Beta-glycosidase (amygdalase and linamarase) from Endomyces fibuliger (LU677): formation and crude enzymes properties.

Applied Microbiology and Biotechnology

Brimer, L.; Nout, M.J.R.; Tuncel, G.

https://doi.org/10.1007/s002530051156

Appl Microbiol Biotechnol (1998) 49: 182–188

ORIGINAL PAPER

L. Brimer · M. J. R. Nout · G. Tuncel

β-Glycosidase (amygdalase and linamarase) from *Endomyces fibuliger* (LU677): formation and crude enzyme properties

Received: 16 September 1997 / Received revision: 6 October 1997 / Accepted: 14 October 1997

Abstract In our previous studies, the yeast *Endomyces fibuliger* LU677 was found to degrade amygdalin in bitter apricot seeds. The present investigation shows that *E. fibuliger* LU677 produces extracellular β-glycosidase activity when grown in malt extract broth (MEB). Growth was very good at 25 °C and 30 °C and slightly less at 35 °C. When grown in MEB of pH 5 and pH 6 with addition of 0, 10 or 100 ppm amygdalin, *E. fibuliger* produced only slightly more biomass at pH 5, and was only slightly inhibited in the presence of amygdalin. Approximately, 60% of the added amygdalin was degraded (fastest at 35 °C) during an incubation period of 5 days. Supernatants of cultures grown at 25 °C and pH 6 for 5 days were tested for the effects of pH and temperature on activity (using amygdalin, linamarin and prunasin as substrates). Prunase activity had two pH optima (pH 4 and pH 6), amygdalase and linamarase only one each at pH 6 and pH 4–5 respectively. The linamarase activity evolved earlier than amygdalase (2 days and 4 days respectively). The data thus indicate the presence of at least two different glycosidases having different pH optima and kinetics of excretion. In the presence of amygdalin, lower glycosidase activities were generally produced. However, the amygdalin was degraded from the start of the growth, strongly indicating an uptake of amygdalin by the cells. The temperature optimum for all activities was at 40 °C. Activities of amygdalase (assayed at pH 4) and linamarase (at pH 6) evolving during the growth of *E. fibuliger* were generally higher in cultures grown at 25 °C and 30 °C. TLC analysis of amygdalin degradation products show a two-stage sequential mechanism as follows: (1) amygdalin to prunasin and (2) prunasin to cyanohydrin.

L. Brimer (✉)
Department of Pharmacology and Pathobiology,
Section of Pharmacology and Toxicology,
The Royal Veterinary and Agricultural University,
13 Bülowsvej, DK-1870 Frederiksberg C (Copenhagen),
Denmark.
Fax: +45 31 35 35 14

M. J. R. Nout
Department of Food Technology and Nutritional Sciences,
Agricultural University, Bomenweg 2,
6703 HD Wageningen, The Netherlands

G. Tuncel
Faculty of Engineering, Ege University,
35100 Bornova, Izmir, Turkey

Introduction

Recent years have brought focus to the use of microorganisms for the degradation of toxins in otherwise valuable foods or feeds of plant origin (Reddy and Pierson 1994). A number of screenings have been published on the ability of microorganisms to degrade cyanogenic glycosides (Flores et al. 1990; Brimer et al. 1993), and on the microbial production of enzymes such as β-glycosidases (Brimer et al. 1994). Microorganisms have been isolated from soils (Flores et al. 1990), others were food-grade organisms (Brimer et al. 1993), or organisms easy to handle in large-scale fermentations (Brimer et al. 1994). Studies of the degradation of toxic substances during traditional fermentations of plant foods also exist (Reddy and Pierson 1994). However, the literature shows only very few examples of systematic screenings of microorganisms followed by food-detoxification studies, and investigations of enzyme production, by the organisms selected (Ikediobi and Onyike 1982; Ikediobi et al. 1985, 1987). The present investigation aims to study the production of β-glycosidase(s) by *Endomyces fibuliger* (syn. *Saccharomycopsis fibuligera*) LU677. A screening of food-grade organisms selected this yeast, together with three other microorganisms (Brimer et al. 1993), for its good ability to degrade amygdalin (Fig. 1), which is present in apricot (*Prunus armeniaca*) seeds (Tuncel et al. 1990, 1995) and in seeds of other Prunus species (Ohtsubo and Ikeda 1994; Mizutani et al. 1991; Frehner et al. 1990). A detoxification trial comparing the efficiency of these four microorganisms, and two strains of *Bacillus* spp. isolated

