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Enzyme-Assisted Processing Increases Antimicrobial and Antioxidant Activity of Bilberry

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The effects of nine cell wall-degrading enzymes on the antimicrobial and antioxidant activities of bilberry were studied. Antimicrobial activity was measured using the human pathogens *Salmonella enterica* sv. Typhimurium and *Staphylococcus aureus* as test strains. Enzyme treatments liberated phenolics from the cell wall matrix, which clearly increased the antimicrobial activity of berry juices, press cakes, and berry mashes on the basis of plate counts. Antibacterial effects were stronger against *Salmonella* than against *Staphylococcus* bacteria. In general, the increase in activity measured as colony-forming units per milliliter was 3–5 logarithmic units against *Salmonella* and 1–2 units against *Staphylococcus* bacteria. Increase in antimicrobial activity was observed only in acidic conditions, which is also the natural environment in various berry products, such as juices. The activity profile of the pectinase preparation affected the chemistry of the phenolics due to the presence of deglycosylating activities in some preparations. The difference in phenolic profiles was reflected in the antimicrobial effects. Bilberry mashes treated with Pectinex Ultra SP-L, Pectinex 3 XL, and Pectinex BE XXL were most efficient against *Salmonella* bacteria, whereas mashes treated with Pectinex Smash, Pectinex BE 3-L, and Biopectinase CCM showed the strongest antimicrobial activity against *Staphylococcus* bacteria. Due to the liberation of phenolics from the cell wall matrix the antioxidant activity measured as radical scavenging activity was also increased on average about 30% by the enzymatic treatments. The highest increase in phenolic compounds was about 40%. Highest increases in anthocyanins and in antioxidant activity were observed in berry mash treated with Pectinex Smash XXL enzyme, and the lowest increase was observed after treatment with Pectinex BE 3-L. Enzyme-assisted processing is traditionally used to improve berry and fruit juice yields. However, enzymatic treatments also have an impact on the functional properties of the products. The increased liberation of phenolics from the cell wall matrix can prolong the shelf life of berry products by limiting the growth of contaminants during processing or storage. The increased amount of phenolic compounds may also have a positive effect on gut well-being.

KEYWORDS: Berry; antimicrobial; *Salmonella*; *Staphylococcus*; antioxidant; phenolic compounds; enzyme; processing

INTRODUCTION

Wild berries are traditionally an important part of the daily diet for many people in the Nordic countries, where almost 40 edible berry species are grown. In Finland the annual crop of bilberry, which is one of the most important wild berries, is as much as 200 million kilograms. Today, wild berries are also an important raw material for the berry-processing industry. In addition to being healthy in general, berries have a long history in folk medicine. Bilberry, for example, has been used as a medicinal herb for the treatment of diarrhea and to improve night vision. Cranberry has been used for the treatment of urinary tract infections. Many health effects of berries are related to phenolic compounds, which the berries contain at high levels (*1, 2*). Flavonoids, phenolic acids, lignans, and complex phenolic polymers (polymeric tannins) are typical components of berries. Many berries, such as bilberry, are rich sources of anthocyanins, which impart the dark red or blue color to the berry fruit.

The antimicrobial activity of berry compounds has attracted interest because recent studies suggest that these compounds may protect against human pathogenic bacteria (*3–9*). Pathogenic bacteria or toxins produced by bacteria often enter the human body via food or drink, causing symptoms or illness with several mechanisms. For example *Salmonella enterica* sv.

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Table 1. Activity Profiles of Enzymes Measured at pH 3.5[a]

activity	Pectinex Ultra SP-L, nkat/mL	Pectinex BE 3-L, nkat/mL	Pectinex 3 XL, nkat/mL	Pectinex Smash, nkat/mL	Pectinex Smash XXL, nkat/mL	Pectinex BE XXL, nkat/mL	Rohapect, nkat/mg	Biopectinase CCM, nkat/mL	Biopectinase Super 8X, nkat/mL
polygalacturonase	29300	11950	11109	34900	220	12300	7267	37000	51000
pectin methyl esterase	2540	2090	1885	7800	0	2110	0	5140	7970
endoglucanase	1650	990	563	1990	83	1010	733	1470	2665
xylanase	900	21630	795	590	86	8840	9833	17620	12930
mannanase	16160	1290	500	30900	0	1210	990	3140	2380
α-arabinosidase	715	3000	2965	775	0	3150	70	1690	3715
β-galactosidase	1460	2800	1210	1910	0	1535	270	690	1127
β-glucosidase	8	340	34	42	0	88	110	136	107

[a] nkat = enzyme activity unit, nmol/s.

