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Purification and characterization of an intracellular β-glucosidase from a new strain of *Leuconostoc mesenteroides* isolated from cassava

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Y. GUEGUEN, P. CHEMARDIN, P. LABROT, A. ARNAUD AND P. GALZY. 1997. The lactic acid bacterium, *Leuconostoc mesenteroides,* when grown on an arbutin-containing medium, was found to produce an intracellular β-glucosidase. The enzyme was purified by chromatofocusing, ion-exchange chromatography and gel filtration. The molecular mass of the purified intracellular β-glucosidase, as estimated by gel filtration, was 360 kDa. The tetrameric structure of the β-glucosidase was determined following treatment of the purified enzyme with dodecyl sulphate (SDS). The intracellular β-glucosidase exhibited optimum catalytic activity at 50°C and pH 6 with citrate–phosphate buffer, and 5·5 with phosphate buffer. The enzyme was active against glycosides with (1→4)-β, (1→4)-α and (1→6)-α linkage configuration. From Lineweaver–Burk plots, K_m values of 0·07 mmol l^{-1} and 3·7 mmol l^{-1} were found for *p*-nitrophenyl-β-D-glucopyranoside and linamarin, respectively. The β-glucosidase was competitively inhibited by glucose and by D-gluconic acid–lactone and a glucosyl transferase activity was observed in the presence of ethanol. The β-glucosidase of *Leuconostoc mesenteroides,* with cyanogenic activity, could be of potential interest in cassava detoxification, by hydrolysing the cyanogenic glucosides present in cassava pulp.

INTRODUCTION

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) constitute a group of well studied hydrolases that have been isolated from members of all three domains of life, i.e. eucaryotes, bacteria and archaea (Woodward and Wiseman 1982). The principal reaction catalysed by this class of enzymes is the hydrolytic cleavage of β-glycosidic linkages of low molecular mass glycosides. The physiological roles postulated for β-glucosidases are extremely diverse: glucoside ceramide catabolism in human tissues, cell wall, pigment and cyanoglucoside metabolism, defence against pathogens in plants, and utilization of oligosaccharide substrates by many fungi and bacteria (Leclerc *et al.* 1987). Recently, this enzyme was also studied for its potential to liberate aroma-rich terpenes. Such aroma precursor compounds are found in different fruits (mango, passion fruit, grapes) (Williams *et al.* 1982; Gunata *et al.* 1990; Vasserot *et al.* 1993) bonded to glucosides and the β-glucosidases were more effective and specific than the acid hydrolysis process for liberating terpenols from terpenylglucosides.

The establishment of a correlation between epidemics of the human central nervous system syndrome 'Konzo' and prolonged intake of cassava products containing residual cyanogenic glucosides (linamarin, lotaustralin) and cyanohydrins (Tylleskaer *et al.* 1992) has focused attention on both the detoxification and analysis of cassava (Ikediobi *et al.* 1980; Legras *et al.* 1990; Okafor and Ejiofor 1990). Cassava is detoxified during processing by the endogenous β-glucosidase, linamarase, present in the enlarged cassava root. The enzyme is released during grating of the roots. Apparently, the quantity of enzyme released is not sufficient to break down the glucoside present in the root completely (Okafor 1977). However, it was therefore thought that the endogenous linamarase of the tuber could be supplemented from a microbial source exogenous to the roots to ensure greater breakdown of the linamarin.

In this paper, we report the purification and detailed biochemical study of an intracellular β-glucosidase with cyano-

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genic activity from *Leuconostoc mesenteroides*, a lactic acid bacteria involved in the fermentation of cassava product.

MATERIALS AND METHODS

Organism

Leuconostoc mesenteroides was isolated in our laboratory from Cassava (*Manihot esculenta* Crantz) from Guadeloupe and identified using the criteria of *Bergey's Manual of Determinative Bacteriology* (Holt 1989).

Culture techniques

The microaerophilic *Leuc. mesenteroides* was grown in Erlenmeyer flasks filled to the maximum of their capacity (4·8 l in 5 l) to obtain microaerophilic conditions. The culture medium was MRS (De Man *et al.* 1960) adjusted to pH 6·3 with 1 mol l^{-1} HCl. The carbon source was arbutin (5 g l^{-1}) and the medium was autoclaved at 120°C for 20 min.

Enzyme extraction

The bacterial cells were harvested by centrifugation at 8000 *g* for 10 min. Following two rinses with phosphate buffer (20 mmol l^{-1}, pH 7), the pellet was re-dispersed in the same buffer and sonicated with a sonifier 250 apparatus (Branson Co., Danburry, CT, USA). The power was 60 W and the cells were submitted to 0·5 s s^{-1} of sonification for 15 min. The resulting suspension was centrifuged at 10 000 *g* for 10 min. The supernatant fluid (S1) was then ultracentrifuged (180 000 *g*, 90 min) and the β-glucosidase was contained in this supernatant fluid (S2).