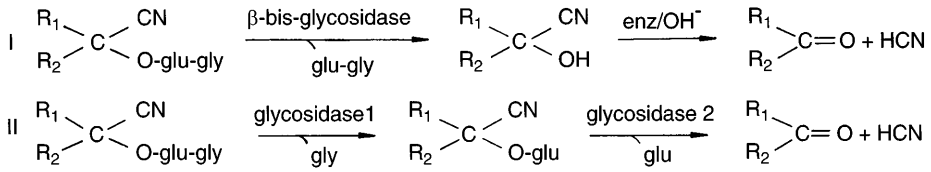

Fig. 1 Different mechanisms for the cleavage of cyanogenic glycosides to release HCN. *I* Cleavage of a glycoside by a β-bisglycosidase (e.g. cleavage of vicianin in *Davallia* species by the endogene plant enzyme). *II* Cleavage of a glycoside by the concerted action of two hydrolytic reactions (e.g. the cleavage of amygdalin by glucosidases A and B in almond). The cleavage of the cyanohydrin formed, to yield HCN and an oxocompound (*I*), has been omitted in this reaction line. The second reaction (*II*) also describes the cleavage of a glucoside (e.g. linamarin or prunasin). *glu* glucose, *gly* a monosaccharide (glucose in amygdalin), *enz*/OH^- enzyme or alkaline pH

from the apricot seeds themselves, pointed to *E. fibuliger* (LU677) as being highly effective in this respect (Nout et al. 1995).

Materials and methods

Microorganism, chemicals and materials

Endomyces fibuliger LU677 (LU code refers to the collection maintained at the laboratory of food microbiology, Department of Food Technology and Nutritional Sciences, Agricultural University, Wageningen, the Netherlands). Amygdalin from apricot (A-6005), prunasin (M-0636), β-glucosidase from almonds (G-0395) and linamarin from cassava (L-9131) were purchased from Sigma, St. Louis, Mo. 63178, USA, and TLC Polygram Ionex 25 SB AC (806023) sheets from Macherey-Nagel, Düren, 5160, Germany. All other chemicals were of analytical quality from Merck, Darmstadt, 64293, Germany. HCN-sensitive detection sheets were made from precoated ion-exchange sheets (Polygram Ionex 25-SB-AC, Macherey-Nagel), which were impregnated by consecutive immersion in two solutions (1) a saturated solution of picric acid in H_2O followed by air drying; (2) a 1 M aqueous Na_2CO_3 solution followed by air drying. Those to be used in the visualization of cyanogens on thin-layer-chromatograms (TLC) were further impregnated in a 2% ethanolic solution of hexadecanol (Brimer et al. 1983).

Media and growth conditions

E. fibuliger LU677 grown in malt extract broth (MEB) at the same temperature as the experiment (25 °C for 3 days, 30 °C for 2 days, 35 °C for 1 day) were inoculated at 3% v/v (9 ml culture/flask) in MEB (Oxoid): 300-ml portions in 500 ml-size conical flasks. Additions of 0, 10, or 100 ppm amygdalin were made (0.3 ml or 3 ml 1% stock solution in sterile water, per flask) and, after sterilization, the pH was adjusted to 5.0 or 6.0 with a predetermined quantity of sterile HCl or NaOH (0.1 M). The cultures were grown under orbital shaking (130 rpm) at 25 °C, 30 °C or 35 °C. Samples were taken: 0, 1, 2, 3, 4 and 5 days after inoculation.

Sampling and dry weight determination

At each sampling time, 40 ml homogeneous culture was taken from each flask by pouring it into a pre-weighed dry plastic centrifuge tube, which was then centrifuged at 3000 rpm for 15 min. The supernatant was decanted into a plastic screw-capped tube with a flat bottom and frozen at −20 °C. The sediment was dried at 80 °C for 24 h and weighed and the amount of dry biomass recorded.

β-Glycosidase enzyme assays

The activity of a broth sample

The cyanogen-degrading activity was determined at a resulting substrate concentration of 1.33 mM, at pH 4.0 (amygdalin) and pH 6.0 (linamarin) and 40 °C, as follows. A 1-ml sample of 66 mM sodium/potassium phosphate buffer and 0.3 ml enzyme solution (supernatant of microbial culture broth) were mixed in test-tubes in a specially designed test rack as described by Brimer (1994). The test-tubes were closed with a HCN-sensitive picrate sheet (Brimer et al. 1983) on which coloured (orange-reddish) spots formed from the reaction between HCN and picrate. The reaction was started by injecting 0.2 ml 10 mM aqueous solution of the cyanogenic substrate through the reagent sheet, and stopped by injection of 0.12 ml 1 M NaOH (which also splits the cyanohydrin formed to release CN^-); 0.4 ml 2 M H_2SO_4 was then injected to convert CN^- to HCN (Brimer et al. 1994; Brimer 1994). The rack was left overnight at room temperature. The intensity of the colour was evaluated against a standard using a green-light reflectometre (NycoCard Reader, NycoMed, Oslo, Norway) (Brimer 1994). Activities were calculated from the initial velocities determined from a plot of time against HCN concentration. For the β-bisglycoside amygdalin the overall reaction, resulting in the formation of the cyanohydrin mandelonitrile, was measured. The relative standard deviation [SD ($n - 1$)] for this picrate-based assay for cyanogens is around 7%–8% (Saka et al. 1997).

pH and temperature optima

Prior to the activity assay on the supernatants (above), the pH optimum was determined for each substrate (amygdalin, prunasin and linamarin), by determining the activity (as above) at 30 °C for one broth (pH 6, 25 °C, no amygdalin) using a range of 66 mM sodium/potassium phosphate buffers (pH 3–8). Subsequently the temperature optimum for the degradation of amygdalin and linamarin was determined at both pH 4 and 6, by determining the activity at the temperatures 30, 40, 50 and 60 °C.

