World Journal of Microbiology & Biotechnology 11, 178–182

Origin of enzymes involved in detoxification and root softening during cassava retting

F. Ampe and A. Brauman*

The origin of root softening during cassava retting was investigated in a natural retting and in a sterile fermentation. Softening only occurred in the natural retting. Although high activities of endogenous pectin methyl esterase were found in cassava extracts from both fermentations, the depolymerizing enzymes polygalacturonase, active at low pH, and pectate lyase were only found in the non-sterile retting. No cellulase or xylanase activity was observed. The role of pectinases in the softening of cassava roots was confirmed *in vitro* using commercial enzymes. Root softening is therefore due to the combined action of endogenous pectin methyl esterase and exogenous bacterial depolymerizing enzymes. Detoxification occurred in both fermentations, confirming that the linamarase responsible for the destruction of cassava cyanide glycosides was mainly endogenous, even though microbial β-glucosidases may help in the detoxication.

Key words: Cassava, detoxification, linamarase, pectinase, softening, traditional fermentation.

Retting, a spontaneous lactic fermentation of cassava roots, is the key step in the preparation of *foo-foo* (cassava flour) and *chickwangue* (cassava bread), the main cassava-based foods of Central Africa. This process has been described (Okafor *et al.* 1984; Oyewole 1990; Brauman *et al.* 1995) and optimized in terms of product quality and retting speed (Ampe *et al.* 1994a). During the process, cyanogenic compounds are degraded, flavour compounds are elaborated and the roots softened. Softening is indispensible for further processing of the roots but the mechanisms involved are not fully understood.

Degradation of all plant cell walls may be due to the combined action of pectic enzymes, as in linen flax (Chesson 1978). This involves the demethoxylating pectinesterase, (EC 3.1.1.11) and depolymerizing enzymes. The latter include polygalacturonases (endopolygalacturonases, EC 3.2.1.15), polygalacturonate lyases (endolyase, EC 4.2.2.2, and exolyase, EC 4.2.2.9), and polymethylgalacturonate lyases (endolyase, EC 4.2.2.10 and an unclassified exolyase). Polygalacturonate lyases (PGL) and polygalacturonases (PG) need de-esterification of the methoxyl group of pectin for activity whereas polymethylgalacturonate lyases (PMGL) can degrade highly methoxylated pectic substances. Pectinesterases (PE) are found in bacteria, fungi and most plants (Baterman & Millar 1966). PG are mainly microbial in origin but sometimes occur in ripening fruits and vegetables. PMGL and PGL have only been described in microbial species (Rexova-Benkova & Markovic 1976).

Previous results have shown that cassava softening is mediated by bacteria (Okafor *et al.* 1984; Oyewole 1990). Sterile roots soaked in sterile water do not ret whereas microorganisms, isolated from a previous retting, can be used to induce cell wall degradation. PE and PGL were only found in cassava inoculated with *Corynebacterium* sp. However, no pectolytic activity and especially no PE were found in fresh roots. More surprisingly, Okafor *et al.* (1984) found no pectolytic activity in rettings inoculated with *Bacillus* sp. although softening occurred. More recently, Oyewole & Odunfa (1992) showed the presence of extracellular pectin methyl esterase during retting. However, no conclusion about the mechanisms of root softening can be made from these results as the origin of the enzymes was not determined.

The other main transformation during retting is the degradation of the cyanogenic compounds in the cassava, mainly linamarin, a β-cyanoglucoside. Detoxification is mostly due to the action of linamarase (linamarin β-D-glucoside glucohydrolase) (Ikediobi & Onyike 1982), which has been extensively studied (Cooke *et al.* 1978; Ketiku *et al.*

F. Ampe is and A. Brauman was with the Laboratoire de Microbiologie et de Biotechnologie, ORSTOM, BP 181-Brazzaville, Congo. A. Brauman is now with the Laboratoire d'Ecophysiologie des Invertébrés, Université Paris XII, Avenue du Général De Gaulle, 94 010 Créteil, France, Fax: (33) 1 45 17 15 05.* Corresponding author.

Table 1. Polygalacturonate lyase, polygalacturonase and pectinesterase activities in extracts from control and sterile fermentations.