Table 2. Calculated Enzyme Activities Associated with a Polygalacturonase Activity of 100 nkat/g of Berry Mash[a]

activity	Pectinex Ultra SP-L	Pectinex BE 3-L	Pectinex 3 XL	Pectinex Smash	Pectinex Smash XXL	Pectinex BE XXL	Rohapect	Biopectinase CCM	Biopectinase Super 8X
polygalacturonase	100	100	100	100	100	100	100	100	100
pectin methyl esterase	9	17	17	22	0	17	0	14	16
endoglucanase	6	8	5	6	38	8	10	4	5
xylanase	3	181	7	2	39	72	135	48	25
mannanase	55	11	5	89	0	10	14	8	5
α-arabinosidase	2	25	27	2	0	26	1	5	7
β-galactosidase	5	23	11	5	0	12	4	2	2
β-glucosidase	0	3	0	0	0	1	2	0	0

[a] nkat = enzyme activity unit, nmol/s.

Typhimurium causes foodborne and waterborne outbreaks of gastrointestinal tract infections in humans, and *Staphylococcus aureus* is a causative agent of food poisoning by producing toxin in food, followed by toxic symptoms in humans. The health-promoting effects of berries are anticipated to be partially due to the high antioxidativity of berry phenolics (*10, 11*). In several studies berries have shown a remarkably high free radical scavenging activity (*12, 13*).

Pectinolytic enzymes are currently used in industrial berry processing to facilitate juice extraction. Most of these commercial pectinase preparations are mixtures of endoacting (carbohydrate backbone cleaving) pectinases together with cellulases and hemicellulases. In addition, exoacting (carbohydrate side-chain cleaving) enzymes are also present in the enzyme mixtures, with potential effects on the chemistry of phenolic glycosides (*14, 15*). The activity profile of the pectinase mixture has a major impact on the chemical composition of the anthocyanins and anthocyanindins in berry juice (*15*). Especially the presence of deglycosylating activities is crucial, as they also effectively hydrolyze certain glycosides to the corresponding less stable aglycones (anthocyanidins). The different phenolic profile can in turn affect the biological activities of berry products.

The aim of the present study was to determine the impact of different commercial pectinase preparations on the antimicrobial and antioxidant activities of bilberry mash, juice, and press cake.

MATERIALS AND METHODS

Berry Material. Frozen berry samples of bilberry (*Vaccinium myrtillus* L.) were obtained from Kiantama Ltd., Suomussalmi, northern Finland. Berries were harvested in 2003 and 2004. Frozen berries were kept in a freezer (−18 °C) until they were processed.

Bacterial Strains and Culture Conditions. Three bacterial strains were used as test organisms in the experiments. *Lactobacillus rhamnosus* VTT E-96666 (ATCC 53103, American Type Culture Collection) is a probiotic bacterium. *S. aureus* VTT E-70045 (Sanitized Testing Laboratory, Burgdorf, Switzerland) and *S. enterica* sv. Typhimurium VTT E-981151 (National Public Health Institute, Finland) are virulent strains. *L. rhamnosus* and *S. enterica* originated from human feces and *S. aureus* from a human wound.

L. rhamnosus GG VTT E-96666 was cultured anaerobically at 37 °C in MRS broth (de Man, Rogosa, Sharpe Broth, Oxoid, Cambridge, U.K.) or on MRS agar (de Man Rogosa Sharpe agar, Oxoid). *Salmonella* and *Staphylococcus* strains were cultured aerobically at 37 °C in nutrient broth (Oxoid) with agitation (150–200 rpm) or on nutrient agar (Oxoid). Frozen stock cultures were maintained at −70 °C. Before experimental use, cultures were transferred to a solid medium and incubated for 1–2 days. Cultures were then subcultured in a liquid medium, incubated for 12–24 h, and used as the source of inoculum for each experiment.

Enzymes. All enzymes used in the study were commercial food grade pectinase mixtures. Pectinex BE XXL, Pectinex BE 3-L, Pectinex Ultra SP-L, Pectinex Smash, Pectinex 3 XL, and Pectinex Smash XXL were supplied by Novozymes (Bagsvaerd, Denmark). Biopectinase CCM and Biopectinase Super were obtained from Quest International (Cork, Ireland), and Rohapect was from AB Enzymes (Rajamäki, Finland). The activity profiles of the enzyme preparations were determined as described previously (*15*) (**Table 1**).