Mechanism of hydrolysis of amygdalin

The mechanism of hydrolysis of amygdalin was investigated by incubating at 40 °C 300 µl culture broth supernatant in 1 ml solution of 2 mM amygdalin in 6.6 mM phosphate buffer (resulting substrate concentration 1.54 mM), pH 4 or 6. Samples of 50 µl were taken at different incubation times (5, 10, 20, 30 min and 1, 2 and 3 h), and the reaction was stopped by the addition of 20 µl 0.2 M H_2SO_4. Analyses were performed by thin-layer chromatography (TLC) by applying, as a 1-cm band, 20 µl stopped sample on (10 × 20 cm) silica gel 60 F_{254} sheets (64271; Merck, Darmstadt, Germany). Standards for the determination of R_F values (40 nmol) were amygdalin and prunasin. The solvent system was ethyl acetate/acetone/chloroform/methanol/water (5:3.75:1.5:1.25:1) and the chromatograms were developed twice (Brimer et al. 1983). The visualization of the reaction products of the spots was carried out by the procedure of Brimer et al. (1983), using a 0.1% aqueous solution of β-glucosidase (almond) as the source of enzyme. The picrate-sheet copies of the chromatograms were analysed by total-spot-area-integrating densitometry, as follows: the sheets were scanned to give a TIFF 256 grey scale (eight-bit) picture file, using an HP Scanjet Plus (HP 9195A/AB) (software: Gallery Plus 5.0) graphic scanner; the density of the spots was measured by analysing

the picture file with the software Quantiscan (Biosoft, Cambridge) (Brimer 1994).

Residual cyanogens in samples

Supernatants of cultures grown with the addition of amygdalin may contain residual cyanogens, i.e. amygdalin, prunasin and mandelonitrile (Fig. 1). At pH values lower than approximately 6.0 the mandelonitrile is relatively stable (Cooke 1978; Fomunyam et al. 1985). Furthermore, HCN formed by cleavage of mandelonitrile may bind reversibly to the sugar in the medium (Chadha et al. 1991). The content of residual cyanogens in these samples was determined as follows. (1) Total cyanogens were assayed by adding 1 ml 66 mM sodium/potassium phosphate buffer, 0.3 ml broth and 0.1 ml 0.5% w/v solution of β-glucosidase (almond) to the test-tubes, which (covered with the picrate sheet) were incubated overnight at 30 °C. (2) Non-glycosidic cyanogens were determined as in the assay for the activity of a broth sample but with no substrate added and stopping the reaction immediately. The HCN released from these residual cyanogens during a time-stopped incubation (activity assay), was determined by performing an activity determination without the addition of substrate.

Results

The yeast grew well at all three temperatures. No general tendency was seen indicating that added amygdalin influenced the growth (Fig. 2). The added amygdalin was degraded (Fig. 3A, B). However, within a period of 5 days of incubation, residual cyanogens still remained detectable in all broths from cultures to which 100 ppm amygdalin had been added. The degradation of the amygdalin, as well as the disappearance of the non-glycosidic cyanogens formed, was fastest when the yeast was grown at 35 °C as compared to 25 °C (Fig. 3A, B).

The pH and temperature dependences, as measured on one selected culture supernatant (5 days of incubation, initial pH 6, no amygdalin added), are shown in Figs. 4 and 5. The plot of activity against pH showed different patterns for the three substrates prunasin, linamarin and amygdalin: the hydrolysis of prunasin had