Enzyme assay

β-Glucosidase activity against GlcβNp was determined by adding 0·1 ml of enzyme solution to 4·9 ml of citrate–phosphate buffer (0·1 mol l^{-1}, pH 6) containing GlcβNp (5 mmol l^{-1} final concentration) (Blondin *et al.* 1983). The reaction mixture was incubated at 30°C. Samples (0·5 ml) were taken at regular intervals and added to 1·0 ml of carbonate buffer (0·2 mol l^{-1}; pH 10·2). Liberated *p*-nitrophenol (pNP) in this mixture was assayed by spectrophotometry at 400 nm. The molar extinction coefficient used was 18 300 mol^{-1} cm^{-1}. One β-glucosidase activity unit (U) was defined as the quantity of enzyme required for hydrolysis of 1 μmol of substrate (GlcβNp) per min (U ml^{-1}) under the above experimental conditions.

When the substrate used did not contain *p*-nitrophenol, β-glucosidase activity was determined by assaying the liberated glucose. In this case, enzyme solution (0·1 ml) and the substrate (0·2 mol l^{-1} final) were added to 2·9 ml of citrate–phosphate buffer (0·1 mol l^{-1}; pH 6). The reaction mixture was incubated at 30°C. Samples (0·2 ml) were taken at regular intervals and added to 0·4 ml of carbonate buffer (0·2 mol l^{-1}; pH 10·2). Liberated glucose was determined using a hexokinase and glucose 6-P-dehydrogenase procedure (Bergmeyer *et al.* 1974).

When detection of β-glucosidase activity was required in a non-denaturing electrophoresis process, the following technique was used: the gel was immersed in a citrate–phosphate buffer, pH 6, containing 4-methylumbelliferyl-β-D-glucoside (5 mmol l^{-1}) for 10 min at 30°C. The plate was observed under u.v. light. The fluorescent band that appeared corresponded to the enzyme activity (Gusakov *et al.* 1991).

Protein assays

Proteins were assayed by the bicinchoninic acid (BCA) method during the purification procedure, using bovine serum albumin as the standard. The A_{280} was used for monitoring protein in some column effluents.

β-Glucosidase purification

During purification, all procedures were carried out at 4°C.

PBE 94 (chromatofocusing). Chromatofocusing was performed using a Pharmacia HR column (10 × 400 mm) equilibrated with piperazine/HCl, pH 5·5 buffer. Proteins were eluted with 400 ml of polybuffer 74 (Pharmacia), diluted 1:10 and adjusted to pH 4·0 with 1 mol l^{-1} HCl. Proteins were eluted in 5 ml fractions at a flow rate of 20 ml h^{-1}.

Q-Sepharose chromatography (ion-exchange chromatography). The Pharmacia HR column (26 × 400 mm) was equilibrated with Tris–HCl buffer (25 mmol l^{-1}, pH 7). Proteins were eluted with a linear gradient of NaCl from 0·25 mol l^{-1} to 0·5 mol l^{-1}, at a flow rate of 200 ml h^{-1}, and the eluent was collected in 10 ml fractions. Active fractions were pooled and concentrated in an Amicon cell with a PM 10 membrane (W.R. Grace and Co., Amicon Division, Danvers, MA, USA).

Sephacryl S-300 chromatography (gel filtration chromatography). The Pharmacia HR column (10 × 1000 mm) was equilibrated and further eluted with Tris–HCl, 10% (p/v) glycerol buffer (25 mmol l^{-1}, pH 7). The elution rate was 17·0 ml h^{-1} and the eluent was collected in 1·7 ml fractions. The column was calibrated using the following molecular weight standards: thyroglobulin 669 kDa, ferritin 440 kDa, β-amylase 200 kDa, aldolase 158 kDa, bovine serum albumin 66 kDa, ribonuclease A cytochrome c 13·7 kDa.

Polyacrylamide gel electrophoresis

For native electrophoresis, precasted slab gels from the Daiichi Pure Chemicals Co. Ltd (Tokyo) were used. Electrophoresis was performed at pH 8·4 with Tris (0·025 mol l^{-1}) glycine (0·192 mol l^{-1}) buffer. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with precasted slab gels. The enzyme and molecular weight standards (BioRad Laboratories) were denatured in the presence of 5% (v/v) β-mercaptoethanol and 2% (w/v) sodium dodecyl sulphate at 100°C for 10 min. Tris (0·025 mol l^{-1}) glycine (0·192 mol l^{-1}), pH 8·4 with SDS 0·1% (w/v) was used. Electrophoreses were carried out at room temperature and at 30 mA constant current on vertical slabs (10 × 8 cm). The protein bands were revealed with a BioRad silver staining kit.

Determination of the molecular mass

The molecular mass was determined by gel filtration on Sephacryl S-300, as described above and by SDS-PAGE. The molecular mass of the subunit was extrapolated from a plot of log (molecular mass) *vs* mobility (BioRad electrophoresis calibration kit).

For each set of data, the results represent the average of two experiments.

Chemicals

All chemicals were analytical grade.

p-Nitrophenyl-β-D-glucopyranoside (GlcβNp), *o*-nitrophenyl-β-D-glucopyranoside (GlcoNp), *p*-nitrophenyl-α-D-glucopyranoside (GlcαNp), *p*-nitrophenyl-β-D-cellobioside (CelβNp), *p*-nitrophenyl-β-D-galactopyranoside (GalpβNp), *p*-nitrophenyl-β-D-fucopyranoside (FucpβNp), *p*-nitrophenyl-α-L-rhamnopyranoside (RhapαNp), *p*-nitrophenyl-α-L-arabinofuranoside (ArafαNp), *p*-nitrophenyl-β-D-xylopyranoside (XylpβNp), arbutin, methyl-β-D-glucopyranoside, cellobiose, gentiobiose, laminaribiose, amygdalin, linamarin and prunassin were all purchased from the Sigma Chemical Co. Soluble starch and maltose were from Merck. Esculin, α-trehalose and salicin were obtained from Fluka Biochemika. Q-Sepharose Fast Flow, Sephacryl S-300 and PBE 94 were from Pharmacia LKB Biotechnology.

