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Degradation of cyanogenic glycosides of bitter apricot seeds (*Prunus armeniaca*) by endogenous and added enzymes as affected by heat treatments and particle size

G. Tunçel,[a] M. J. R. Nout,[b]* & L. Brimer[c]

[a]*Food Engineering Department, Ege University, Bornova, Izmir, Turkey*
[b]*Department of Food Technology and Nutritional Sciences, Agricultural University, Wageningen, The Netherlands*
[c]*Department of Pharmacology and Pathobiology, Section of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen, Denmark*

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Bitter apricot (*Prunus armeniaca*) seeds (kernels) are by-products of the apricot processing industry. They contain approximately 50–150 μMol/g (dry weight basis) of potentially toxic cyanogenic glycosides, mainly amygdalin and prunasin. The present paper deals with the degradation of these glycosides by endogenous and added enzymes in raw and blanched seeds of different particle sizes. A hot water blanching treatment of 20 min at 100°C was adequate to inactivate endogenous β-glucosidase activity in raw bitter apricot seeds. In addition to raw seeds, such blanched seeds were used as an experimental model to investigate the effect of particle size and added individual enzyme preparations on the degradation of cyanogenic glycosides. Finely ground (< 2 mm) fractions showed increased glycoside degradation, supporting the hypothesis that particle size is a limiting factor for enzymic degradation. Our hypothesis that added pectinase activity would enhance degradation of glycosides by improving enzyme–substrate contact could not be affirmed. Furthermore, it was observed that substantial enzyme addition (β-glucosidase) is required to fully degrade residual glycoside levels in raw and/or blanched seeds.

INTRODUCTION

Bitter apricot (*Prunus armeniaca*) seeds (kernels) are by-products of the apricot processing industry. They are used as a substitute for bitter almonds to produce 'persipan' for the bakery industry. The oil (53% in the seed) is used, in e.g. cosmetics (Hallabo *et al.*, 1975), as a cheaper substitute for bitter almond oil. The seeds can also be of interest as a food or feed ingredient because of their high crude protein content (20–25% w/w, dry weight basis). Bitter apricot seeds originate from the variety *Prunus armeniaca* var. *amar* (El-Adawy *et al.*, 1994). However, depending on the specific cultivar (Femenia *et al.*, 1995), they contain different concentrations of the potentially toxic (Lasch and El Shawa, 1981) amygdalin and prunasin. Thus, concentrations of approximately 50–150 μMol/g (dry weight basis) have been reported (Abd El-Aal *et al.*, 1986; Tunçel *et al.*, 1990; Femenia *et al.*, 1995). Degradation of these glycosidic cyanogens (GLY) will result in the formation of mandelonitrile and finally free CN^-, both molecules being non-glycosidic cyanogens (NGC). We reported earlier (Tunçel *et al.*, 1990) on prospects for detoxification by biotechnological means, i.e. by using the endogenous enzymes combined with microbial fermentation. The tempe fermentation process using the fungus *Rhizopus oligosporus* as inoculant enabled a removal of around 70% of total cyanide potential. As we considered this inadequate from a toxicological point of view, we screened different microbial strains for their ability to degrade amygdalin by hydrolysis. Some strains showed degradation activity > 95% of initial levels of amygdalin (Brimer *et al.*, 1993; Nout *et al.*, 1995).

On the other hand, results by Nout *et al.* (1995) and Tunçel *et al.* (1995) showed that endogenous β-glucosidase activity causes significant degradation of

*To whom correspondence should be addressed. Fax: +31-317-484893.

amygdalin in soaked ground seeds, and that the rate of degradation is increased when smaller particle sizes of apricot seed are used. In spite of great reductions, none of the wet seed products reach levels of total cyanogenic potential as low as those set for cassava flour and gari from cassava, not even after cooking (Tunçel *et al.*, 1995).

The present paper addresses the question what factor(s) limit the efficacy of endogenous β-glucosidases in detoxifying apricot glycosides. Our hypothesis is that degradation of apricot seed cell walls is a major factor enabling contact between β-glucosidases and cyanogenic glycosides. This would explain the higher rate and extent of degradation when using smaller particle sizes. In addition, pectin-degrading enzymes might favour the action of β-glucosidases by improving the contact between substrate and enzymes.