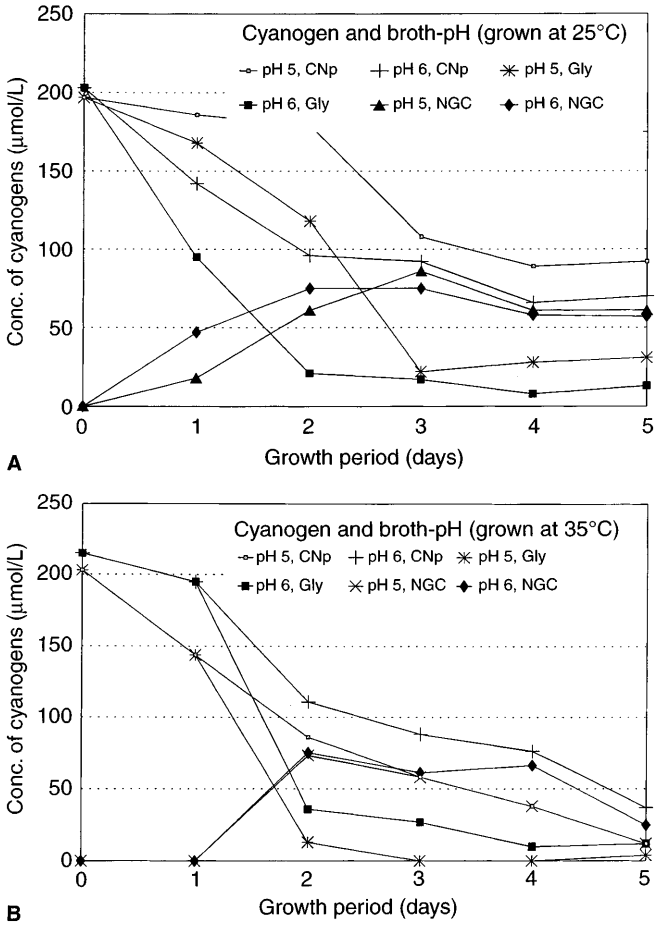

Fig. 3A, B Amygdalin degradation by *E. fibuliger* (LU677) during growth in MEB + 100 ppm (220 µmol l^{-1}) amygdalin, at different initial pH (5 or 6) and incubation temperatures (25 °C or 35 °C). *CNP* total cyanogens, *Gly* cyanogenic glycosides = amygdalin + prunasin, *NGC* non-glycosidic cyanogens = mandelonitrile + HCN

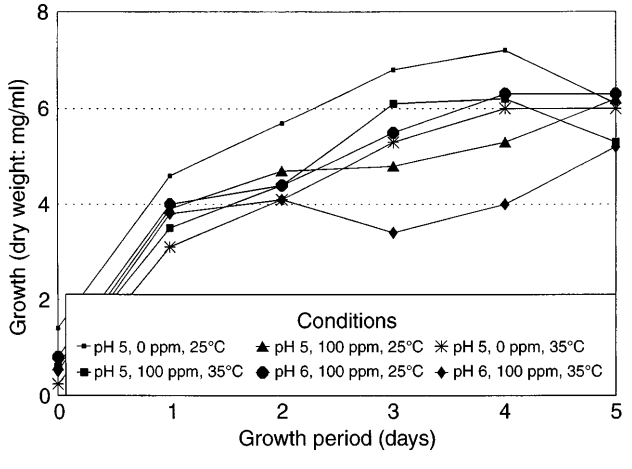

Fig. 2 Selected growth curves of *Endomyces fibuliger* (LU677) in malt extract broth (MEB) without or with added amygdalin (100 ppm), at different incubation temperatures and initial pH (5 or 6)

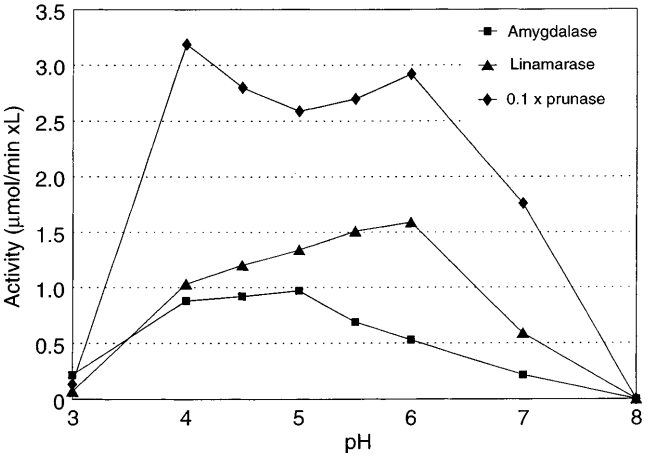

Fig. 4 Degradative activities as a function of pH of assay. Measured at 30 °C and a substrate concentration of 1.33 mM, using supernatant of culture grown at pH 6 and 25 °C for 5 days (no amygdalin added during growth)

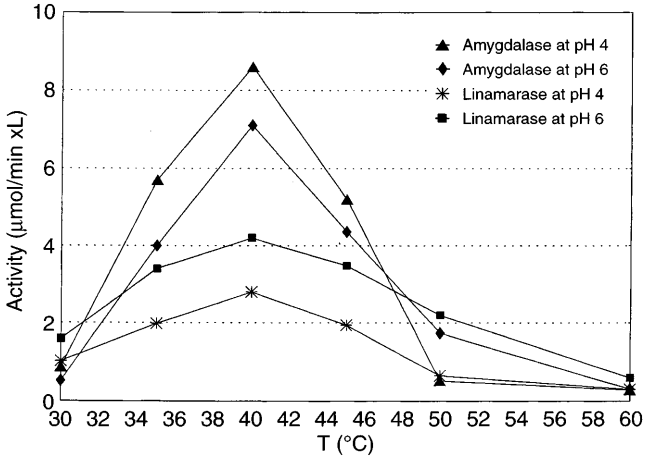

Fig. 5 Degradative activities as a function of temperature of assay. Measured at a substrate concentration of 1.33 mM, using supernatant of culture grown at pH 6 and 25 °C for 5 days (no amygdalin added during growth)

two maxima at pH around 4 and 6, while the optima for linamarin and amygdalin were 6 and 4–5, respectively (Fig. 4). Hence, the measurements of activity at different temperatures were done at both pH 4 and 6, using the two substrates linamarin and amygdalin. The amygdalase activity was highest when measured at pH 4, and the linamarase when assayed at pH 6, over most of the temperature interval (Fig. 5), thus confirming the aforementioned pH optima (Fig. 4). Both activities had their temperature optimum at 40 °C irrespective of the pH (Fig. 5).