RESULTS

Location and biosynthesis of the β-glucosidase

After submerged growth on arbutin (5 g l^{-1}) as the carbon source, the cells were sonicated. The resulting homogenate showed a β-glucosidase activity which was also found in the supernatant fluid following the centrifugation (S1) and ultracentrifugation (S2) steps. No activity was detected in the culture medium supernatant fluid even after 100-fold concentration with the Amicon cell. The enzyme is considered as being endocellular.

During submerged cell growth on arbutin (5 g l^{-1}), several sample aliquots were taken. Following sonication, the β-glucosidase activity was measured in the S1 fractions (Fig. 1). The production of this activity appeared to be maximum towards the end of the growth phase.

Different carbon sources were used to grow the cells. The resulting activities measured are given in Table 1 and do not indicate any substantial repression or induction. This β-glucosidase appeared to be a constitutive enzyme. The organism was, however, unable to use cellobiose (1→4-β glucose dimer) as carbon source.

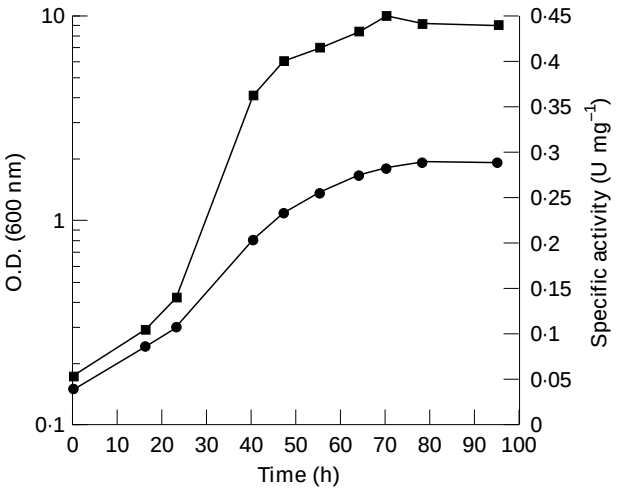

Fig. 1 Growth (●) and β-glucosidase (■) activity of *Leuconostoc mesenteroides* in culture with arbutin (5 g l^{-1}). Experimental procedures are described in Materials and Methods

Table 1 Biosynthesis of the β-glucosidase of *Leuconostoc mesenteroides* during growth with different carbon sources (β-glucosidase activity was determined at 30°C, using 5 mmol l^{-1} *p*-nitrophenyl-β-D-glucopyranoside as the substrate)

Carbon sources	Specific activities*
Glucose	
0·5%	0·20 (46)
1%	0·24 (53)
1·5%	0·26 (55)
2%	0·27 (56)
Salicin 0·5%	0·31 (71)
Arbutin 0·5%	0·45 (100)
Cellobiose 0·5%	no growth

* Values in parentheses are relative activities with the β-glucosidase activity of cells grown on 0·5% arbutin taken as 100.

Purification of the β-glucosidase

The S1 supernatant fluid was centrifuged at 180 000 *g* for 90 min, the supernatant fluid (S2) was fractionated on a chromatofocusing column. The enzyme eluted as a single activity peak from the column at a pH of 4·05. Fractions 34–38 were pooled and fractionated on a Q-Sepharose column (Fig. 2). The aryl-β-glucosidase was eluted as a single peak in fractions 47–57 at 0·37 mol l^{-1} NaCl. These two columns separated from the majority of contaminating proteins. Fractions 50–54 were pooled and concentrated in an Amicon cell with a PM 10 membrane and chromatographed on a Sephacryl S-300 column (Fig. 2). The enzyme was found in fractions 24–30 which give the purified β-glucosidase preparation. The data for the purification steps are summarized in Table 2. At this stage, the enzyme was purified 172·2-fold with a recovery of 10·7% of the total activity.

