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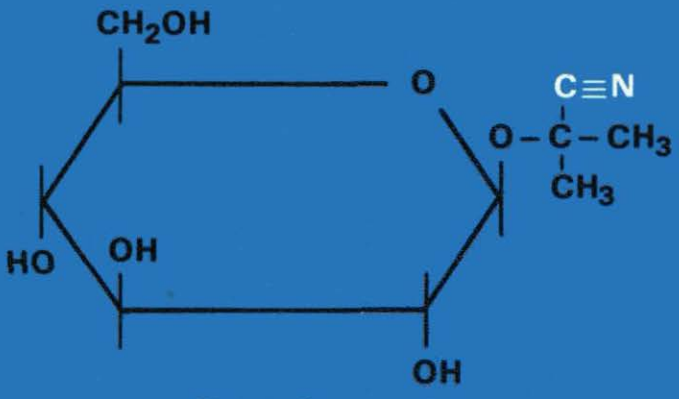

Linamarin

Enzymatic assay for determining the cyanide content of cassava and cassava products

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CASSAVA INFORMATION CENTER
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ENZYMATIC ASSAY FOR DETERMINING THE CYANIDE CONTENT OF CASSAVA AND CASSAVA PRODUCTS

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Abstract

A new methodology is given for the preparation and assay of linamarase and its use in determining total and free cyanide contents in cassava. The enzymatic assay is reproducible, faster and more sensitive than earlier quantitative methods. The method for purifying linamarase from the peel permits one technician to prepare in 2 days sufficient enzyme for 5000 assays. The preparation of assay extracts from parenchymal tissue, peel or leaves is described; one technician can easily handle 40 samples/day. Cyanogenic glucosides are hydrolysed by short incubation with the purified enzyme, and the cyanide released is measured colorimetrically. The cyanide detection limit is <0.01 mg/100 g fresh wt of peeled root. Chemicals and solutions required and representative calculations of cyanide contents are given in the Appendix.

I. Introduction

Cassava (*Manihot esculenta* Crantz) is not only an important source of calories for more than 200 million people, but also its use as an animal feed is increasing (8). The utilisation of cassava in human and animal

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nutrition is, however, limited by the possibility of chronic and acute cyanide toxicity (6) resulting from continuous consumption. Cassava contains the cyanogenic glucosides linamarin and lotaustralin, which produce hydrocyanic acid when the action of the enzyme linamarase is initiated by crushing or otherwise damaging the cellular structure of the plant (1).

Although the toxicity of free (non-glucosidic) cyanide has clearly been established, the degree of toxicity of the cyanogenic glucosides is uncertain (7). Defining the extent of this problem is complicated by the difficulties of the usual quantitative cyanide-determination methods. These methods are tedious, irreproducible and inaccurate (9) since they employ lengthy hydrolysis or autolysis of the glucosides followed by distillation or aspiration of the released cyanide.

Recently, an enzymatic assay for determining the cyanide content of cassava was developed (2,4), which obviates the need for steam distillation or aspiration. It is reproducible, faster and more sensitive than earlier quantitative methods. The cassava sample is extracted in acid which inactivates endogenous linamarase, allowing measurement of the proportion of cyanide present in the sample as cyanogenic glucoside and free cyanide. The assay can be applied to parenchymal tissue (the peeled root), the peel itself and the leaves, as well as to cassava products since it relies on the purified exogenous enzyme, unlike the autolytic methods. The cyanide detection limit is <0.01 mg/100 g fresh weight of peeled root. Using the methods described in detail in Section II, 40 samples per day can be handled easily by one technician.

The method for purifying linamarase from the peel is simple and permits a technician to prepare in two days sufficient enzyme for about 5000 assays from 500 g of peel. The cyanogenic glucosides present in the cassava extracts are hydrolysed by a short incubation (15 min) with this purified enzyme, and the free cyanide produced is then measured colorimetrically.

The different responses of the cyanogenic glucoside and the free cyanide to simple processing were investigated in a preliminary study using this new methodology (3). The assay has also been applied to a

preliminary study of a breeding programme for producing cassava lines with low cyanide contents (5).