Enzymes	Time (h)	Activity in control fermentation		Activity in sterile fermentation	
		Total (μmol/min 100 g dry matter)	Specific (μmol/min mg protein)	Total (μmol/min 100 g dry matter)	Specific (μmol/min mg protein)
Pectate lyase	0	0	0	0	0
	9.5	0	0	0	0
	20	184	0.38	0	0
	27	125	0.29	0	0
	44	79	0.24	0	0
Polygalacturonase	0	71	0.09	0	0
	9.5	0	0	0	0
	20	135	0.34	0	0
	27	133	0.44	0	0
	44	92	0.33	0	0
Pectinesterase	0	465	0.56	211	0.41
	9.5	335	0.53	191	0.54
	20	328	0.82	173	0.59
	27	301	0.99	132	0.47
	44	196	0.70	170	0.68

1978; Yeoh 1989) and is present in both the cassava root (Cooke *et al.* 1978) and in the bacteria involved in retting (Okafor & Ejiofor 1985; Giraud & Raimbault 1992). The relative contribution to retting of the linamarases from the two sources is still controversial (Maduagwu 1983; Okafor & Ejiofor 1990).

The present study attempts to define the role and origin of pectolytic enzymes and linamarase in retting, to elucidate the mechanism of root softening and the origin of detoxification. The eventual aim is to produce a bacterial starter culture for this traditional fermentation.

Materials and Methods

Origin of the Plant Material

Cassava roots (*Manihot esculenta* var. MM86, known as 'Ngansa') were harvested near Brazzaville (Congo) 18 months after planting.

Fermentation Conditions

Two controlled rettings were performed. A 'natural' retting in a bioreactor was used as a control (CF) in which pH and dissolved O_2 were monitored. For the sterile retting (SF), the cubed cassava roots were sterilized with 0.1% (w/v) $HgCl_2$ in ethanol, according to Okafor *et al.* (1984), and then soaked in sterile water. The pH and partial O_2 pressure of the SF were set to that of the CF by addition of 1 M HCl and N_2, respectively. For both fermentations, 1.5 kg peeled roots, cut into cubes (1 cm^3), were soaked in 2.5 l sterile water in 4.5-l bioreactors (SET 004M; Setric, Toulouse, France). The temperature was maintained at 32°C and agitation performed by liquid recirculation at 2.5 l/h (using a peristaltic pump).

Penetrometry Index

A previous study had shown that a penetrometry index of 3 mm/s corresponded to the end of a retting, as traditionally evaluated (Ampe *et al.* 1994a). A penetrometer (model 10-SUR; PNR, Berlin) was used on six randomly chosen root sections; each section was tested six times.

Enzyme Assays

Eighty ml of 0.1 M citrate buffer, pH 6.5, were added to 40 g cassava mash which was then homogenized in a Waring Blender, held overnight at 4°C and centrifuged at 12,000 × *g* for 30 min. The supernatant was lyophilized and resuspended in 0.1 vol. of citrate buffer.

β-Glucosidase. Activity of β-glucosidase was measured using *p*-nitrophenol-β-D-glucopyranoside (PNPG), (20 mM) in 0.1 M Na-phosphate buffer, pH 6.8, for 1 h at 25°C. The reaction was stopped by addition of an equal volume of 0.2 M sodium borate, pH 9.8, and *p*-nitrophenol was determined spectrophotometrically at 400 nm (Hosel & Barz 1975).

Linamarase. Linamarase was assayed with linamarin as substrate and measuring the appearance of CN^- (Giraud & Raimbault 1992): 100 μl 50 mM linamarin in 0.1 M citrate buffer, pH 6, were added to 400 μl of extract. At regular intervals, 50 μl aliquots were each added to 50 μl 0.1 M NaOH to stop the reaction and stored at 4°C. Cyanide was liberated by addition of 50 μl 0.1 M H_2SO_4 and 850 μl distilled water to each aliquot and was measured using Spectroquant (Merck, Darmstadt, Germany). One unit of linamarase was defined as the amount of enzyme which released 1 μmol of CN^- per min.

Pectinesterase. Pectin pectylhydrolase (EC 3.1.1.11) activity was assayed by titration of 1 ml of extract in 1% pectin (74% dry matter; Grindsted), 0.1 M NaCl and 1 mM NaN_3 to pH 7 with 0.01 M NaOH at 30°C. One unit was defined as the amount neutralizing 1 μmol of COO^-/min.