Taking these results into consideration, the activity was then measured for all samples from the growing experiments, using the following two assays: (1) degradation of amygdalin to cyanohydrin at pH 4 and (2) degradation of linamarin to cyanohydrin at pH 6. The results were corrected for the HCN released (from residual cyanogens) during the time-stopped incubations. Figure 6A, B shows the results for selected supernatants.

Monitoring the degradation of amygdalin, as a function of reaction time, by TLC analysis showed (example Fig. 7) that both a supernatant grown at 25 °C for 3 days and one grown at 30 °C for 5 days (both at pH 6 and with no amygdalin added) degraded amygdalin by a two-step (sequential) mechanism. This was the case no matter whether the incubation was done at pH 4 or 6. The intermediate product prunasin accumulated to a significant level during the degradation. The highest concentration was found between 1 h and 2 h of incubation.

Discussion

While the growth of *E. fibuliger* LU677 was relatively unaffected by the temperature (Fig. 2), the enzyme activities were found to be significantly higher in broths grown at 25 °C and 30 °C than at 35 °C. The influence of incubation temperature was apparently stronger for

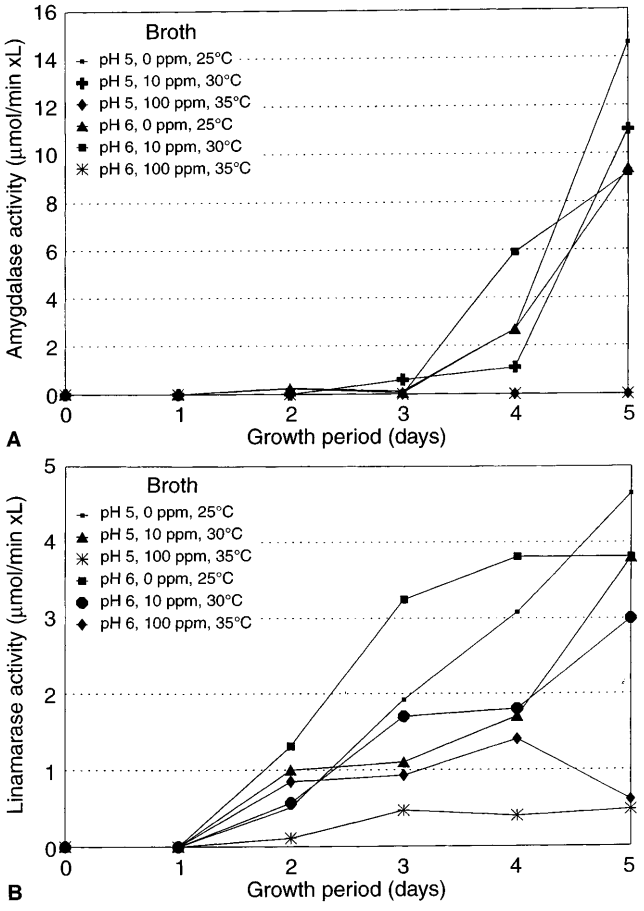

Fig 6A, B Amygdalin- (**A**) and linamarin-degrading (**B**) activities in selected supernatants of *E. fibuliger* (LU677) grown at different initial pH (5 or 6) and incubation temperatures (25 °C, 30 °C and 35 °C) in MEB without or with added amygdalin (10 ppm or 100 ppm). Activities measured at 40 °C using a substrate concentration of 1.33 mM. Linamarase measured at pH 6, amygdalase at pH 4

the "amygdalase" activity than for the "linamarase". This phenomenon, together with the initial observation of the two different pH optima (Fig. 4), could point to the existence of two different β-glycosidases. Moreover, this hypothesis is supported by a number of other observations. Thus, the growth/activity correlation for "linamarase" (measured at pH 6) shows a different pattern from that of amygdalin measured at pH 4 (at all three growth temperatures). While the linamarase activity is detectable after 48 h, increasing slowly through the next 3 days of growth, the amygdalin-degrading activity is not detected until day 4, increasing significantly between days 4 and 5 (Fig. 6A, B). Amygdalin added to the growth medium at a concentration of 100 ppm was degraded to form NGC (cyanohydrin + cyanide) from day 1 of incubation (Fig. 3A, B), in spite of the fact that only a negligible "amygdalase" activity could be detected in the supernatant after 24, 48 and even 72 h of growth (Fig. 6A). During the incubation the total amount of cyanogens steadily decreased. These observations indicate that the amygdalin is taken up by