II. Experimental procedures

A. Preparation of linamarase from the peel

Diced (0.2 x 0.5 cm) cassava peel (200 g) is homogenized in a Waring blender for 3 min at maximum speed in 0.1 *M* acetate pH 5.5 (8 batches of 25 g peel each in 200 ml acetate). The homogenate is centrifuged at 10,000 *g* for 30 min and the supernatant (ca. 1600 ml) brought to 60% saturation of ammonium sulphate and held at 4°C for 16 hours (overnight). The precipitate obtained by centrifugation at 10,000 *g* for 1 hour is dissolved in 150 ml 0.1 *M* phosphate pH 6.0 and dialysed (3 x 50 vol) against this buffer solution. The solution should be stored in 20-ml lots (for convenience) at -18°C; it will lose ca. 10% of its activity after 2 months' storage.

B. Determination of linamarase activity

The activity of the linamarase solution must be determined prior to its use in cyanide determinations. Enzyme aliquots (0.1 ml) are added to tubes containing 5 m*M* linamarin (0.5 ml). The tubes are incubated at 30°C for 30 min; the reaction is stopped by adding 0.6 ml of 0.2 *M* NaOH.

The cyanide present in each tube is determined spectrophotometrically as follows: 2.8 ml of 0.1 *M* phosphate pH 6.0 is added to each tube, followed by 0.2 ml of chloramine T. The solutions are mixed and the tubes placed in iced water for about 5 min. Then 0.8 ml of the pyridine/pyrazolone reagent is added to each tube, followed by thorough mixing. The blue colour that has developed after 90-min storage at room temperature is measured at 620 nm; this absorbance does not change (<5%) between 60 and 120 min after adding the colour reagent. Chloramine T incubation times between 3 and 15 min do not vary the final absorbance.

The rate of HCN release in the range 0 to 1.2 A_{620}/30 min is proportional to the quantity of enzyme added. Potassium cyanide dried over concentrated H_2SO_4 is used to calibrate the absorbance values; 2.5 μg of KCN in the final 5-ml volume of the assay usually gives about 0.84 A_{620} (i.e.,1 μMol gives 21.8 A_{620}). The absorbance measured in the enzyme assay is used to calculate the activity in units/ml of enzyme solution, where one unit is defined as that which produces 1 μMol of HCN/min at 30°C. When prepared as described above and diluted 600 times with phosphate buffer, the enzyme usually gives an absorbance of about 1.0 (i.e., ca. 9 units activity/ml of undiluted enzyme solution), depending of course on the age, condition and variety of cassava used. The enzyme activity required to hydrolyse totally the cyanogenic glucosides present in the extracts (in the system described in Section C) is 0.3 units; i.e., 0.1 ml of such an enzyme solution diluted 3 times.

C. Preparation of cassava extracts for cyanide analysis

Diced 1-cm cubes of parenchymal tissue (30-100 g) are homogenized at room temperature in a Waring blender for 15 s at low speed in 160 ml of 0.1 *M* orthophosphoric acid, followed by 2 one-min homogenizations (at a one-min interval) at high speed. This timing procedure minimises overheating and wear and tear of the blender. If the peel or the leaves are being analysed, 2-10 g of peel (diced 0.5-cm squares) or 1-4 g of leaves (whole or sliced) are used.

The resultant homogenate is filtered on GF/A paper (15-cm diameter)*, using a Buchner funnel. The homogenizer vessel is rinsed with 0.1 *M* orthophosphoric acid (60 ml), which is filtered in the same way. After the final volume has been measured, the filtrate is transferred to a screw-cap bottle, using 10 ml of distilled water to rinse the Buchner flask. This acid extract should be stored at 4°C and will remain stable for at least 4 days at this temperature.

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* Whatman's Ltd., Maidstone, Kent ME 14 2 LE, UK

Instead of the glass fibre filtration (GF/A) stage, the extracts can be centrifuged at 10,000 *g* for 30 min, but filtration is usually faster. Centrifugation is recommended only for boiled root products since the gelatinised starch hinders filtration.

D. Determination of the total cyanide and free cyanide of the extracts

Four aliquots (0.1 ml) of the foregoing extracts (diluted if necessary with 0.1 *M* phosphoric acid) are added to 4 Quickfit-stoppered test tubes containing 0.4 ml of 0.2 *M* phosphate buffer adjusted to pH 7.0 with 5 *M* NaOH. An aliquot (0.1 ml giving an activity of at least 0.3 units, as described in Section IIB) of linamarase (suitably diluted in 0.1 *M* phosphate pH 6.0) is added to 2 of the tubes to give total cyanide data; and 0.1 ml of 0.1 *M* phosphate pH 6.0 is added to the other 2 tubes to obtain free cyanide data.