Polygalacturonate Lyase. PGL activity was assayed by the procedure of Starr *et al.* (1977), which does not differentiate between endo-PGL [poly(1,4-α-D-galacturonide)lyase, EC 4.2.2.2] and expo-PGL [poly(1,4-α-D-galacturonide)exolyase, EC 4.2.2.9]. One unit of PGL was defined as the amount forming of 1 μmol of one unsaturated bond in the galacturonide between C4 and C5.

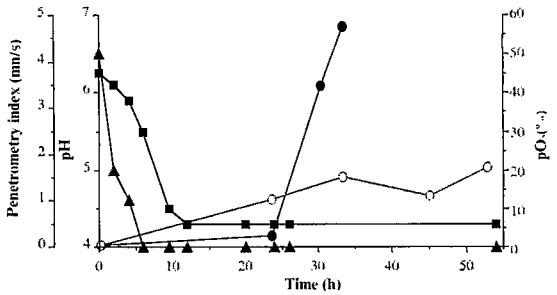

Figure 1. Evolution of root softening (in terms of penetrometry index) in the control (●) and sterile (○) fermentations, and pH (■) and pO_2 (▲) profiles during both fermentations.

Polygalacturonase. Poly(1,4-α-D-galacturonide)glycanohydrolase (EC 3.2.1.15; PG) was assayed by viscometry: 0.5 ml of extract was added to 40 ml of 1% pectin in 100 mM acetate buffer, pH 4.7, and the rate of reduction in viscosity measured at 25°C in a viscosimeter at a rotation of 150.93 s^{-1}. One unit was defined as the amount releasing 1 μmol of hexose/min. Total activities are expressed as U/100 g cassava.

Cellulase and Xylanase. Cellulase and xylanase were assayed at 37°C in McIlvaine buffer, pH 5.8, (McIlvaine 1921) using the Somogyi procedure (Somogyi 1945). The substrates were microcrystalline cellulose (100 mg) and xylan (18 mg/ml), respectively.

Electrophoresis

For pectinase activities, non-denaturing PAGE was performed in 7% acrylamide gels containing 1% pectin using a slab cell (Hoefer Scientific Instruments) operating at 250 V (20 mA) for 80 min. Gels were stained with Ruthenium Red (Cruickshank & Wade 1980), which stains polygalacturonic acid; the action of PG was seen as the presence of clear zones in the gel.

Other Analytical Methods

Total and free cyanides were assayed using the method of Cooke *et al.* (1978) and protein was determined using a modification of Lowry's procedure (Bensadoun & Weinstein 1976).

Action of Pectic Enzymes In Vivo

Sterilized slices of cassava were inoculated with 50 μl enzyme extract or 5 μl purified pectolytic enzymes [endopolygalacturonase from *Aspergillus niger*; pectolyase from *A. japonicum* and/or pectinesterase from orange peel (all Sigma)] and placed in sterile beakers containing 10 ml of 0.01 M citrate buffer, pH 5. Penetrometer readings were estimated after 24 and 48 h at 30°C.

Results

Softening

In the CF, retting was complete in 35 h, but no softening was obtained in the SF (Figure 1). Despite the physicochemical changes imposed on the SF (pH decrease, anaerobiosis), the walls of cassava cells from it did not appear degraded when viewed under the microscope. Significant PE activity was found in fresh cassava roots (405 U/100 g of cassava dry matter; Table 1). Total activity remained fairly constant in the SF but decreased with time in the CF. Specific activity in the CF was slightly higher than in the SF. PG was only found in the CF from 20 h to the end of the fermentation (Table 1). The total activity of PG in the CF ranged from 92 to 135 U/100 g cassava dry matter.

Non-denaturing electrophoresis of extracts followed by specific staining with Ruthenium Red confirmed the presence of PG. Clear zones were very intense for samples from 20 h to the end of the CF, whereas no clearing was obtained in samples from the SF or fresh cassava. Migration was similar for all positive samples, and very slow compared with the control enzyme from *A. niger*.

PGL activity was found in the CF from 20 h to the end of the process (Table 1). No such activity was detected in SF.

In vivo lytic activity of pectic enzymes on cassava cells was confirmed by inoculating fresh sterile cassava with commercial enzymes. After 24 h at 30°C, tissues inoculated with PE plus PG, PE plus PGL and PE plus PGL and PG were almost totally degraded.

No xylanase or cellulase activity was found in either retting (data not shown).