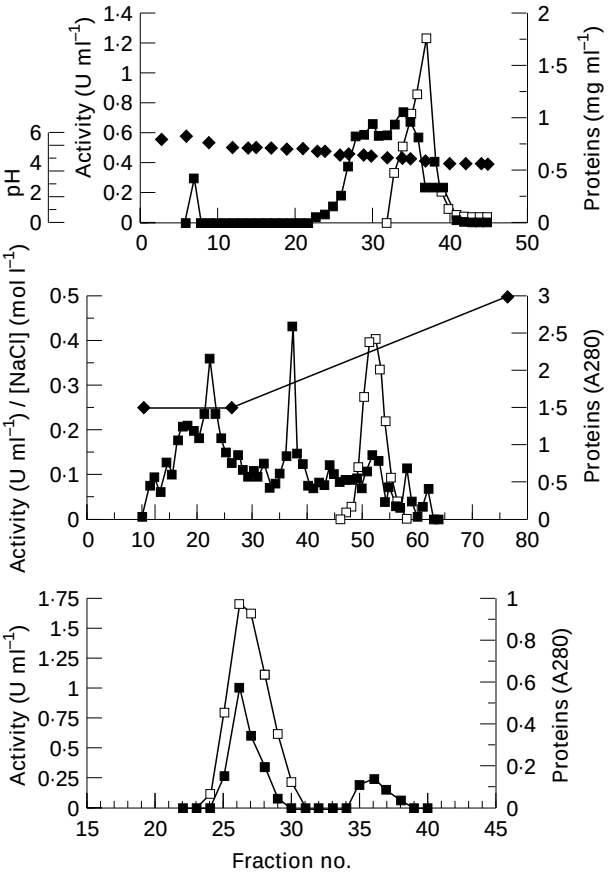

Fig. 2 Purification of the β-glucosidase of *Leuconostoc mesenteroides*. Fractions after isoelectrofocusing on PBE 94 containing high β-glucosidase activity (□) were collected and rechromatographed using a QSFF anion exchange column. Resulting peak fractions containing β-glucosidase activity were further purified by gel filtration chromatography on a Sephacryl S-300 column. pH and NaCl gradients (◆) and proteins (■). β-Glucosidase activity was determined at 30°C, using 5 mmol l^{-1} Glcβ*Np* as the substrate. Experimental procedures are described in Materials and Methods

The purity of the β-glucosidase preparation was confirmed by electrophoresis on native polyacrylamide gel. A single band was observed. The purity was also confirmed by the presence of a single band upon SDS-PAGE.

Physical and chemical properties of the β-glucosidase

The properties of the enzyme were determined using a purified extract.

The molecular mass as estimated by Sephacryl S-300 chromatography was 360 kDa. The denatured enzyme showed one major band (molecular mass 88 kDa) on SDS-PAGE. These results suggested that the β-glucosidase of *Leuc. mesenteroides* was composed of four identical subunits.

The denaturation of the enzyme was monitored by measuring its activity against Glcβ*Np* at 30°C, pH 6, following incubation at several temperatures for various time periods. The calculated enzyme inactivation energy determined from the Arrhenius plots was 190·8 kJ mol^{-1}. The value is similar to those generally reported for enzymes, in which the inactivation energy lies between 150 and 400 kJ mol^{-1}. These values are high, but can be explained by the necessity of breaking, simultaneously, an important number of bindings. The enzyme was not very heat resistant with rapid inactivation at 50°C and above. The half-life of the enzyme was 15 min at 45°C and 5 min at 50°C.

Kinetic properties of the purified β-glucosidase

Influence of pH on activity. The influence of the pH on enzymatic activity is shown in Fig. 3. The optimum pH depends on the buffer used. In phosphate buffer (0·1 mol l^{-1}), it was 5·5 and in citrate–phosphate buffer (0·1 mol l^{-1}), it was 6.

Influence of temperature on activity. The influence of the temperature on activity has been studied with citrate–phosphate buffer (0·1 mol l^{-1}, pH 7) and is shown in Fig. 3. The measurements of activity were done at several temperatures for 4 min. The activity plot for Glcβ*Np* gives an optimal temperature of 50°C. The thermal activation energy determined from the Arrhenius plots is 55·5 kJ mol^{-1}.

Enzyme specificity. The action of the purified β-glucosidase was tested over a large number of substrates with α and β configurations. The results summarized in Table 3 show that the β-glucosidase was active against (1→4)-β configurations (alkyl-β-glucosides, aryl-β-glucosides) but inactive against

Table 2 Summary of the purification procedure for *Leuconostoc mesenteroides* β-glucosidase (β-glucosidase activity was determined at 30°C, using 5 mmol l^{-1} *p*-nitrophenyl-β-D-glucopyranoside as the substrate)

Purification step	Total volume (ml)	Total protein (mg)	Specific activity*	% Total activity (U)	Purification factor†
S1	80	180·6	0·45	100	1
S2	79	125·8	0·63	98·8	1·4
PBE 94	24	4·3	4·60	24·3	10·1
Q-Sepharose	3·3	0·23	46·95	13·1	104·3
Sephacryl S-300	9·1	0·11	77·5	10·7	172·2

* Expressed as μmol *p*-nitrophenyl-β-D-glucopyranoside hydrolysed min^{-1} mg^{-1} protein.
† The purification factor is defined as the ratio of the specific activity to that of S1.

(1→4)-β diglucosides (cellobiose). However, the enzyme was able to hydrolyse α-linkages (GlcαNp, RhapαNp), and with most microbial β-glucosidases, the *Leuc. mesenteroides* β-glucosidase exhibited β-xylosidase activity.

In addition, and because of its ability to hydrolyse two different types of cyanoglucosides (prunassin and linamarin), this enzyme shows a broader specificity than cyanogenic β-glucosidases from plants (Hosel and Conn 1982; Legras *et al.* 1989).

