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DETERMINING LINAMARIN IN CASSAVA, USING THE ENZYME-IMMOBILIZED MICROPLATE METHOD

H.-H. Yeoh and C.-K. C. Tan

Abstract

Glutaraldehyde and polyethylene imine were used to bind cassava leaf ß-glucosidase (linamarase) onto the inner walls of a 96-well microplate. The enzyme microplates were easy to prepare and could be stored at 4 ^{o}C until needed. For linamarin determination, cassava roots were homogenized in 0.1 M *o*-phosphoric acid, and the filtrate was adjusted to a pH of 6 with NaOH before being added to the wells. The cyanide released was then determined with a spectrophotometer. As little as 1 nmol of linamarase could be detected. The microplate method would be suitable for the rapid analysis of a large number of samples.

Introduction

Cassava is widely cultivated in the tropics for its starchy, edible roots. Linamarin, a cyanoglucoside, is found in both leaves and roots (Bradbury and Holloway 1988).

Various methods have been developed to determine the linamarin content in cassava roots (Bradbury and Egan 1992; Bradbury et al. 1991; Cooke 1978; Nambisan and Sundarasan 1984; Yeoh 1993; Yeoh and Truong 1993). Several procedures incorporate the use of exogenous linamarase to hydrolyse the cyanoglucoside, then measure the cyanide released with either a spectrophotometer (Cooke 1978; Nambisan and Sundarasan 1984) or with a cyanide ion-selective electrode (Yeoh 1993; Yeoh and Truong 1993).

To analyse large numbers of samples, these procedures can be time consuming and tedious unless they are automated (Narinesingh et al. 1988; Rao and Hahn 1984). Moreover, spectrophotometry generally uses large volumes of reagents, particularly pyridine, which is both toxic and expensive. We therefore examined alternative methods of determining cyanogen that could handle large numbers of samples, and reduce the concentration of pyridine. We first studied a procedure involving enzyme-immobilized microcentrifuge tubes (Yeoh and Tan 1994b), then an enzyme-linked microplate, which may be more efficient in handling large numbers of samples (Yeoh and Tan 1994a). In this paper, we describe the preparation of an enzyme-immobilized microplate, and discuss the protocol for determining linamarin in cassava roots.

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Materials and Methods

Enzyme preparation

Cassava leaf ß-glucosidase (linamarase) was prepared as described by Yeoh (1989).

Enzyme-microplate immobilization

The enzyme was immobilized to a 96-well microplate, using the protocol for immobilizing the ß-glucosidase to Hybond-N nylon described by Yeoh (1993), but with some modifications. The following steps were carried out at room temperature (24-25 ^{o}C): each well was activated with 50 µL 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 15 min. The activated well was then coupled with 50 µL 5% (w/v) polyethylene imine in 0.1 M phosphate buffer (pH 7.5) for 60 min, followed by reactivation with 50 µL 2.5% (w/v) glutaraldehyde for 15 min. At the end of each treatment, the well was washed with deionised water. Cassava leaf ß-glucosidase, on citrate buffer (pH 6, 50 µL, 130 nkat/mL), was then added to the activated well and left overnight at 4 ^{o}C. The enzyme-bound well was then washed with deionised water, followed by a solution of 0.5 M NaCl and deionised water. Citrate buffer (pH 6, 50 µL) was then pipetted into the well and the microplate stored at 4 ^{o}C.

Preparing cassava extract

The parenchymal tissue of freshly harvested roots was homogenized in 0.1 M *o*-phosphoric acid at the ratio of 1 g fresh wt to 10 mL extraction medium. The homogenate was filtered and the filtrate adjusted to pH 6 with NaOH (Yeoh and Truong 1993). The linamarin standard was also prepared in 0.1 M *o*-phosphoric acid and its pH adjusted to 6 with NaOH.

Determining linamarin content

Aliquots (5, 10, and 20 µL) of the cassava extract were added to the enzyme-bound microplate wells. The final volume was made up to 20 µL. Linamarin standards of about 130 nmol were also added to the wells. The microplate was then incubated for 10 min at 30 ^{o}C. A spectrophotometer (Nambisan and Sundarasan 1984) was then used to measure the cyanide that was released. This was carried out by adding 8 µL each of 0.2 M NaOH, 0.2 M HCl, and

1% (w/v) chloramine T. After 1 min, 24 μL of barbituric acid-pyridine reagent was added, followed by 125 μL of deionised water. The microplate was left to stand at room temperature for 10 min and the absorbance at 570 nm read with a microplate reader.

