the concentration of linamarin in the extracts. This method is rapid, direct and high sensitivity method for analysis of quantity and quality of linamarin in the extracts. This method does not require the enzymatic hydrolysis of linamarin, does not have the problem of insufficient linamarase to hydrolyze the linamarin, does not result in the incomplete hydrolysis of linamarin in the extracts, and prevents the lost of the cyanide liberated during the reaction. Thus, the HPLC is a good method for quantitative and qualitative measurement of linamarin

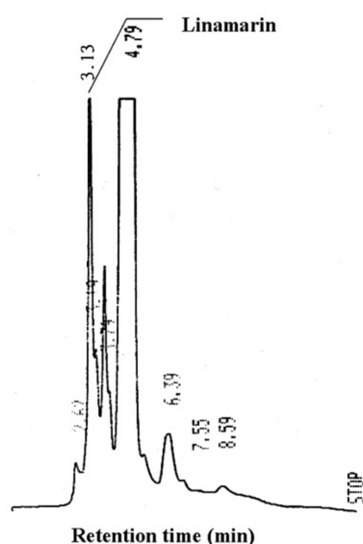

Fig. 1. Chromatogram of crude linamarin extracted from cassava root cortex using 0.10 M H_2SO_4.

Table 2
Concentrations of linamarin from crude extracts of cassava root cortex using various concentrations of H_2SO_4

Concentration of H_2SO_4 (M)	Linamarin (g)/kg fresh weight of tissue	Linamarin (g)/kg dry weight of tissue
0.05	7.10 ± 0.15	26.18 ± 0.57
0.10	7.71 ± 0.03	28.40 ± 0.14
0.25	9.46 ± 0.19	34.86 ± 0.68
0.50	7.84 ± 0.11	28.91 ± 0.37
1.00	6.59 ± 0.06	24.23 ± 0.24

The values are means ± standard deviations from five independent experiments.

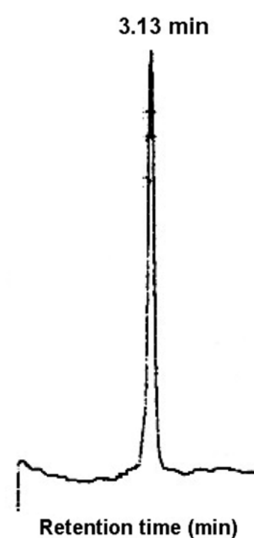

Fig. 2. HPLC chromatogram of the purified linamarin.

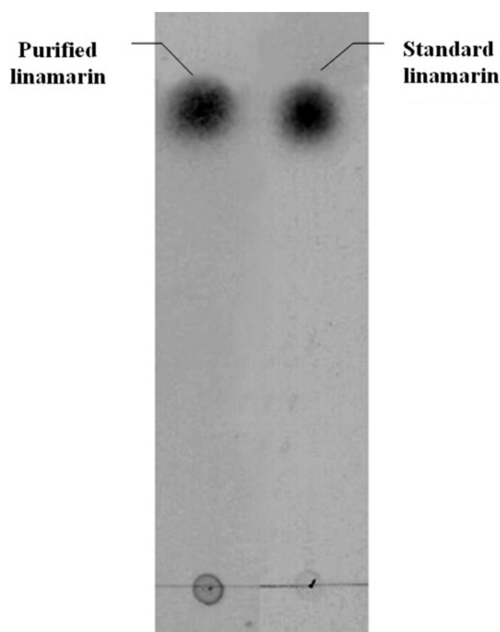

Fig. 3. TLC of the purified linamarin.