The results of the study of the enzyme affinities for different substrates are given in Table 4. Among the β-glucosides tested, linamarin was the only substrate with an aglycone moiety that was not an aromatic compound. The β-glucosidases showed weak affinity for this compound ($K_m = 3·7$ mmol l^{-1}) as compared to prunassin ($K_m = 0·8$ mmol l^{-1}) and GlcβNp ($K_m = 0·7$ mmol l^{-1}), but this value is average compared to those reported in the literature for linamarase activity: $K_m = 0·51$ mmol l^{-1} for the β-glucosidase from *Fusarium equisety* (Ikediobi *et al.* 1987) and $K_m = 25$ mmol l^{-1} for the linamarase of *Leuc. mesenteroides* (Okafor and Ejiofor 1985).

Enzyme inhibition. The effect of various cations was tested. The enzyme was inhibited by Hg^{2+}, Ag^{+} and Zn^{2+}. The action of some effectors such as EDTA (10 mmol l^{-1}), a chelating agent, allowed us to conclude that the intracellular β-glucosidase of *Leuc. mesenteroides* was not dependent on a metallic cofactor at its active site. The inhibition by *N*-bromosuccinimide, at a concentration of 10 mmol l^{-1}, indicates that a tryptophan residue of the enzyme molecule is important in its catalytic action. *p*-Chloromercuribenzoate, a well-known SH blocking agent, had a moderately inhibitory effect on enzyme activity. This result, in combination with the observed high sensitivity towards Hg^{2+}, may indicate the presence of important sulphydryl groups. Iode (5 mmol l^{-1}), an agent reacting with tyrosine, strongly inhibited the catalytic activity.

Glucose inhibition was studied using GlcβNp as substrate. As is the case for other β-glucosidases (Legras *et al.* 1989; Christakopoulos *et al.* 1994; Gueguen *et al.* 1995), the enzyme was inhibited competitively by glucose. The K_i obtained at the intersection of the lines on the Dixon plot was 14·3 mmol l^{-1}. Moreover, the enzyme was competitively inhibited by D-gluconic acid–lactone ($K_i = 0·06$ mmol l^{-1}), which is known to have a structural similarity with an intermediary compound of the reaction (Conchie *et al.* 1967). The comparison of K_i values indicates that gluconolactone inhibited the enzyme 250 times more strongly than glucose.

Activation. We studied the effect of alcohols on β-glucosidase activity, using GlcβNp as substrate (Fig. 3). Methanol and ethanol increased the activity of the β-glucosidase by 1·3- and 1·1-fold, while propanol and butanol increased the activity by 1·6- and 2-fold, respectively. However, ethanol, propanol and butanol inhibited the activity of this enzyme at or above 1–2 mol l^{-1}. Activation by ethanol has been observed for β-glucosidases from *Candida entomophila* (Gueguen *et al.* 1994) and *Fusarium oxysporum* (Christakopoulos *et al.* 1994). Such a phenomenon, as described by Pemberton *et al.* (1980), is the result of a glycosyl transferase activity. At higher concentrations, the enzymes were inhibited by ethanol, probably because of protein denaturation.

DISCUSSION

Leuconostoc mesenteroides β-glucosidase was obtained in a high state of purity, as judged from gel electrophoretic data and its high specific activity. The molecular mass of the purified β-glucosidase was 360 kDa. The gel filtration and the electrophoretic data suggested that the β-glucosidase was a tetrameric enzyme with a monomer molecular mass of 88 kDa. It is interesting to note that most β-glucosidases purified from eubacteria and archaea, e.g. *Streptomyces reticuli*, *Clostridium thermocellum* or *Clostridium stercorarium*, have been reported to be monomeric (Aït *et al.* 1982; Bronnenmeier *et al.* 1988; Heupel *et al.* 1993). However, β-glucosidases from eubacteria