Detoxification

Half (50%) of the total cyanogenic compounds were degraded in the SF and 97% in the CF (Figure 2). Enzyme assays confirmed endogenous linamarase activity (Table 2). Linamarase activity was first estimated by its ability to degrade PNPG, as previous authors have shown 65% analogy between assays performed with linamarin and PNPG (Mkpong *et al.* 1990; Okafor & Ejiofor 1990). β-Glucosidase activity in the CF was significant in fresh roots (specific activity 9.4 IU/mg protein) but decreased after a few hours (Table 2). In the SF, total activity remained constant but low. Consistency of these results was confirmed by assay of linamarase activity using linamarin as substrate (data not shown).

To try to differentiate between plant and microbial β-glucosidase, the pH-activity profiles of the enzyme extracts were determined (Figure 3). The enzymes were active over a wide pH range (4 to 8), with an optimum at 6.0 to 7.5 but no significant difference was observed between the two profiles.

Discussion

From these results, pectolytic enzymes of microbial origin are clearly indispensible for the softening to be completed. The significant activity of PE in fresh cassava roots, and its stability throughout the fermentation, demonstrates its plant origin and indicates its involvement in softening. This enzyme is bound to cell walls by strong ionic bonds but may be partly released by solvation of the roots in retting

Table 2. β-Glucosidase activities in control and sterile fermentations.

	Activity in control fermentation		Activity in sterile fermentation	
Time (h)	Total (μmol/min 100 g dry matter)	Specific (μmol/min mg protein)	Total (μmol/min 100 g dry matter)	Specific (μmol/min mg protein)
0	9.12	9.4	2.15	4.7
9.5	5.58	7.5	2.55	6.6
20	6.10	12.5	1.75	5.1
27	7.68	17.6	2.30	6.2
44	7.24	21.8	1.38	6.5

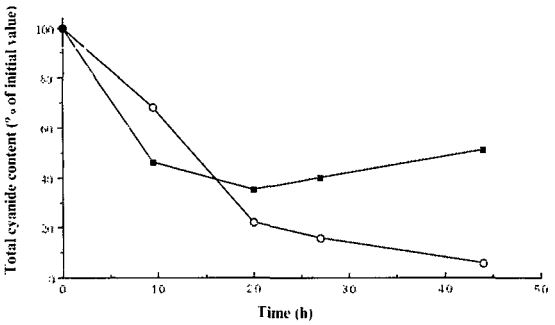

Figure 2. Total cyanide content in the control (○) and sterile (■) fermentations. The initial cyanide content was 207 mg. Kg $^{-1}$.

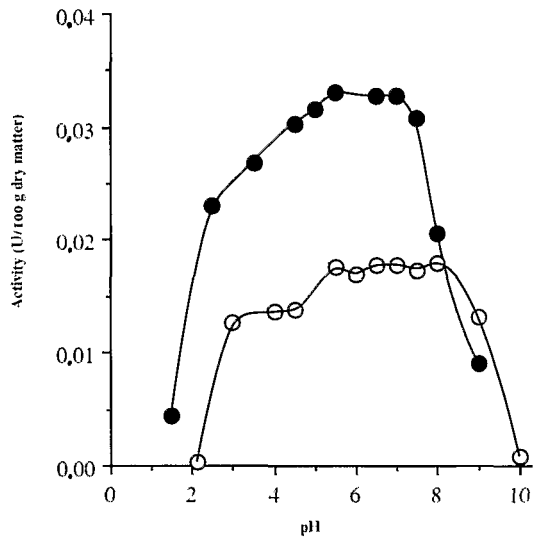

Figure 3. β-Glucosidase activity versus pH profiles of the crude enzyme extracts taken from the control (●) and sterile (○) fermentations.

juice. In contrast, the depolymerizing enzymes (PG and PGL) were not detected in fresh roots or the SF. In CF, significant activities were detected after 20 h of fermentation, when the microbial populations were fully established (Brauman *et al.* 1995), thus demonstrating their microbial origin. The absence of lyase activity in SF is in agreement with the results of Fogarty & Ward (1974), who found that PGL and polymethylgalacturonate lyases were of microbial origin.

PG activity has an optimum pH close to that of retting juice (pH 5) and predominated throughout the retting, whereas PGL only retained 10% of its maximal activity at pH 5 (Ampe *et al.* 1994b; unpublished work). However, as the pH of the retted roots is higher than the retting juice (Brauman *et al.* 1995) and PGL is generally considered to be more characteristic of bacterial pectolytic enzymes (Chesson 1978), the role of the PGL in softening could be important, and must be investigated further.