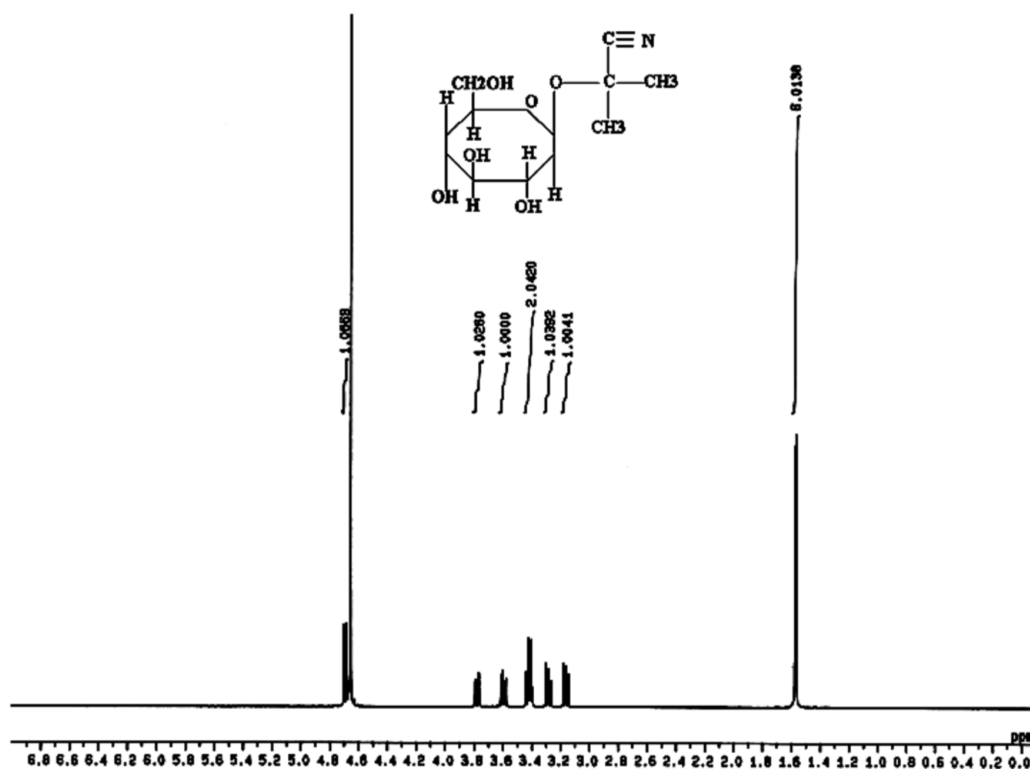

Fig. 4. ^{1}H NMR spectra of the purified linamarin.

in the extracts. Furthermore, we studied the optimum concentration of H_2SO_4 for extraction of linamarin from the root cortex. The chilled 0.05, 0.10, 0.25, 0.50 and 1.00 M H_2SO_4 were used for extraction of linamarin from the root cortex. The results are shown in Table 2. It showed that the highest concentration of linamarin was obtained by using 0.25 M H_2SO_4, followed by 0.50, 0.10, 0.05 and 1.00 M H_2SO_4 at 34.86 ± 0.68, 28.91 ± 0.37, 28.40 ± 0.14, 26.18 ± 0.57 and 24.23 ± 0.24 g linamarin kg^{-1} dry weight of tissue. Based on these results, 0.25 M H_2SO_4 is the best concentration for extraction of linamarin from cassava root cortex.

3.2. Purification and purity of linamarin

The pooled fractions of linamarin of the first step HPLC extracted from cassava root cortex using chilled 0.25 M H_2SO_4 was purified by second step HPLC. One peak of linamarin was detected by HPLC (Fig. 2). After that TLC was used pre-checking for the purity of linamarin. The collected fractions which contained high concentration of linamarin were presented only one spot on the plate (Fig. 3).

The purified linamarin was further characterized by CHN elemental analysis. The experimental values of carbon, hydrogen and nitrogen were 47.04%, 6.65% and 5.48%, respectively, corresponding to the chemical formula of linamarin ($C_{10}H_{17}O_6N$). NMR analysis (Fig. 4) also showed that only ^{1}H chemical shifts of linamarin were observed in ^{1}H NMR spectra. These results revealed that the sample had only one substance (linamarin) without any impurity. These results suggested that, the collected sample is linamarin and purified. Moreover, after purification of linamarin by HPLC, the linamarin was $91.54 \pm 0.36\%$ recovered (31.91 ± 0.67 g/kg dry weight) compared to the extract of cassava root cortex with chilled 0.25 M H_2SO_4, which contained 34.86 ± 0.68 g linamarin/kg dry weight.

Therefore, these acid extraction and HPLC methods should be suitable for extraction, detection and purification of linamarin from cassava root cortex for further applications.

Acknowledgements

The authors gratefully acknowledge the financial support given by the Royal Golden Jubilee Ph.D. Program of the Thailand Research Fund, the National Research Council of Thailand and King Mongkut's University of Technology Thonburi, Thailand.

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