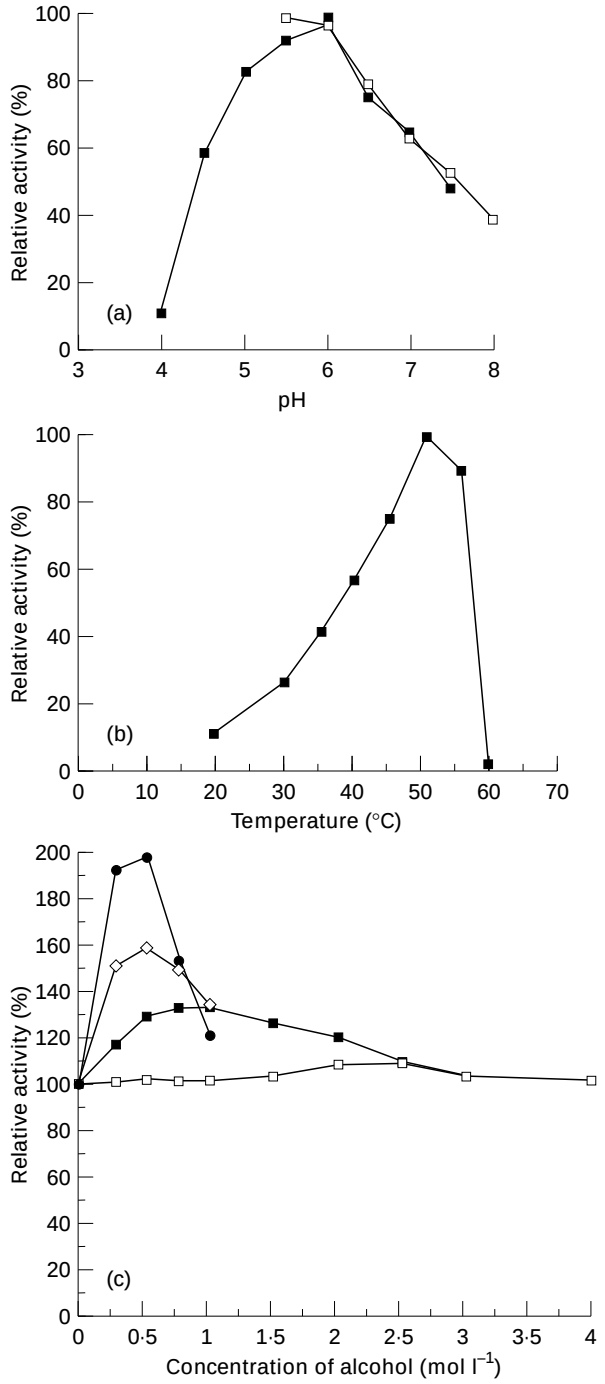

Fig. 3 pH, temperature and alcohol profiles of enzyme activity. Purified β-glucosidase was incubated at the indicated pH (citrate–phosphate buffer (■) and phosphate buffer (□), for the pH ranges 4–7·5 and 5·5–8, respectively), at 30°C (a), at the indicated temperature, at pH 6 (b), at the indicated concentration of methanol (□), ethanol (■), propanol-1 (◇) and butanol-1 (●) at pH 6 and 30°C (c). β-Glucosidase activity was determined, with 5 mmol l^{-1} GlcβNp, via 4-min assays

and archaea were described as dimeric (Legras *et al.* 1989) and tetrameric structures (Kengen *et al.* 1993). The pH_i (4·05) of *Leuc. mesenteroides* β-glucosidase is quite similar to that (4·3) for the β-glucosidase of *Streptomyces lividans* (Mihoc and Klueppel 1990). β-Glucosidases from bacteria generally have acid pH_is (Coughlan 1985). Both pH and temperature optimum are similar to those reported for other purified bacterial β-glucosidases (Sakai *et al.* 1986; Mihoc and Klueppel 1990; Ozaki and Yamada 1991).

The β-glucosidase of *Leuc. mesenteroides* was active against alkyl and aryl glucosides with (1→4)-α or (1→4)-β configurations. Morever, a (1→6)-α-arabinofuranosidase activity was described, but was very low compared to the activity towards aryl-β-D-glucosides like GlcβNp. It is not uncommon for β-glucosidase to be described with a very broad specificity. Glycosidases may be divided into three groups on the basis of substrate specificity: aryl-β-glucosidases (which hydrolyse exclusively aryl-β-glucosides), cellobiases (which hydrolyse oligosaccharides only) and broad specificity β-glucosidases (which show activity on both substrate types and form the most commonly observed group in cellulolytic micro-organisms). The results presented here demonstrate that the β-glucosidase of *Leuc. mesenteroides* preferentially hydrolysed 1→4-linked-aryl-β-glucosides. However, the enzyme hydrolysed, with only limited efficiency, 1→4-linked-alkyl-β-glucosides, 1→4-linked-α-glucosides and 1→6-linked-α-glucosides. Thus, the β-glucosidase of *Leuc. mesenteroides* lacked specificity towards the aglycone moiety and hydrolysed GlcβNp as well as other aryl-β-glucosides, such as salicin and arbutin. The ability to attack α-glycosides demonstrated also a low specificity towards the anomeric configuration of the glycosidic bonds. β-Glucosidases with a broad substrate specificity have been isolated from other micro-organisms. For example, *Aspergillus niger* yielded an enzyme with combined β-D-glucosidase, β-D-xylosidase, α-L-arabinase and β-D-galactosidase activities (Rodionova *et al.* 1987) and *Pyrococcus furiosus* (Kengen *et al.* 1993) exhibited β-D-glucosidase, β-D-xylosidase, β-D-galactosidase and some β-mannosidase activities. The substrate specificity of the *Leuc. mesenteroides* enzyme, in conjunction with the kinetic parameters, especially the K_m values, justify its classification as a β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21).

Fermented products of cassava constitute a major part of the daily diet of many inhabitants of West Africa. The most popular of these products are gari, fufu and lafun. Lactic acid bacteria are the common microflora involved in their production processes. Oyewole and Odunfa (1990) reported that *Lactobacillus* spp. and to a lesser degree *Streptococcus* spp. were responsible for acid production and flavour development in gari, while Okafor (1977) identified a *Leuconostoc* sp. as the dominant organism involved in gari production. To solve the problem of detoxification of cassava pulp, several

Table 3 Substrate specificity of purified β-glucosidase of *Leuconostoc mesenteroides* (dependent on the type of substrate, activity was determined by measuring the release of either *p*-nitrophenol (400 nm) or glucose (glucose oxidase method), at 30°C, as described in Materials and Methods)