No other hydrolase was found during cassava retting. Moreover, softening could be performed by inoculating commercial pectinesterase and depolymerizing pectolytic enzymes onto fresh and sterile cassava roots. Therefore, we suggest that, like linen flax (Chesson 1978), the combination of demethoxylating and depolymerizing pectic enzymes alone enables plant cell separation and softening. However, further studies should be undertaken to determine the contribution of each pectic enzyme to softening.

Various bacteria are able to produce pectinases. Some lactic acid bacteria which are important in retting, such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides*, produce PG or PGL (Juven *et al.* 1985; Sakellaris *et al.* 1989). Other bacteria involved in the process, such as clostridia (Brauman *et al.* 1995), may be responsible for pectin degradation, as previously described for softening of potatoes (Lund 1972).

The activity of pectic enzymes in cassava retting was reported by Okafor *et al.* (1984) and Oyewole & Odunfa (1992). The present study is the first evidence for the plant origin of the pectinesterase and for the *in vivo* activity of depolymerizing enzymes.

Results of CN^- measurements indicated that endogenous cassava linamarase, measured as β-glucosidase activity, is mainly responsible for the observed detoxication. The difference in the β-glucosidase activities in the fresh roots from the SF and CF (Table 2) may be due to the inhibitory effect of the $HgCl_2$ used to sterilize the SF roots. Microbial linamarase present in cassava retting is thought to assist detoxification (Okafor & Ejiofor 1985, 1990). However, as nearly 25% (Table 2) of the total β-glucosidase activity present in the sterile roots can degrade more than 50% of

the total cyanide content of the fresh roots, it can be assumed as previously indicated (Maduagwu 1983; Vasconselos *et al.* 1990), that the level of linamarase activity present in intact roots is sufficient to detoxify the roots without the help of microbial linamarase. Nevertheless, even if bacteria do not directly detoxify cassava roots, they could help the degradation of linamarin through the destruction of the plant cell walls.

Linamarase activity was found to be still very high at pH 4; this conflicts with the results of Ikediobi & Onyike (1982) but agrees with those of Yeoh (1989) who found an optimal pH of 6 to 7.3 and 85% activity at both pH 5 and 8 with linamarin or PNPG as substrate. The specific activity of the endogenous linamarase in the present study is comparable with that measured by Cooke *et al.* (1978) and Ikediobi & Onyike (1982).

In conclusion, retting of cassava is a spontaneous fermentation in which endogenous and microbial enzymes act together to soften the roots and to degrade endogenous cyanogenic compounds. The first step is probably the degradation of plant cell walls by endogenous pectin esterase located within the plant and released by the decreasing pH, followed by microbial PG and PGL activities that depolymerize the pectic chains. Degradation of linamarin seems to be mainly due to the endogenous cassava linamarase.

Acknowledgements

The authors acknowledge the technical assistance of E. Avouampo, A. Agossou and G. Eboungabeka. This work was supported by EEC program STD 2 from DG XII, grant TS2A-0226.

References

Ampe, F., Agossou, A., Trèche, S. & Brauman, A. 1994a Cassava retting: Optimization of a traditional fermentation by experimental research methodology. *Journal of the Science of Food and Agriculture* **65**, 335–361.

Ampe, F., Robert, H., Agossou, A., Keleke, S. & Brauman, A. 1994b Enzymatic aspects of root softening. In *Cassava Food Transformation*, eds Brauman, A., Trèche, S. & Agbor-Egbé, T., in press. Paris: ORSTOM.

Baterman, D.F. & Millar, R.L. 1966 Pectic enzymes in tissue degradation. *Annual Review of Phytopathology* **4**, 119–146.

Bensadoun, A. & Weinstein, D. 1976 Assay of protein in the presence of interfering materials. *Analytical Biochemistry* **70**, 241–250.

Brauman, A., Kéléké, S., Miambi, E. 1995 Étude cinétique du rouissage traditionnel des racines de manioc en Afrique Centrale (Congo). In *Cassava Food Processing*, eds Agbor-Egbé, T., Brauman, A., Griffon, D. & Trèche, S., in press. Paris: ORSTOM.

