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Changes in Activities of Cell Wall Degrading Enzymes during Fermentation of Cassava (*Manihot esculenta* Crantz) with *Citrobacter freundii*

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ABSTRACT

The cell wall degrading enzymes polygalacturonase (EC 3.2.1.15), pectinase (EC 3.1.1.11), cellulase (EC 3.2.1.4) and xylanase (EC 3.2.1.8), as well as α-amylase (EC 3.2.1.1) and phosphorylase (EC 2.4.1.1), were monitored during fermentation of cassava (Manihot esculenta *Crantz) with* Citrobacter freundii. *All the enzymes were detected in cassava at the start. During fermentation, initial decreases in polygalacturonase, cellulase and xylanase were followed by increases which peaked as the tissue softened. There were significant* ($P<0.05$) *increases in pectinase, xylanase, cellulase, polygalacturonase and phosphorylase in inoculated cassava and the fermentation medium relative to controls (uninoculated cassava and medium) during the softening period. The control cassava did not ferment, indicating that the textural changes in inoculated cassava were due to enzymes secreted by* C freundii. *Studies on the effect of enzyme inhibition on fermentation showed that the pectic enzymes and cellulase were of primary importance and that inhibition of α-amylase and phosphorylase had no effect on the process.*

Key words: Cassava fermentation, *Citrobacter freundii*, cell wall enzymes.

1 INTRODUCTION

The mature underground tuberous roots of cassava (*Manihot esculenta* Crantz) provide an essential source of calories for several millions of people in tropical Africa and Central America (Nartey 1978; Bourdoux *et al* 1980). Because of the presence of the toxic cyanogens linamarin and lotaustralin (Nartey 1978; Bourdoux

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et al 1980), the roots are usually processed to yield products with safe levels of residual HCN (Nartey 1978) before consumption. In Nigeria, one of the processing methods involves the fermentation of the roots in water for about three days during which period they soften. They are then washed, sieved and strained through cloth bags to produce a whitish, semi-solid meal popularly called *akpu*. This is cooked in boiling water, pounded into *fufu* and served with soup. Virtually nothing is known about the biochemical changes that bring about this process of cassava root softening which leads to the production of *akpu*. However, it is well known that the activities of cell wall degrading enzymes increase during the textural changes associated with the ripening of many fruits, a process very similar to cassava root softening. Also it is well established that many plant pathogens elaborate enzymes capable of degrading the cell wall polysaccharides of host plants. Previous work has identified the microflora involved in *akpu* fermentation (Okpokiri *et al* 1986) but the enzymes associated with the process have hitherto not been studied.

This paper reports the detection and variation in activities of polygalacturonase, pectinase, phosphorylase, cellulase, α-amylase and xylanase during the controlled fermentation of cassava with *Citrobacter freundii*.

2 EXPERIMENTAL

Freshly harvested cassava tubers (Udukanana, Igbo variety) were obtained from a farm in Benin City. The tubers were about 18 months old at the time of harvest. They were used a few hours after harvesting.

2.1 Pre-treatment of tubers

Healthy, unwounded tubers were washed in tap water and surface-sterilised by immersing in absolute ethanol for 5 min. The bark (outermost brown covering) was peeled off with a sterilised knife to expose the cortex which was also surface-sterilised in absolute ethanol. The phelloderm was then removed with a sterilised knife and, operating in a sterile hood, semi-circular sections of approximately equal size cut out from the 'middle' of the tubers into sterile trays.