Results and Discussion

A linear relationship was obtained between nmol of linamarin and absorbance at 570 nm for linamarin up to 30 nmol. Linamarin as low as 1 nmol could also be detected. The absorbance values were observed to decrease slightly with time, but the relationship between the absorbance values and nmol of linamarin remained linear. Linamarin standards should therefore be included in every microplate assay.

A suitable protocol for linamarin determination in cassava roots was thus developed, bearing in mind the limits of linamarin detection by the enzyme microplate. For this purpose, we selected one cultivar (PRC 60a) that was high in linamarin content, and two others (PRC 443 and PRC 476) that were low (Yeoh and Truong 1993). Cultivars with such wide-ranging linamarin contents were chosen to facilitate the design of a suitable protocol.

Results showed that PRC 60a had a linamarin content of 2,460 $\pm$ 0.38 mg/g fresh wt and, as expected, cvs. PRC 443 and PRC 476 had lower contents (640 $\pm$ 0.19 and 460 $\pm$ 0.08 mg linamarin per gram fresh wt, respectively). These values compared reasonably well with those previously reported for these cultivars (Yeoh and Truong 1993), given that linamarin concentrates in longitudinal and radial fashion in cassava roots varies in content from root to root in the same plant (Bradbury et al. 1991; Cooke 1978), and is affected by environmental conditions.

Because each microplate contains 96 wells, the microplate method should be useful for large numbers of samples.

Another advantage is that the microplates can be stored at 4^{o} C until needed. So far, we have not observed any decrease in enzyme activity in the microplate during storage.

The procedure for linamarin determination is not only simple to carry out, but it also requires small samples and small volumes of reagent. The use of barbituric acid-pyridine reagent is greatly reduced: from 3 mL (Nambisan and Sundarasan 1984) to 24 μL per sample. Although the microplate method still requires the addition of many reagents to measure the cyanide released, this task is easily carried out with multichannel pipettes. The chore of measuring the absorbance values of individual samples is also eliminated with the use of the microplate reader. Overall, this assay is both cost and time effective.

References

Bradbury JH; Egan SV. 1992. Rapid screening assay of cyanide content of cassava. Phytochem Anal 3:91-94.

Bradbury JH; Holloway WD. 1988. Chemistry of tropical root crops: significance for nutrition and agriculture in the Pacific. Australian Centre for International Agricultural Research (ACIAR), Canberra, A.C.T., Australia.

Bradbury JH; Egan SV; Lynch MJ. 1991. Analysis of cyanide in cassava using acid hydrolysis of cyanogenic glucosides. J Sci Food Agric 55:277-290.

Cooke RD. 1978. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). J Sci Food Agric 29:345-352.

Nambisan B; Sundarasan S. 1984. Spectrophotometric determination of cyanoglucosides in cassava. J Assoc Off Anal Chem 67:641-643.

Narinesingh D; Jaipersad D; Chang-Yen I. 1988. Immobilization of linamarase and its use in the determination of bound cyanide in cassava using flow injection analysis. Anal Biochem 172:89-95.

Rao P; Hahn SK. 1984. An automated enzymatic assay for determining the cyanide content of cassava (*Manihot esculenta* Crantz) and cassava products. J Sci Food Agric 35:426-436.

Yeoh H-H. 1989. Kinetic properties of ß-glucosidase from cassava. Phytochemistry 28:721-724.

Yeoh H-H. 1993. Nylon-linamarase electrode for rapid determination of linamarin. Biotechnol Tech 7:761-764.

Yeoh H-H; Tan C-KC. 1994a. An enzyme-immobilized microplate for determination of linamarin for large number of samples. Biotechnol Tech 8:337-338.

Yeoh H-H; Tan C-KC. 1994b. Determination of linamarin in cassava using enzyme-sensitised microcentrifuge tubes. J Sci Food Agric (in press.)

Yeoh H-H; Truong VD. 1993. Quantitative analysis of linamarin in cassava using a cassava ß-glucosidase electrode. Food Chem 47:295-298.