Chesson, A. 1978 The maceration of linen flax under anaerobic conditions. *Journal of Applied Bacteriology* **45**, 219–230.

Cooke, R.D., Blake, G.G. & Battershill, J.M. 1978 Purification of cassava linamarase. *Phytochemistry* **17**, 381–383.

Cruickshank, R.H. & Wade, G.C. 1980 Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* **107**, 177–181.

Fogarty, W.M. & Ward, O.P. 1974 Pectinases and pectic polysaccharides. *Progress in Industrial Microbiology* **13**, 59–119.

Giraud, E. & Raimbault, M. 1992 Degradation of the cassava linamarin by lactic acid bacteria. *Biotechnology Letters* **14**, 593–598.

Hosel, W. & Barz, W. 1975 β-Glucosidases from *Cicer arientum* L. *European Journal of Biochemistry* **57**, 607–616.

Ikediobi, C.O. & Onyike, E. 1982 Linamarase activity and detoxication of cassava (*Manihot esculenta*) during fermentation for gari production. *Agricultural and Biological Chemistry* **46**, 1667–1669.

Juven, B.J., Lindner, P. & Weisslowicz, H. 1985 Pectin degradation in plant material by *Leuconostoc mesenteroides*. *Journal of Applied Bacteriology* **58**, 533–538.

Ketiku, A.O., Akinyele, I.O., Keshinro, O.O. & Akinnawo, O.O. 1978 Changes in the hydrocyanic acid concentration during traditional processing of cassava into 'gari' and 'lafun'. *Food Chemistry* **3**, 221–226.

Lund, B.M. 1972 Isolation of pectolytic clostridia from potatoes. *Journal of Applied Bacteriology* **35**, 609–614.

McIlvaine, T.C. 1921 A buffer solution for colorimetric comparison. *Journal of Biological Chemistry* **49**, 183–188.

Maduagwu, E.N. 1983 Differential effects on the cyanogenic glycoside content of fermenting cassava root pulp by β-glucosidase and microbial activities. *Toxicology Letters* **15**, 335–339.

Mkpong, O.E., Yan, H., Chism, G. & Sayre, R.T. 1990 Purification, characterization, and localization of linamarase in cassava. *Plant Physiology* **93**, 176–181.

Okafor, N. & Ejiofor, M.A.N. 1985 The linamarase of *Leuconostoc mesenteroides*: production, isolation and some properties. *Journal of the Science of Food and Agriculture* **36**, 669–678.

Okafor, N. & Ejiofor, M.A.N. 1990 Rapid detoxification of cassava mash fermenting for garri production following inoculation with a yeast simultaneously producing linamarase and amylase. *Process Biochemistry International* **June**, 82–86.

Okafor, N., Ijioma, B. & Oyolu, C. 1984 Studies on the microbiology of cassava retting for foo-foo production. *Journal of Applied Bacteriology* **56**, 1–13.

Oyewole, O.B. 1990 Optimization of cassava fermentation for fufu production: effects of single starter cultures. *Journal of Applied Bacteriology* **68**, 49–54.

Oyewole, O.B. & Odunfa, S.A. 1992 Extracellular enzyme activities during cassava fermentation for *'fufu'* production. *World Journal of Microbiology and Biotechnology* **8**, 71–72.

Rexova-Benkova, L. & Markovic, O. 1976 Pectic enzymes. *Advances in Carbohydrate Chemistry and Biochemistry* **33**, 323–385.

Sakellaris, G., Nokolaropoulos, S. & Evangelopoulos, A.E. 1989 Purification and characterization of an extracellular polygalacturonase from *Lactobacillus plantarum* strain BA 11. *Journal of Applied Bacteriology* **67**, 77–85.

Somogyi, M. 1945 Determination of blood sugar. *Journal of Biological Chemistry* **160**, 61–68.

Starr, M.P., Chatterjee, A.K., Starr, P.B. & Buchanan, G.E. 1977 Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. *Journal of Clinical Microbiology* **6**, 379–386.

Vasconselos, A.T., Twiddy, D.R., Westby, A. & Reilly, P.J.A. 1990 Detoxification of cassava during gari preparation. *International Journal of Food Science and Technology* **25**, 198–203.

Yeoh, H.-H. 1989 Kinetic properties of β-glucosidase from cassava. *Phytochemistry* **28**, 721–724.

(Received in revised form 30 September 1994; accepted 13 October 1994)