The experiments were carried out with raw (non-blanched) as well as blanched seeds. The use of raw seeds with active endogenous β-glucosidases enables an assessment of the additional effect of microbial β-glucosidases and cell wall- and/or pectin-degrading enzymes. However, the study of the effect of an added enzyme, or enzyme mixture, on the degradation of the cyanogenic glycosides in ground tissues (e.g. seeds) is difficult. Thus, the level of endogenous β-glucosidase(s) may vary between seed batches, as may the degree of release of both these enzyme(s) and the glycosides during the comminution. In addition, crude microbiological preparations will contain a mixture of β-glucosidase(s), pectinase(s) and cellulase(s), of which the tissue and cell degrading enzymes will affect the above mentioned release further.

In order to study the effect of a number of crude microbiological preparations with considerable β-glucosidase activity as measured using amygdalin, the use of a simplified system was found to be essential. Hence for this purpose we used blanched seeds in which the endogenous enzyme activities had been inactivated, as a substrate.

MATERIALS AND METHODS

Materials

Bitter apricot seeds were obtained from Izmir, Turkey. Pectinase (EC 3.2.1.15) from *Rhizopus* sp. (P 2401) and β-glucosidase from almond (G-0395) were purchased from Sigma (St Louis, MO, USA). Olivex (batch G-509) was obtained from NOVO, Denmark. Picrate reagent sheets were prepared according to Brimer *et al.* (1983). *Candida guilliermondii* strain LU 120 (LU codes refer to the collection maintained at the laboratory of food microbiology, Agricultural University, Wageningen, The Netherlands), and *Endomyces fibuliger* LU677 originate from fermented foods. *Aspergillus niger* LU1500 was isolated by G. Tunçel from Turkish soil.

Seed processing

Blanching conditions required to inactivate endogenous β-glucosidases were determined as follows: whole apricot seeds (portions of 100 g) were wrapped in cheese cloth and blanched by immersion in boiling water for 5, 10, 20 or 30 min; one portion was autoclaved at 121°C for 15 min. The control group was not blanched. Seeds were ground in a kitchen cutter (Krups type 708 A, Ireland). The particle size was determined using Karl Kolb (Germany) test sieves.

Determination of blanching conditions

Residual activity of β-glucosidases after heat treatments was assessed by comparison of levels of glycosidic and non-glycosidic cyanide in coarsely ground seeds immediately after heating, and after soaking (25°C for 2 h), as follows: (a) 20 g coarsely ground heated seeds were extracted with 180 ml of 0.1 M H_3PO_4; (b) 20 g coarsely ground heated seeds were soaked in beakers with 60 ml of tap water at 25°C for 2 h. Then the total content of beaker was poured into a coned paperfilter (Whatman ashless 41), and the filter cake and filter paper were extracted into 180 ml of 0.1 M H_3PO_4.

Cyanogen analysis

All samples were analysed for total cyanogenic potential (GLY + NGC) and for non-glycosidic cyanogens (NGC), as described by Tunçel *et al.* (1995). From these results the content of cyanogenic glycosides (amygdalin + prunasin) was calculated. This result is reported as 'GLY'. Just prior to analysis, unstable working solutions were prepared from 5 ml of the stable phosphoric acid homogenates, adding phosphate buffer (0.1 M Na_3PO_4/H_3PO_4, pH 7) to give solutions with a resulting pH of 6.5 (dilution factor was noted). Calibration curves were produced by hydrolysis of aliquots of a 1 mM aqueous solution of amygdalin, a 0.2% w/v solution of pectinase being used as source of hydrolytic enzymes for both standards and samples to be analysed for total cyanogenic potential (Brimer and Rosling, 1993). Sample hydrolysis was performed in microtitre plates (Brimer *et al.*, 1993). Each well contained 1000 μl of buffer pH 7 + 50, 30, 10 or 5 μl supernatant of extract (2 wells/plate). One hundred microlitres of pectinase were added to half of the wells to measure cyanogenic potential. Plates were covered with picrate sheets and incubated at 25°C overnight. The density of the red–brown spots on a yellow background typical of positive reactions was assessed by measuring the relative colour intensity, expressed as K/S (absorption/scatter) in arbitrary units, with a Nycocard Reader Model V 1.0 (manufactured by Nycomed Pharma AS, Norway). Calculations were expressed as μmol/g fresh weight of seeds processed. The detection level was 1 μmol HCN/g (Tunçel *et al.*, 1995). Duplicate extractions were made

of each treatment, and of each acidic homogenate, a dilution series was made and each dilution tested in duplicate wells.