Substrates	Configuration of glycoside linkage	Concentration (mmol l^{-1})	Specific activity (U mg^{-1}) at pH 6
(a) *p*-Nitrophenyl-β-D-glucopyranoside	(1→4)-β	5	73·5
o-Nitrophenyl-β-D-glucopyranoside	(1→4)-β	10	20·4
p-Nitrophenyl-α-D-glucopyranoside	(1→4)-α	10	4·1
Salicin	(1→4)-β	20	67·2
Arbutin	(1→4)-β	20	80·6
Methyl-β-D-glucopyranoside	(1→4)-β	10	10·8
Prunassin	(1→4)-β	5	57·1
Linamarin	(1→4)-β	5	33·6
(b) Cellobiose	(1→4)-β	5	0
(c) *p*-Nitrophenyl-β-D-xylopyranoside	(1→4)-β	10	6·9
p-Nitrophenyl-α-L-rhamnopyranoside	(1→6)-α	5	4·8

No activity was detected against *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, gentiobiose, laminaribiose, esculin, maltose, amygdalin, α-trehalose and soluble starch.
(a) Aryl and alkyl-D-glucosides; (b) diglucosides; (c) others.

Table 4 Kinetic constants of the β-glucosidase

Substrates	K_m (mmol^{-1})	V_{max} (μmol^{-1} min^{-1} mg^{-1})
p-Nitrophenyl-β-D-glucopyranoside	0·07	71·4
Arbutin	2·66	909
Prunassin	0·8	714
Linamarin	3·7	364

These values were extracted from Lineweaver–Burk plots on the basis of regression lines. Dependent on the type of substrate, activity was determined by measuring the release of either *p*-nitrophenol (400 nm) or glucose (glucose oxidase method), at 30°C, as described in Materials and Methods.

studies have been undertaken, either by plant variety selection or by modifying the traditional processing technology. Ikediobi and Onyike (1982a, b) showed that the addition of exogenous β-glucosidase was effective in reducing the amounts of residual nitrile compounds. On this basis, we decided to study the properties of the β-glucosidase of *Leuc. mesenteroides*. To our knowledge, this study is the first purification and characterization of the β-glucosidase of *Leuc. mesenteroides*. Okafor and Ejiofor (1985) previously demonstrated the presence of linamarase activity. The results presented here concerning the property of the β-glucosidase differ from those of Okafor and Ejiofor (1985) in terms of values concerning molecular mass, optimum temperature and K_m.

In conclusion, it would be interesting to study a possibility of collaborative roles of microbial and cassava linamarase and/or β-glucosidase during hydrolysis of the glycosides of cassava tissue. Indeed, if adequately associated, this can further enhance accelerated detoxification of the tissues.

REFERENCES

Aït, N., Creuzet, N. and Cattaneo, J. (1982) Properties of β-glucosidase purified from *Clostridium thermocellum*. *Journal of General Microbiology* **128,** 569–577.

Bergmeyer, H.V., Bernt, E., Schmitt, E. and Stork, H. (1974) D-Glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis* ed. Bergmeyer, H. V. pp. 1196–1201. New York: Academic Press.

Blondin, B., Ratomahenina, R., Arnaud, A. and Galzy, P. (1983) Purification and properties of the β-glucosidase of a yeast capable of fermenting cellobiose to ethanol: *Dekkera intermedia*. *European Journal of Applied Microbiology and Biotechnology* **17,** 1–6.

Bronnenmeier, K. and Staudenbauer, W.L. (1988) Purification and properties of an extracellular β-glucosidase from the cellulolytic thermophile *Clostridium stercorarium*. *Applied Microbiology and Biotechnology* **28,** 380–386.

Christakopoulos, P., Goodenough, P.W., Kekos, D., Macris, B.J., Claeyssens, M. and Bhat, K. (1994) Purification and characterisation of an extracellular β-glucosidase with transglycosylation and exo-glucosidase activities from *Fusarium oxysporum*. *European Journal of Biochemistry* **224,** 379–385.

Conchie, J., Gelman, A.L. and Levy, G.A. (1967) Inhibition of glucosidases by aldonolactones of corresponding conformation. The C-4 and C-6 specificity of β-glucosidase and β-galactosidase. *Journal of Biochemistry (Tokyo)* **103,** 609–615.

Coughlan, M.P. (1985) The properties of fungal and bacterial cel-

lulases with comment on their production and application. *Biotechnology and Genetic Engineering Review* **3,** 39–109.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of Lactobacilli. *Journal of Applied Bacteriology* **23,** 130–135.

Gueguen, Y., Chemardin, P., Arnaud, A. and Galzy, P. (1994) Purification and characterisation of the endocellular β-glucosidase of a new strain of *Candida entomophila* isolated from fermenting agave (*Agave* sp.) juice. *Biotechnology and Applied Biochemistry* **20,** 185–198.

Gueguen, Y., Chemardin, P., Arnaud, A. and Galzy, P. (1995) Purification and characterisation of an intracellular β-glucosidase from *Botrytis cinerea*. *Enzyme and Microbial Technology* **17,** 900–906.

Gunata, Y.Z., Bayonove, C.L., Cordonnier, R.E., Arnaud, A. and Galzy, P. (1990) Hydrolysis of grape monoterpenyl-β-D-glucosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. *Journal of the Science of Food and Agriculture* **50,** 499–506.