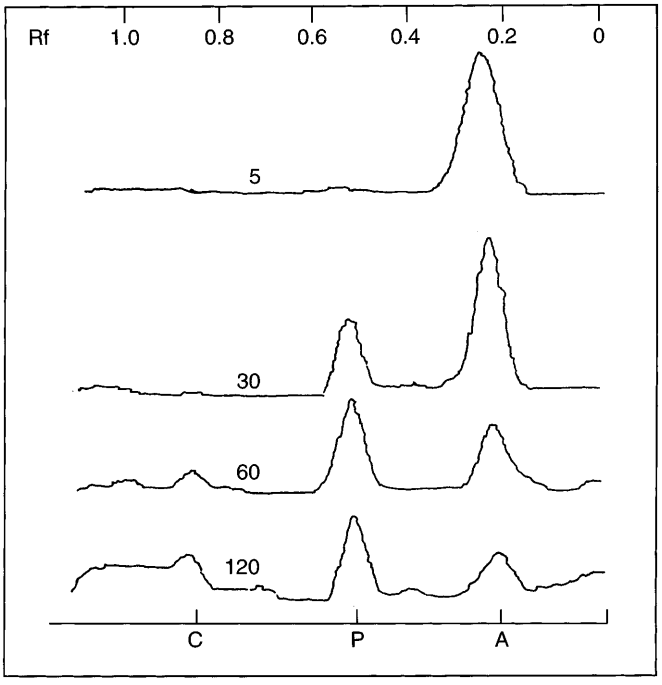

Fig. 7 Densitograms of TLC lanes representing 5, 30, 60 and 120 min of amygdalin degradation by a supernatant from a culture of *E. fibuliger* (LU677). *A* Amygdalin peaks, *P* prunasin peaks, *C* cyanohydrin (mandelonitrile) peaks

the cells, hydrolysed intracellularly and then the non-glycosidic cyanogens and glucoside (prunasin) are excreted. To the best of our knowledge, no experiments have previously been published that report the uptake of cyanogens prior to their degradation by microorganisms. The product of this first reaction may be either cyanohydrin (Fig. 1, I) or prunasin (Fig. 1, II), depending on the nature of the intracellular enzyme(s). If prunasin is formed, this is further degraded by a glucosidase to form cyanohydrin (mandelonitrile). The enzyme with a pH optimum of 4, which facilitates the degradation of amygdalin, catalysing the reaction amygdalin to prunasin as mentioned in Results and shown in Fig. 7, could be such an intracellular enzyme. Between days 4 and 5 it may be released upon cell lysis (Fig. 6A), when the growth has reached its maximum (Fig. 2). As already pointed out, this investigation strongly suggests that the microorganism produces two glycosidases with different pH optima and substrate affinities. The enzyme with $pH_{opt} = 6$, which acts on both linamarin and prunasin, is found in significant concentrations in the supernatant after 48 h of growth (Fig. 6B), and thus could be a true extracellular enzyme. If prunasin, as a result of an intracellular degradation of amygdalin, is released in the medium, this enzyme thus will be able to facilitate its further degradation to mandelonitrile (cyanohydrin), as was observed during the first days of growth (Fig. 3A, B). The above interpretation also allows us to explain why *E. fibuliger* LU677 effectively degrades both amygdalin and prunasin present in ground apricot seeds (Nout et al. 1995).

The pH and temperature optima for most plant β-glycosidases are around 5.5–6.5 and 55–60 °C respectively. In contrast, the pH optima reported for microbial glucosidases vary from approximately 3.0 (*Botrytis cinerea*; Sasaki and Nagayama 1995) to 6 (several), while the temperature optimum may be as high as approximately 80 °C (Nucci et al. 1995). Having the temperature optimum at 40 °C, and being able to degrade both amygdalin and linamarin at a pH ranging from 4–7, *E. fibuliger* LU677 (and its enzymes) appear well suited to the degradation of these and possibly other toxic glycosides in food and feed, at ambient temperatures. Hence, this investigation confirms the results from the earlier food detoxification study (Nout et al. 1995).

Like the fungal myrosinase produced by *Aspergillus sydowi* (Ohtsuru et al. 1969), a number of microbial β-glucosidases have been produced in good yield only after induction. To this end, different β-glycosides such as methyl β-glucoside, amygdalin, salicin and phenyl β-glucoside have been used (Sternberg et al. 1977). The addition of surfactants may also enhance the yield, as shown by Ikediobi et al. (1985), who could enhance the linamarase activity in the supernatant of *A. sydowi* and *F. equiseti* by adding Tween 80 and Triton X-100. However, linamarin and *p*-nitrophenyl β-D-glucopyranoside were ineffective. Okafor and Ejiofor (1985) found that *L. mesenteroides* only produced linamarase when grown on linamarin or when linamarin was added (0.15%) as an inducer, and Flores et al. (1994), while testing the influence of salicin, *p*-nitrophenyl β-D-glucoside, methyl β-D-glucoside and linamarin on the production of linamarase by *A. oryzae*, found only linamarin to be an effective inducing agent. They also demonstrated the production of this enzyme by the inclusion of linamarin-containing cassava cortex powder in the medium (Flores et al. 1994). In the present investigation, both enzymes seem to be constitutive, and both are depressed by the addition of amygdalin, the effect being strongest at a concentration of 100 ppm (Fig. 6A, B).