2.2 Fermentation flasks

Conical flasks (500 ml) were used. These contained 200 ml of distilled water containing 0·20 g of peptone. The flasks were fitted with rubber bungs bearing two glass delivery tubes whose ends were plugged with cotton wool and wrapped with foil. Before use the flasks and contents were sterilised by autoclaving at 121°C for 15 min. On cooling to room temperature (~28°C) 10 cassava sections (each weighing ≈ 3·0 g) were introduced into each flask in the sterile hood. The flasks were then inoculated from a 24-h culture of *C freundii* on nutrient agar; uninoculated flasks containing cassava served as controls. Air was drawn through each flask by a water pump connected to one of the delivery tubes, via the second tube plugged with cotton wool which had been soaked with 0·10% (w/v) $HgCl_2$. A total of 12 flasks were set up, six tests and six controls. The flasks were left at room temperature (28°C). At the start, and subsequently at 12-h intervals, one inoculated and one

control flask were removed for enzyme extraction and assay. The cassava sections, separated from the incubation liquor, were extracted at room temperature. Incubation liquor was used unmodified for enzyme assay.

2.3 Extraction and assay of xylanase, polygalacturonase and cellulase

Enzyme extractions were carried out according to a modification of the method described by Bateman *et al* (1969). Tissue (2·0 g) was homogenised for 10 min in a mortar with 15 ml of cold distilled water. The homogenate was centrifuged at $10\,000 \times g$ for 10 min and the resultant clear supernatant was used for the assay. Release of reducing groups at 30°C from 0·10 g per 100 ml sodium polypectate, 0·10 g per 100 ml oat spelts xylan, and 0·10 g per 100 ml carboxymethylcellulose in 0·10 M buffer (see Bateman *et al* 1969) was measured according to the method of Nelson (1944) as modified by Somogyi (1952). Enzyme activities were expressed in terms of reducing sugar equivalents liberated (μmol min^{-1} ml^{-1} liquor/extract).

2.4 Extraction and assay of pectinase

Pectinase was extracted by the procedure outlined by Awad and Young (1979). Assay was by a slight modification of the procedure of Rouse and Atkins (1955): supernatant (1·0 ml) was added to 5·0 ml of polygalacturonic acid-*D*-methyl ester solution in 0·5 g per 100 ml of 0·10 M NaCl. The mixture was immediately brought to a pH of 7·58. After 10 min incubation at room temperature, the release of carboxyl groups was monitored by titration with 0·02 M NaOH. Pectinase activity was expressed as equivalent of ester hydrolysed min^{-1} ml^{-1} liquor/extract.

2.5 Extraction and assay of α-amylase

Tissue (2·0 g) was homogenised in a mortar with 30 ml of 2 mM sodium acetate buffer, pH 6·8, for 10 min. The homogenate was centrifuged at $6000 \times g$ for 15 min. The supernatant fraction was then incubated for 15 min at 70°C to inactivate β-amylase. Thereafter it was immediately cooled to room temperature (28°C) and used for the assay of α-amylase according to the method of Bidderback (1971) by measuring the decrease in staining power of starch with iodine reagent at 620 nm. α-Amylase activity was expressed as decrease in absorbance min^{-1} ml^{-1} of liquor/extract.

2.6 Extraction and assay of phosphorylase

Phosphorylase was extracted by the method of Bulpin and Rees (1978) and assayed according to the method of Crabtree and Newsholme (1972) as modified by Ugochukwu *et al* (1977). Release of inorganic phosphate from glucose-1-phosphate was monitored by the method of Kuttner and Lichtenstein (1930). Phosphorylase activity was expressed in terms of inorganic phosphate released (μg min^{-1} ml^{-1} of liquor/extract).

2.7 Enzyme inhibition

α-Amylase and phosphorylase were inhibited by maltose (Long *et al* 19??) and glucose (Long *et al* 1971), respectively. Cellulase was inhibited with N-

bromosuccinimide (Hurst *et al* 1977), and pectinase and polygalacturonase were inhibited with *p*-hydroxybenzoic acid (Soni and Bhatia 1981). The inhibitors were added to separate fermentation flasks immediately after autoclaving. Each inhibitor was added to give a concentration of 20 mM. On cooling to room temperature the flasks were used for cassava fermentation with *C freundii* as previously described. After 72 h the contents of each flask were examined. The physiological marker for the degree of inhibition was the extent of transformation of the hitherto hard tuber into softened tissue.