The influence of added enzyme preparations and particle size

Raw and blanched seeds (20 min at 100°C) were ground to obtain coarse (2–4 mm) and fine (< 2 mm) fractions as described above. For both particle sizes the following treatments were applied: (A) immediate extraction (20 g fresh weight in 180 ml of 0.1 M H_3PO_4); (B) 20 g fresh weight + 55 ml water + supernatant (amygdalase activity 1.4 μmol/h/ml) of *Candida guilliermondii* strain LU120 grown for 72 h at 30°C in Malt Extract Broth, incubate 37°C for 3 h, filter, extract in 180 ml of 0.1 M H_3PO_4; (C) same, but with supernatant (act. 4.8 μmol/h/ml) of *Endomyces fibuliger* LU 677 grown for 72 h at 30°C in Malt Extract Broth; (D) same, but with supernatant (act. 67.4 μmol/h/ml) of *Aspergillus niger* LU1500 grown for 72 h at 30°C in Malt Extract Broth; (E) same, but with 0.3% Olivex (act. 0.8 μmol/h/ml); (F) same, but with 0.3% β-glucosidase (act. 14.8 mmol/h/ml); (G) same, but with mix of Olivex + β-glucosidase, each of equal amygdalase activity; H: same, but with mix of Olivex + supernatant LU1500 each of equal amygdalase activity.

Sample hydrolysis was performed in microtitre plates. Each well contained 1000 μl of buffer pH 7 + 50 and 20 μl supernatant of extract (2 wells/plate). One hundred microlitres of pectinase were added to half of the wells. Plates were covered with picrate sheets and incubated at 25°C overnight. Samples were analysed for total cyanogenic potential and for non-glycosidic cyanogens, as described above.

RESULTS AND DISCUSSION

The effect of blanching on degradation of cyanogenic glycosides (amygdalin + prunasin) by endogenous β-glycosidases is shown in Table 1. Seed β-glucosidase activity causes significant degradation of cyanogenic glycosides in ground seeds without blanching (Tunçel *et al.*, 1990; Nout *et al.*, 1995). These plant enzymes gain access to the glucoside after physical disruption of the cell. They will act at about 20–40°C and are readily destroyed by heat. However, at this particle size (2–4 mm) the non-blanched seed fraction still had about 50% residual glycosides (Table 1); this shows the limited impact of endogenous β-glucosidases. In order to test the effects of a smaller particle size and of enzyme additions we decided to carry out part of the experiment in blanched seeds, thus eliminating the interference caused by the endogenous seed enzymes. We found that after the 20 min blanching treatment, endogenous activities had been stopped adequately, whereas sufficient residual cyanogenic glycosides were present in the seeds to enable further degradation experiments. The general decrease in total recoverable cyanide is mainly due to diffusion to the blanching water.

Table 1. The effect of hot water blanching on endogenous β-glucosidase activities in bitter apricot seeds (*Prunus armeniaca*)

Heat treatment	Immediately after heat treatment		After 2 h 25°C soaking of 2–4 mm fraction	
	GLY[a]	NGC[a]	GLY	NGC
None (raw seed)	42; 37[b]	0; 0	21; 20	20; 23
5 min 100°C	20; 33	0; 0	7; 10	16; 13
10 min 100°C	29; 23	0; 0	30; 25	1; 0
20 min 100°C	29; 32	0; 0	27; 28	0; 0
30 min 100°C	26; 31	0; 0	22; 32	0; 0
15 min 121°C	19; 24	0; 0	18; 13	0; 0

[a]GLY = glycoside; NGC = non-glycosidic cyanide.
[b]Data are of duplicate samples; μmol CN eq/g seed fresh weight.