Representatives of several microbial genera such as *Mucor* and *Penicillium* for example (Brimer et al. 1993, 1994, 1996), *Rhizopus* and *Aspergillus* (Brimer and Rosling 1993; Brimer et al. 1995) and *Zygosaccharomyces* (Gueguen et al. 1995), can hydrolyse a number of both mono and disaccharide cyanogenic glycosides of aliphatic as well as aromatic aglycones. The activity on a monoglucoside generally depends on the nature of the aglycone. Further, it has been shown that a number of microorganisms can hydrolyse the bitter principle of several citrus juices, such as naringin [4′,5,7-trihydroxyflavanone-7-*O*-(α-rhamnopyranosyl) β-D-glucopyranoside; Esaki et al. 1993], and enhance the content of monoterpenes in wine. The latter occurs by hydrolysis of a number of disaccharide glycosides, including β-rutinosides (6-*O*-α-L-rhamnopyranosyl β-D-glucopyranosides) and 6-*O*-α-L-arabinofuranosyl β-D-glucopyranosides of geraniol, nerol and linalol; Williams et al. 1982; Günata et al. 1985). This releases the genins

(Grossman et al. 1987, 1989, 1990a; Aryan et al. 1987; Shoseyov et al. 1990). Also, for a number of these latter enzymes, a dependence on the nature of the genin has been established (Grossmann et al. 1987; Günata et al. 1990a, b).

In spite of this, the mechanisms of microbial degradation of disaccharide glycosides remain relatively uninvestigated, except for the following examples. Naringin was shown to be hydrolysed by commercial naringinase from *Penicillium decumbens* to rhamnose and prunin, and the latter was further degraded to glucose and naringenin (Romero et al. 1985). A sequential hydrolysis may be the most important for the release of aroma terpenes from disaccharide terpene glycosides, using commercial Pectinol VR (Rohm, Darmstadt, Germany) or broth from a culture of *Candida molischiana* (Günata et al. 1990a). Very recently we demonstrated a sequential hydrolysis of the cyanogenic gentiobioside amygdalin (Brimer et al. 1996) by *Mucor circinelloides* and *Penicillium aurantiogriseum*. In contrast, Shoseyov et al. (1990) claim to have isolated, and immobilized, an endo-β-glucosidase, from a strain of *Aspergillus niger* that would release the complete disaccharide from its glycoside. In this study, rutinose would have been cleaved from geranyl β-rutinoside, but there was no evidence for the formation of intact rutinose.

The results from our present investigation clearly prove that the main route of amygdalin degradation by the *E. fibuliger* is the sequential one with prunasin as the intermediate product. The roles of the two or more glycosidases present require further investigations.

Acknowledgements The technical contribution of Birgit Hasenack, Department of Food Technology and Nutritional Sciences, Agricultural University, Wageningen, is gratefully acknowledged. This project was supported by NATO Collaborative Research Grant 920009.

References

Aryan AP, Wilson B, Strauss CR, Williams PJ (1987) The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible applications in enzymology. Am J Enol Vitic 38: 182–188

Brimer L (1994) Quantitative solid-state detection of cyanogens: from field test kits to semi-automated laboratory systems allowing kinetic measurements. Acta Hortic (Wageningen) 375: 105–116

Brimer L, Rosling H (1993) A microdiffusion method with solid state detection for determination of cyanogenic glycosides from cassava in human urine. Food Chem Toxicol 31: 599–603

Brimer L, Brøgger Christensen S, Mølgaard P, Nartey F (1983) Determination of cyanogenic compounds by thin-layer chromatography. 1. A densitometric method for quantification of cyanogenic glycosides, employing enzyme preparations (β-glucuronidase) from *Helix pomatia* and picrate-impregnated ion-exchange sheets. J Agric Food Chem 31: 789–793

Brimer L, Tuncel G, Nout MJR (1993) Simple screening procedure for microorganisms to degrade amygdalin. Biotechnol Tech 7: 683–687

Brimer L, Cicalini AR, Federici F, Petruccioli M (1994) Production of beta-glycosidases (linamarase and amygdalase) and pectolytic enzymes by *Penicillium* spp. World J Microbiol Biotechnol 10: 203–206

Brimer L, Cicalini AR, Federici F, Petruccioli M (1995) β-Glycosidase activity as a side activity in commercial pectinase preparations of fungal origin. The hydrolysis of cyanogenic glycosides. Ital J Food Sci 7: 387–394.

Brimer L, Cicalini AR, Federici F, Nout MJR, Petruccioli M, Pulci V (1996) Two-step hydrolysis of amygdalin in molds. Riv Biol/Biol Forum 89: 493–496

Chadha RK, Bryce F, Lawrence JF, Conacher HBS, Arnold D (1991) Toxicity of potassium cyanide added to fresh fruit and juice. Food Chem Toxicol 29: 681–684

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

Esaki S, Ohishi A, Katsumata A, Sugiyama N, Kamiya S (1993) Synthesis of α-mannopyranosyl-containing disaccharides and phenols as substrates for the α-L-mannosidase activity of commercial naringinase. Biosci Biotechnol Biochem 57: 2009–2103

Flores DM, Koizumi Y, Yanagida F, Garcia VV (1990) Screening, isolation and identification of microorganisms with linamarase activity. J Jpn Soc Food Sci Technol 37: 230–233

Flores DM, Garcia VV, Hasegawa T, Koizumi Y, Yanagida F (1994) Production of linamarase in *Aspergillus oryzae* using cassava cortex. J Jpn Soc Food Sci Technol 41: 436–439