3 RESULTS

Xylanase, cellulase, polygalacturonase, pectinase, phosphorylase and α-amylase were detected in cassava tissue at the start. During fermentation, initial decreases in cellulase (Fig 1), xylanase (Fig 2), and polygalacturonase (Fig 3) were followed by increases which became maximal at the appearance of softness between the 48th and 60th hours. Figure 4 shows α-amylase activity during the fermentation period.

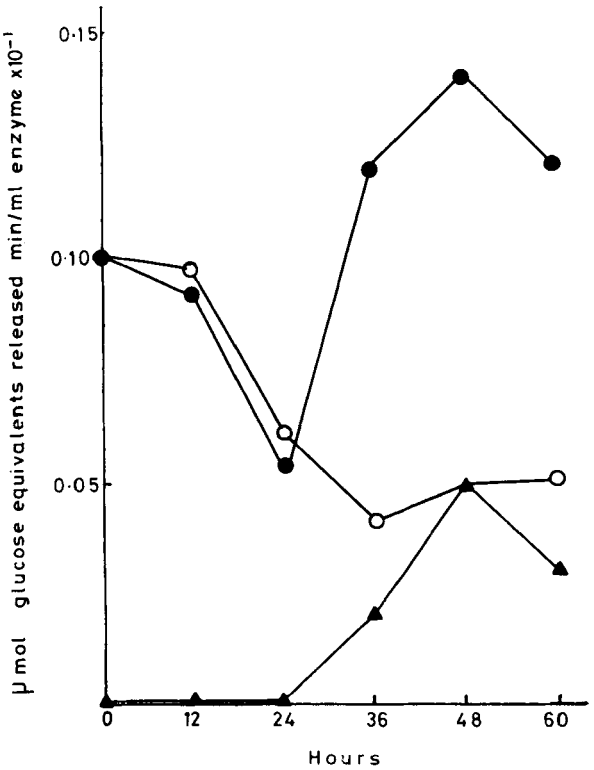

Fig 1. Cellulase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

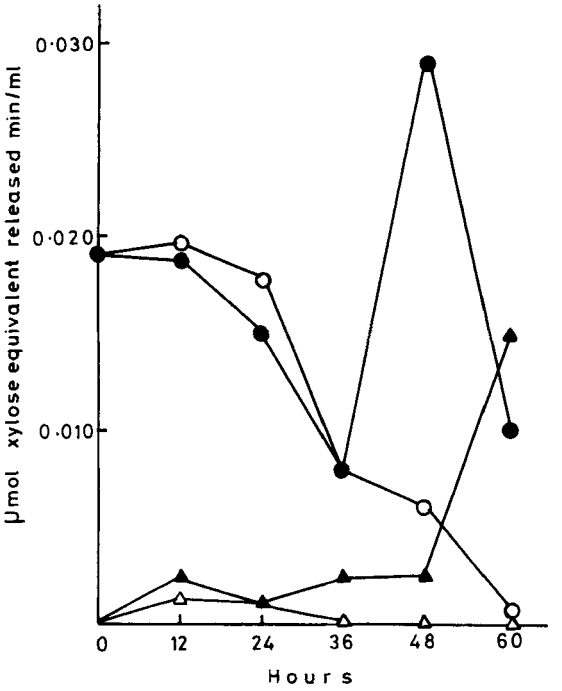

Fig 2. Xylanase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

There was a steady decline in the activity of this enzyme in the cassava itself from both inoculated and control flasks. There was a significant increase ($P < 0{\cdot}05$) in α-amylase activity in the liquor from the inoculated flasks compared with that from control flasks during the softening period. Phosphorylase (Fig 5) also decreased initially but subsequently increased in inoculated cassava tissue only, peaking at the 60th hour. Phosphorylase activity in the fermentation medium rose steadily and peaked with softening. Figure 6 shows that maximum pectinase activity in inoculated cassava tissue was obtained at the 36th hour; 12 h after this enzyme peaked in the fermentation medium. At the softening stage, there were significant increases ($P < 0{\cdot}05$) in the activities of pectinase, polygalacturonase, xylanase and cellulase in inoculated cassava tissue relative to controls. None of the enzymes, with the exception of α-amylase, was detected in the fermentation medium at the start, but all became detectable by the 12th hour and peaked with softening. The initial decreases in enzyme activities were maintained in uninoculated cassava tissue; there was little enzyme activity in the uninoculated medium throughout the 60 h of observation. α-Amylase showed progressive increases in uninoculated medium.