The tubes are incubated at 30°C for 15 min; the reaction is stopped by adding 0.2 *M* NaOH (0.6 ml). The cyanide present in each tube is then determined by the modified chloramine T, pyridine/pyrazolone method described in Section IIB. The free and total cyanide contents in the cassava material are then calculated from the weight of material sampled and the volume of extract produced. (Appendix II gives a representative calculation).

The free cyanide content of fresh cassava material is normally $\leq$10% of the total cyanide content; therefore, it is usually more accurate to use aliquots $>$0.1 ml for the free cyanide determination. A common scheme used is 0.5 ml of the extract, followed by 1.0 ml of the 0.2 *M* NaOH to ensure that the pH is $>$10 after this addition. Then the phosphate buffer 0.1 *M* pH 6.0 (2.5 ml) is added, followed by the chloramine T, pyridine/-pyrazolone reagents as described in IIB.

The total cyanide content of the aliquot assayed should be $\leq$1.5 μg HCN (i.e., $A_{620} \leq$ 1.2); dilution of the extracts may be

necessary in some cases. The sensitivity of the assay can be extended by adding 0.1-0.3 ml of acid extract to the 0.2 *M* phosphate pH 7 for a final volume of 0.5 ml. The pH of the incubation mixture is in the range of 6 ± 0.6, depending on the volume of extract taken; closer control of pH or phosphate molarity is unnecessary. This obviates the need for preliminary neutralisation of the extracts unless 0.5 ml of the extract is used.

Open tubes can be utilised instead of the sealed *Quickfit* tubes, but recovery is usually reduced by ca. 10%, depending on the percentage of total cyanide present as free cyanide. Nevertheless, the use of ordinary tubes considerably shortens assay time, and reduced recoveries may be acceptable in some cases.

References cited

1. CONN, E.E. 1969. Cyanogenic glycosides. J. Agric. Food Chem. 17(3):519-526.

2. COOKE, R.D. 1978. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). J. Sci. Food Agric. 29:345-352.

3. ——— and MADUAGWU, E.N. 1978. The effects of simple processing on the cyanide content of cassava chips. J. Food Technol. 13:299-306.

4. ——— BLAKE, G.G. and BATTERSHILL, J.M. 1978. Purification of cassava linamarase. Phytochemistry 17:381-383.

5. ——— HOWLAND, A.K. and HAHN, S.K. 1978. Screening cassava for low cyanide using an enzymatic assay. Exp. Agric. 14(4):367-372.

6. COURSEY, D.G. 1973. Cassava as food; toxicity and technology. *In* Nestel, B. and MacIntyre, R., eds. Chronic Cassava Toxicity; proceedings of an interdisciplinary workshop, London, 29-30 Jan. 1973. International Development Research Centre, Ottawa, IDRC-010e. pp.27-36.

7. MONTGOMERY, R.D. 1969. Cyanogens. *In* Liener, I.E., ed. Toxic Constituents of Plant Foodstuffs. Academic Press, New York. pp.143-157.

8. NESTEL, B. 1973. Current utilization and future potential for cassava. *In* Nestel, B. and MacIntyre, R., eds. Chronic Cassava Toxicity; proceedings of an interdisciplinary workshop, London, 29-30 Jan. 1973. International Development Research Centre, Ottawa, IDRC-010e. pp. 11-26.

9. ZITNAK, A. 1973. Assay methods for hydrocyanic acid in plant tissues and their applications in studies of cyanogenic glycosides in *Manihot esculenta*. *In* Nestel, B. and MacIntyre, R., eds. Chronic Cassava Toxicity; proceedings of an interdisciplinary workshop, London, 29-30 Jan. 1973. International Development Research Centre, Ottawa, IDRC-010e. pp.89-96.

APPENDIX

I. Chemicals and solutions required

A. Orthophosphoric acid, phosphate and acetate buffers

1. 0.1 *M* orthophosphoric acid: 5.6 ml of orthophosphoric acid/*l* of distilled water (depending on the purity of the acid)
2. 0.1 *M* phosphate buffer pH 6.0: Solution A1is adjusted to pH 6.0 with 5 *M* NaOH
3. 0.2 *M* phosphate buffer pH 7.0: 11.0 ml of orthophosphoric acid/*l* of distilled water, subsequently adjusted to pH 7.0 with 5 *M* NaOH

   (Solutions A2 and A3 can be made from mono- or di-sodium phosphate followed by pH adjustment, but care must be taken that the degree of hydration stated on the bottle is correct since some of these salts are hygroscopic.)
4. 0.1 *M* acetate buffer pH 5.5:5.8 ml glacial acetic acid/*l* of distilled water, subsequently adjusted to pH 5.5 with 5 *M* NaOH.