Fomunyam RT, Adegbola AA, Oke OL (1985) The stability of cyanohydrins. Food Chem 17: 221–225

Frehner M, Scalet M, Conn EE (1990) Pattern of the cyanide-potential in developing fruits. Plant Physiol (Bethesda) 94: 28–34

Grossmann M, Rapp A, Rieth W (1987) Enzymatische freisetzung gebundener aromastoffe in wein. Dtsch Lebensm Rundsch 83: 7–12

Gueguen Y, Chemardin P, Arnaud, Galzy P (1995) Comparative study of extracellular and intracellular β-glucosidases of a new strain of *Zygosaccharomyces bailii* isolated from fermenting agave juice. J Appl Bacteriol 78: 270–280

Günata YZ, Bayonove CL, Baumes RL, Cordonnier RE (1985) The aroma of grapes. I. Extraction and determination of free and glycosidally bound fractions of some grape aroma components. J Chromatogr 331: 83–90

Günata YZ, Biron C, Sapis JC, Bayonove C (1989) Glycosidase activities in sound and rotten grapes in relation to hydrolysis of grape monoterpenyl glycosides. Vitis 28: 191–197

Günata YZ, Bayonovo CL, Cordonnier RE, Arnaud A, Galzy P (1990a) Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamnii* β-glucosidases. J Sci Food Agric 50: 499–506

Günata YZ, Bayonove CL, Tapiero C, Cordonnier RE (1990b) Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. J Agric Food Chem. 38: 1232–1236

Ikediobi CO, Onyike E (1982) The use of linamarase in gari production. Process Biochem 17: 2–5

Ikediobi CO, Ogundu EC, Ukoha AI (1985) Production of linamarase by *Aspergillus sydowi* and *Fusarium equiseti*. Process Biochem 20: 99–102

Ikediobi CO, Ibrahim S, Ogbonna AI (1987) Linamarase from *Fusarium equiseti*. Appl Microbiol Biotechnol 25: 327–333

Mizutani F, Hirota R, Amano S, Hino A, Kadoya K (1991) Changes in cyanogenic glycoside content and β-cyanoalanine synthetase activity in flesh and seeds of Japanese plum (*Prunus salicina* Lindl.) during development. J Jpn Soc Hortic Sci 59: 863–867

Nout MJR, Tuncel G, Brimer L (1995) Microbial degradation of amygdalin of bitter apricot seeds (*Prunus armeniaca*). Int J Food Microbiol 24: 407–412

Nucci R, D'Auraria S, Febbraio F, Vaccaro C, Morana A, De Rosa M, Rossi M (1995) A thermostable β-glycosidase from *Sulfolobus solfataricus*: temperature and SDS effects on its functional and structural properties. Biotechnol Appl Biochem 21: 265–274

Ohtsubo T, Ikeda F (1994) Seasonal changes of cyanogenic glycosides in Mume (*Prunus mume* Sieb. et Zucc.) seeds. J Jpn Soc Hortic Sci 62: 695–700

Ohtsuru M, Tsuruo I, Hata T (1969) Studies on fungous myrosinase. Part 3. On the β-glucosidase activity of fungous myrosinase and the relationship of fungous and plant myrosinases to β-glucosidases. Agr Biol Chem 33: 1320–1325

Okafor N, Ejiofor MAN (1985) The linamarase of *Leuconostoc mesenteroides*: production, isolation and some properties. J Sci Food Agric 36: 669–678

Reddy NR, Pierson MD (1994) Reduction in antinutritional and toxic components in plant foods by fermentation. Food Res Int 27: 281–290

Romero C, Manjón A, Bastida J, Iborra JL (1985) A method for assaying the rhamnosidase activity of naringinase. Anal Biochem 149: 566–571

Saka JDK, Mhone ARK, Brimer L (1997) An improved microdiffusion method with solid-state detection for the determination of total cyanogens (CNp) in fresh cassava. Comparison to the method of Cooke (1978). J Sci Food Agric (in press)

Sasaki I, Nagayama H (1995) Purification and characterization of β-glucosidase from *Botrytis cinerea*. Biosci Biotechnol Biochem 59: 100–101

Shoseyov O, Bravo BA, Siegel D, Goldman A, Cohen S, Shoseyov L, Ikan R (1990) Immobilized endo-β-glucosidase enriches flavor of wine and passion fruit juice. J Agric Food Chem 38: 1387–1390

Sternberg D, Vijayakumar P, Reese ET (1977) β-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. Can J Microbiol 23: 139–147

Tuncel G, Nout MJR, Brimer L, Göktan D (1990) Toxicological, nutritional and microbiological evaluation of tempe fermentation with *Rhizopus oligosporus* of bitter and sweet apricot seeds. Int J Food Microbiol 11: 337–344

Tuncel G, Nout MJR, Brimer L (1995) The effect of soaking, grinding and cooking on the degradation of amygdalin of bitter apricot seeds. Food Chem 53: 447–451

Williams PJ, Strauss CR, Wilson B, Massy-Westropp RA (1982) Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. Phytochemistry 21: 2013–2020