The inhibition of polygalacturonase, pectinase and cellulase caused cessation of fermentation, whereas the inhibition of α-amylase and phosphorylase had no effect on the fermentation process. It was also observed that the contents of the uninoculated flasks did not ferment.

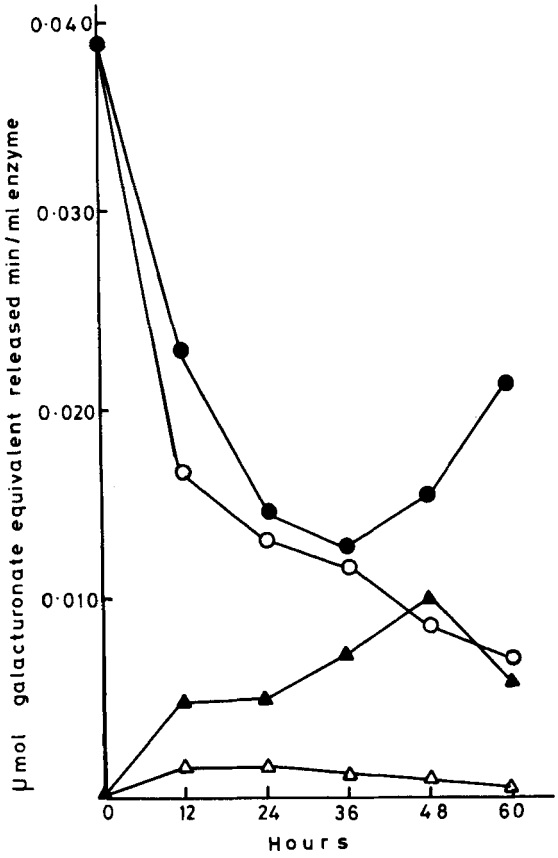

Fig 3. Polygalacturonase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

4 DISCUSSION

The *akpu* meal produced from an uncontrolled fermentation of cassava is usually associated with an undesirable odour, probably caused by the presence of some microbial metabolites. In preliminary studies on the production of a more acceptable product (Okolie P N, Ugochukwu E N unpublished) it was found that controlled fermentation of cassava with *C freundii* resulted in a product devoid of the undesirable smell. Post-fermentation streaking of cassava mash on sterile agar plates and subsequent incubation at 37°C showed that *C freundii* was the predominant microorganism at the end of fermentation. This is highly desirable in fermentation-related enzyme studies, because then it is possible more validly to ascribe enzyme activities to a specific microorganism; this is difficult in mixed-culture fermentations. In the same studies, histological examinations of fermenting cassava sections revealed that the fermentation process involved progressive cell