B. Sodium hydroxide

1. 5 *M* NaOH: 20p g of NaOH/*l* of distilled water
2. 0.2 *M* NaOH: 40 ml of solution B1 diluted to 1 litre with distilled water.

C. Linamarin*

5m*M* linamarin: 36 mg of linamarin are dissolved in 30 ml of 0.1 *M* phosphate pH 6.0.

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* Calbiochem, 10933 N. Torrey Pines Rd., La Joya, CA 92037

D. Colour development reagents for estimating free cyanide

1. Chloramine T: 0.5 g dissolved in 100 ml of distilled water. Should be made up fresh every day.
2. Pyridine/pyrazolone reagent: 0.2 g of bispyrazolone[3,3'-dimethyl-1,l'-diphenyl-(4,4'-bi-2-pyrazolone) -5,5'-dione] and 1.0g of 3-methyl-1-phenyl-5-pyrazolone are dissolved in 200 ml of analytical grade pyridine.
   This reagent should be made up fresh every 3 days and stored in dark-glass bottles.

E. Potassium cyanide for calibrating absorbance values

KCN is dried for at least 12 hours over concentrated H_2SO_4 in a dessicator. Just prior to use, 125 mg dissolved in 500 ml of 0.2 *M* NaOH in a volumetric flask is diluted 100 times in 0.1 *M* phosphate pH 6 to give a solution of 2.5 μg/ml.

F. Ammonium sulphate

The extracts of crude enzyme (Section IIA) are raised to 60% concentration of $(NH_4)_2SO_4$ by dissolving 460 g of finely ground $(NH_4)_2SO_4$ per litre of extract at room temperature (~20°C), using a glass rod or magnetic agitator.

II. Representative calculation of the cyanide content of cassava extracts

A. KCN calibration of absorbance values

If 2.6 μg of dried KCN in the 5-ml final assay volume (IIB) produces an absorbance of 0.8 at 620 nm under the experimental conditions used; i.e., 2.6 μg KCN, which is the equivalent of 1.08 µg HCN produces 0.8 A_{620}
therefore, 1 µg HCN produces an absorbance of 0.741 . . . . *(a)*

A smaller range of linearity in the increase in absorbance with HCN concentration has been found with the use of less pure (reagent)

grades of pyridine, approximating 0 to 0.8 A_{620} for both the enzyme assay or KCN calibration (instead of 0 to 1.2 A_{620} stated in IIB, IID). A KCN calibration of absorbances should be done to check the upper limit of absorbance linearity. Use of this restricted absorbance range may also be appropriate with low-cost spectrophotometers,which are less exact above absorbances of 0.7.

B. Cyanide content in extracts

Assuming that (i) Xg of cassava material (roots, leaves, peel, etc.) are extracted as described in Section IIC and produce a total orthophosphoric extract of Vml, and (ii) 0.1 ml of this extract is assayed as described in Section IID and at 620 nm produces an absorbance of *A*, the cyanide present in this Vml of extract is

$$\frac{10V \times A}{0.741}\ \mu g \text{ of HCN} \quad \ldots\ldots (b)$$

This was derived from Xg of cassava material, hence the concentration of cyanide in this material is

$$\frac{10V \times A}{0.741} \times \frac{100}{X}\ \mu g \text{ HCN/100 g of cassava}$$

$$\frac{10V \times A}{0.741} \times \frac{100}{X} \times \frac{1}{1000}\ mg \text{ HCN/100 g of cassava}$$

$$\frac{1.35V \times A}{X}\ mg \text{ HCN/100 g} \quad \ldots (c)$$

If 0.5 ml of the extract was sampled (as is often the case for the free cyanide measurements), then this becomes

$$\frac{0.27\ V \times A}{X} \text{ mg HCN/100 g . . . . .} (d)$$

The precise size of the coefficient in these formulae will vary slightly from laboratory to laboratory since the KCN calibration of the absorbance at 620 nm (*a* above) will vary slightly, depending on the purity of the chemicals used and the efficiency of the spectrophotometer. For example, if (i) 68 g of parenchymal tissue gave 230 ml of the extract and 0.1 ml of this extract assayed after enzyme incubation (Section IID) gave an absorbance of 0.569, the total cyanide content is

2.60 mg/100 g of tissue or 26.0 ppm

or if (ii) 0.5 ml of this extract was assayed for free cyanide (Section IID) and gave an absorbance of 0.097, the free cyanide content is

0.089 mg/100 g of tissue or 0.89 ppm.