Gusakov, A.V., Oksana, V.P., Chernoglasov, V.M., Sinistyn, A.P., Kovaysheva, G.V., Shpanchenko, O.O.V. and Ermolova, O.V. (1991) Transglycosylation activity of cellobiohydrolase I from *Trichoderma longibrachiatum* on synthetic and natural substrates. *Biochimica et Biophysica Acta* **1073,** 481–485.

Heupel, C., Schlochtermeier, A. and Schrempf, H. (1993) Characterisation of an intracellular β-glucosidase from *Streptomyces reticuli*. *Enzyme and Microbial Technology* **15,** 127–132.

Holt, J.G (ed.) (1989) *Bergey's Manual of Systematic Bacteriology*. Vol. 4. Baltimore: Williams and Wilkins.

Hosel, W. and Conn, E.E. (1982) The aglycone specificity of plant β-glucosidases. *Trends in Biochemical Sciences* **7,** 219–221.

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

Ikediobi, C.O. and Onyike, E. (1982b) The use of linamarase in gari production. *Process in Biochemistry* **17,** 2–5.

Ikediobi, C.O., Onyia, G.O.C. and Eluwa, C.E. (1980) A rapid and inexpensive enzymatic assay for total cyanide in cassava (*Manihot esculenta* Crantz) and cassava products. *Agricultural and Biological Chemistry* **44,** 2803–2808.

Ikediobi, C.O., Ibrahim, S. and Ogbonna, A. (1987) Linamarase from *Fusarium equiseti*. *Applied Microbiology and Biotechnology* **25,** 327–333.

Kengen, S.W.M., Luesink, E.J., Stams, A.J.M. and Zehnder, A.J. (1993) Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *European Journal of Biochemistry* **213,** 305–312.

Leclerc, M., Arnaud, A., Ratomahenina, R. and Galzy, P. (1987) Yeast β-glucosidases. *Biotechnology and Genetic Engineering Review* **5,** 269–295.

Legras, J.L., Kaakeh, M.R., Arnaud, A. and Galzy, P. (1989) Purification and properties of the β-glucosidase from a nitrile hydratase-producing *Brevibacterium* sp. R312. *Journal of Basic Microbiology* **29**(10), 655–659.

Legras, J.L., Jory, M., Arnaud, A. and Galzy, P. (1990) Detoxification of cassava pulp using *Brevibacterium* sp. R312. *Applied Microbiology and Biotechnology* **33,** 529–533.

Mihoc, A. and Klueppel, D. (1990) Purification and characterisation of a β-glucosidase from *Streptomyces lividans* 66. *Canadian Journal of Microbiology* **36,** 53–56.

Okafor, N. (1977) Micro-organisms associated with cassava fermentation for gari production. *Journal of Applied Bacteriology* **42,** 279–284.

Okafor, N. and Ejiofor, A.O. (1985) The linamarase of *Leuconostoc mesenteroides*. Production, isolation and some properties. *Journal of the Science of Food and Agriculture* **36,** 669–678.

Okafor, N. and Ejiofor, A.O. (1990) A rapid detoxification of cassava mash fermenting for gari production following inoculation with a yeast simultaneously producing linamarase and amylase. *Process in Biochemistry* **25,** 82–86.

Oyewole, O.B. and Odunfa, S.A. (1990) Characterization and distribution of lactic acid bacteria in cassava fermentation during fufu production. *Journal of Applied Bacteriology* **68,** 145–152.

Ozaki, H. and Yamada, K. (1991) Isolation of *Streptomyces* sp. producing glucose-tolerant β-glucosidases and properties of the enzymes. *Agricultural and Biological Chemistry* **55**(4), 979–987.

Pemberton, M.S., Brown, R.D. and Emert, G.H. (1980) The role of β-glucosidase in the bioconversion of cellulose to ethanol. *Canadian Journal of Chemical Engineering* **58,** 723–729.

Rodionova, N.A., Tavobilov, M.I., Martinovich, L.I., Buachidze, I.S.H., Kvesitadze, G.I. and Bezborodov, A.M. (1987) β-Glucosidases from cellulolytic fungi *Aspergillus terreus*, *Geotrichum candidum* and *Trichoderma longibrachiatum* as typical glycosidases. *Biotechnology and Applied Biochemistry* **9,** 239–250.

Sakai, K., Tachiki, T., Kumagai, H. and Tochikura, T. (1986) Isolation and characterization of two β-glucosidases from *Bifidobacterium breve* 203. *Agricultural and Biological Chemistry* **50**(9), 2287–2293.

Tylleskaer, T., Banea, H., Bikagi, N., Cooke, R.D., Pouter, N.H. and Rosling, H. (1992) Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet* **339,** 208–211.

Vasserot, Y., Arnaud, A. and Galzy, P. (1993) Evidence for marc monoterpenol glycosides hydrolysis by free or immobilized yeast β-glucosidase. *Bioresource Technology* **43,** 269–271.

Williams, P.J., Strauss, C.R., Wilson, B. and Massy-Westropp, R.A. (1982) Studies on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene β-D-glucosides rationalizing the monoterpene composition of grapes. *Journal of Agricultural and Food Chemistry* **30,** 1219–1223.

Woodward, J. and Wiseman, A. (1982) Fungal and other β-D-glucosidases. Their properties and applications. *Enzyme and Microbial Technology* **4,** 73–79.