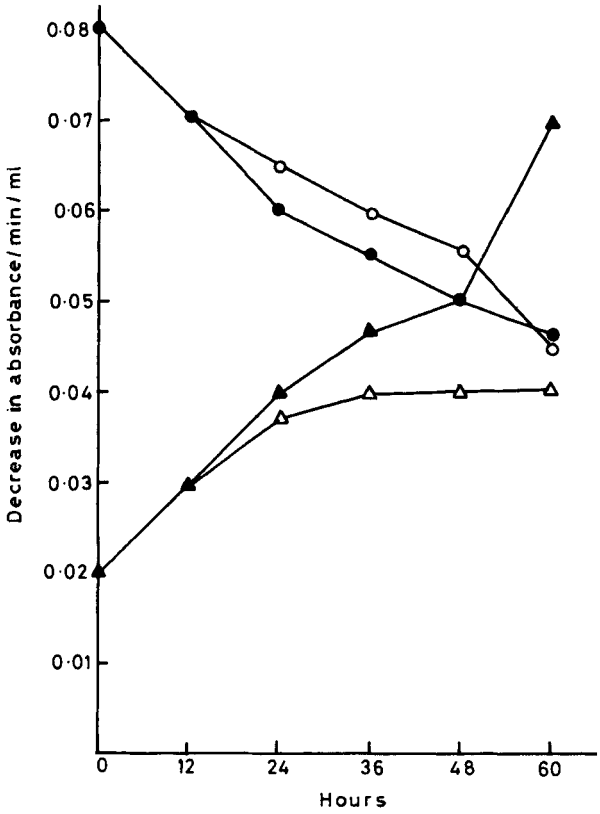

Fig 4. α-Amylase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

wall disruption. Maximal disruption was seen at a stage corresponding to the appearance of softness. Cell wall disruption strongly suggests the involvement of cell wall degrading enzymes in cassava fermentation. In the present study, the cell wall enzymes xylanase, cellulase and polygalacturonase showed maximal activities with tuber softening, whereas pectinase peaked just before this stage. These results, in addition to those obtained in enzyme inhibition studies, show that cellulase, pectinase, xylanase and polygalacturonase are of key importance in the fermentation-dependent softening of cassava tubers. Thus cassava softening is brought about by the activities of cell wall degrading enzymes. This finding is consistent with the primary roles assigned to pectin methyl esterase and polygalacturonase in host/pathogen interactions (Bateman and Miller 1966; Byrde and Fielding 1968). These enzymes have been shown to cause tissue maceration, electrolyte losses and even cell death (Bateman and Miller 1966; Byrde and Fielding 1968; Goribaldi and Bateman 1971). In addition, it has been reported that cellulase appears to be the principal factor involved in the ripening of avocado fruit (Awad 1977; Awad and Young 1979), whereas polygalacturonase is the sole softening

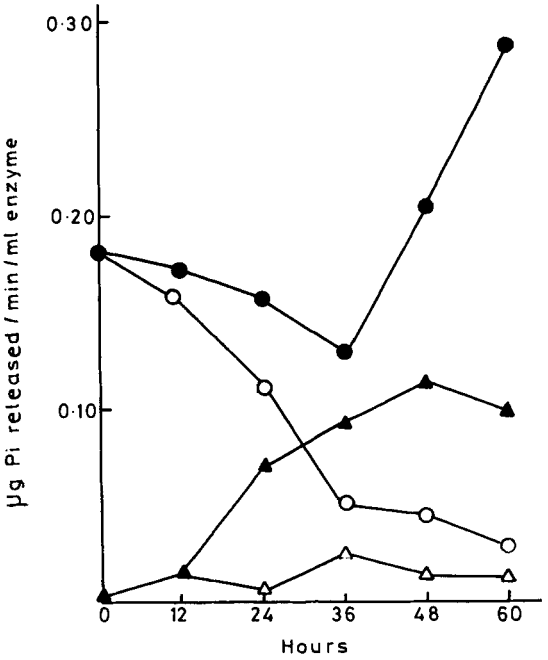

Fig 5. Phosphorylase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

factor in tomatoes (Hobsen 1963). However, a positive correlation has been established between increases in cellulase (Awad 1977; Awad and Young 1979), polygalacturonase (Hultin and Levine 1965; Raymond and Phaff 1966; Barash and Khazzan 1970; Zauberman and Schiffmann-Nadel 1972; Awad and Young 1979) and the softening of avocado fruit. Increases in polygalacturonase have also been reported in ripening Bartlett pears (Ahmed and Labavitch 1980). In the softening of many fruits, pectinase has been shown to increase (Hobsen 1963; Hultin and Levine 1965) or decrease (Zauberman and Schiffmann-Nadel 1972; Awad and Young 1979). Awad and Young (1979) and Zauberman and Schiffmann-Nadel (1972) have reported an inverse relationship between polygalacturonase and pectinase activities during the ripening of avocado fruit, with softening occurring when polygalacturonase was maximal and pectinase minimal. However, our results show that although polygalacturonase in inoculated cassava started to increase at the 36th hour, with onset of decrease in pectinase, softening commenced at the 48th hour, when pectinase was still high, and was complete by the 60th hour when polygalacturonase activity was maximal and pectin methyl esterase minimal.