Table 2 shows the effects of added enzyme preparations to seeds of different particle sizes, i.e. 2–4 mm and < 2 mm fractions. The reason for this experiment was to find out whether the cyanogen residue observed in non-blanched seeds could be reduced further in both types of seeds by the addition of enzymes. In the finely ground fraction, only blanched seeds were used as the endogenous enzyme activity in raw seeds would leave too little glycoside to achieve detectable degradation by the added enzymes. It was interesting to note that in raw seeds of 2–4 mm dimensions, only treatments C and F result in more degradation compared with the control as indicated by lower GLY and higher levels of NGC. The culture supernatants of LU120 and LU1500 did not give significantly increased degradation. We had expected a strong effect from Olivex that is used in the olive processing industry because of its high pectinase activities. Apparently, pectinases are not the enzymes required to stimulate glycoside release from apricot seeds. In blanched seeds of 2–4 mm particle size, only treatment F gave complete degradation.

The question of particle size as a limiting factor was addressed using blanched seeds. Table 2 shows the effects of added enzyme preparations on coarse (2–4 mm) and fine (< 2 mm) milling fractions of blanched apricot seeds. If finely ground seeds (< 2 mm) were used, better enzymatic degradation could be obtained. The degradation of glycosides proved to be significantly ($p < 0.05$) more efficient in finely ground seeds. However, even in the fine grind most tested preparations yielded poor results (less than about 15% of total CN as NGC). Only at very high activity levels of β-glucosidase (Sigma) could it be shown that 100% conversion of GLY into NGC is possible. Combinations of β-glucosidase and Olivex (having high pectinase activity) as in treatment G and H again show that the effect of β-glucosidase is not enhanced by added pectolytic activity. These data would suggest that particle size is not the only critical factor. The substrate

Table 2. Effect of particle size and added enzyme preparations on degradation of cyanogenic glycosides in apricot seeds (*Prunus armeniaca*)

Treatment	Enzyme origin	Added amygdalase activity (μmol/h/g seed fresh weight)	Incubation (h/°C)	Coarse fraction (2–4 mm)				Fine fraction (< 2 mm)	
				Raw (non-blanched)		Blanched 20 min 100°C		Blanched 20 min 100°C	
				GLY[a]	NGC[a]	GLY	NGC	GLY	NGC
A	No addition	Not applicable	3/37	24; 27[b]	8; 11	29; 32	0; 0	32; 31	0; 0
B	LU120, supernatant	7	3/37	26; 22	11; 15	30; 31	0; 1	30; 25	2; 5
C	LU677, supernatant	24	3/37	19; 17	19; 20	29; 28	1; 1	28; 24	2; 7
D	LU1500, supernatant	33.7	3/37					30; 30	0; 1
		101.3						29; 29	1; 3
		337		24; 27	13; 10	31; 25	0; 6	29; 26	3; 4
E	Olivex, NOVO	4	3/37	29; 28	11; 8	28; 29	2; 2	27; 22	3; 9
		37.5						28; 24	3; 5
		112.5						26; 23	4; 6
F	β-Glucosidase, Sigma	37	3/37					30; 28	1; 2
		111						23; 22	7; 10
		74 000		15; 16	20; 18	1; 1	30; 32	0; 1	29; 30
G	Olivex + β-Glucosidase	37	3/37					30; 28	1; 2
		112						28; 27	2; 2
H	Olivex + LU1500	34	3/37					31; 30	0; 1
		102						30; 28	2; 2

[a]GLY = glycoside; NGC = non-glycosidic cyanide.
[b]Data are of duplicate samples; μmol CN eq/g seed fresh weight.

affinity of the tested enzymes is possibly inadequate, and other potential limitations (e.g. absence of other required cell wall-degrading enzymes, and/or feed-back inhibition) may play a role. A further study aimed at characterizing the kinetics of the enzymes discussed here could be helpful to understand the nature of the enzyme-related limitations of amygdalin degradation. Additional tests with a series of cell wall-degrading enzymes could be useful.

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