As might be expected, α-amylase and phosphorylase do not seem to play any significant part in the fermentation of cassava. This is in agreement with the key role status already proposed for the cell wall degrading enzymes. Thus the increases in phosphorylase activity between the 36th and 60th hours of fermentation may have been induced by the increasing presence of starch granules released by cell wall degradation.

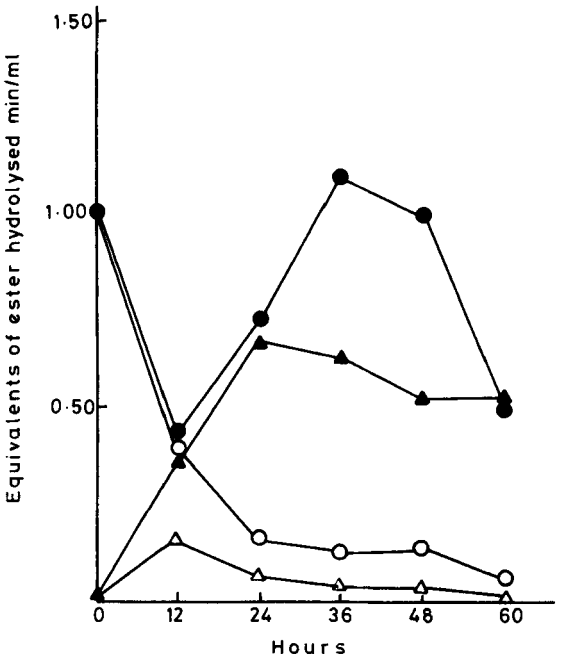

Fig 6. Pectinase activity. ●——● Enzyme activity in inoculated cassava; ○——○ enzyme activity in uninoculated (control) cassava; ▲——▲ enzyme activity in inoculated medium; △——△ enzyme activity in uninoculated (control) medium. Each datum point is the mean of 5 determinations.

The absence of fermentation in the uninoculated flasks suggests that cassava fermentation is not due to endogenous cell wall degrading enzymes. Because of the initial decreases in the activities of these enzymes in cassava, the activities detected in the medium would appear to be due to leaching from cassava. This may be true for initial α-amylase activities in control medium, but it seems unlikely in the fermentation medium. This is because enzyme activities in the fermentation medium at various periods between the 24th and 60th hours were significantly higher ($P < 0{\cdot}05$) than those of controls. Secondly, the initial activity decreases in cassava (inoculated and uninoculated) were followed by simultaneous increases in inoculated cassava and inoculated medium only. Moreover the peak activities of cellulase, pectinase, xylanase and phosphorylase in inoculated cassava exceeded their corresponding values at 0 h. These 'net' increases are unlikely to be due to synthesis of new enzymes, since the cassava tissue at that stage of processing cannot be regarded as living. They are most probably due to microbial enzyme elaboration. Similarly the enzyme activities detected in the fermentation medium are due to secretions of *C freundii*. This is consistent with the finding that microbial and fungal pathogens degrade plant cell walls by enzymes elaborated into their growth medium (Wood 1960; Byrde and Fielding 1968; Albersheim *et al* 1969; Bateman *et al* 1969; Goribaldi and Bateman 1971). The initial enzyme activity decreases in cassava might be due to their conversion to inactive forms, or inhibition by metabolites of *C freundii*. On the other hand, it might be that elaboration of enzymes by *C freundii* in some way suppresses the activities of similar enzymes endogenous to cassava.

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