Comparison of Different Enzymes. To compare the effects of different enzyme preparations, berry mash was treated separately with nine different enzymes (**Table 1**). Enzyme treatments were carried out according to the method of Buchert et al. (*15*). Briefly, frozen bilberries (50 g) were first thawed and then mashed for 8 s in a multifunctional chopper (Multitrio, Moulinex, Ireland). Enzyme solution was added, and the berry mash was incubated for 2 h at 45 °C. Enzymes were dosed on the basis of an endopolygalacturonase (PG) activity of 100 nkat/g (**Table 2**). Reference treatments were carried out correspondingly either without enzymes or using heat-denaturated enzymes (the enzymes were kept in boiling water for 5 min). After the treatment, the berry mashes were centrifuged at 7000 rpm for 20 min. The supernatants were stored at −20 °C and used for analyses.

Enzyme-Assisted Juice Pressing. In juice-pressing experiments two pectinex preparations, i.e. Pectinex BE XXL and Pectinex BE 3-L, were used. Enzyme treatments were carried out as described above. Enzymes were dosed on the basis of endopolygalacturonase activity corresponding to 10 and 50 nkat/g in the treatment. Reference treatments were carried out correspondingly either without enzymes or using heat-denaturated enzymes (the enzymes were kept in boiling water for 5 min). After

incubation, the berry mash was pressed using TA-Hdi texture analyzer equipment (Stable Micro Systems, Goldalming, U.K.). The berry juice and press cake samples were stored at −20 °C and used as such for analysis.

Antimicrobial Activity. The antimicrobial activity of berry materials on the bacterial strains was measured in liquid cultures by a plate count method according to the method of Puupponen-Pimiä et al. (*6*). Berry juice, press cake, or centrifuged mash (supernatant) was used in these liquid culture experiments. Briefly, 10 mL of fresh growth medium was inoculated with 1% overnight culture. Berry material was added as such to the culture media, without pH adjustment, to give final concentrations of 100 mg/mL (wet weight). All of the cultures were shaken well and incubated as described above for each bacterial strain. Bacterial growth was followed by taking samples from the cultures at the beginning of cultivation and after 3, 7, and 24 h. The samples were diluted in peptone saline (Maximal Recovery Diluent, Laboratory M) and plated. The plates were incubated as described above, and the bacterial counts were recorded. Increase in inhibition caused by the enzyme treatments was measured by comparing growth curves of non-enzyme-treated reference samples with those obtained from enzyme-treated samples. A control bacterial growth curve, without any berry material, was also measured.

The same experiments were repeated in adjusted pH conditions. The pH of all cultures containing berry material was adjusted to pH 6 at the beginning of cultivation. This was the pH value of the control bacterial growth medium.

Analysis of Total Phenolic and Anthocyanin Content. Berry press cake or mash supernatant (1 g) was extracted by stirring in 10 mL of methanol in the dark for 1 h and centrifuging for 5 min at 8000 rpm (Biofuge *Primo R*, Heraeus, Germany). The supernatant was collected and the pellet re-extracted as above, and the mash supernatants were combined. The extracts were stored in the dark at 5 °C, and radical scavenging activity was measured within 2 days. Duplicate extracts were prepared from each sample.

Total phenolics were measured from the berry juices or extracts described above spectrophotometrically according to the Folin−Ciocalteu procedure (*16*). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of fresh press cake or in grams per liter of berry juice or supernatant. Three replicate analyses were carried out for each extract. Total anthocyanin content was measured using the spectrophotometric European Pharmacopeia method (*17*). Three replicate analyses were carried out for each extract.

Radical Scavenging Activity. Antioxidant activity was evaluated using the DPPH radical scavenging method (*18*) with minor modifications. Berry extracts prepared from mash supernatant were first diluted with methanol (1:20, 1:50, 1:100, 1:200, 1:500). The DPPH solution (23.8 mg of DPPH/100 mL of methanol) was diluted 1:5 with methanol, and 0.5 mL of the diluted solution was pipeted into 1 mL cuvettes. Diluted berry extracts (0.5 mL) were added to DPPH solution. Samples were incubated at room temperature in the dark for 30 min. The absorbance of the samples was measured at A_{515nm} in a spectrophotometer (U-2000, Hitachi Ltd.). Radical scavenging activity was expressed as EC_{50}, the amount of antioxidant needed to decrease the initial DPPH radical concentration by 50%. Thus, the higher the EC_{50} value, the lower the antioxidant activity. Three replicate analyses were carried out for each extract.

Statistics. Pearson's correlation coefficients and regression were used to study the relationships between antimicrobial and radical scavenging activity, total phenolics, and anthocyanin contents.

RESULTS

Effects of Pectinase Preparations on Antimicrobial and Antioxidant Activities of Mash Supernatant. Bilberry mash (harvested in 2004) was treated with nine different pectinase preparations (100 nkat/g of berries), which had different activity profiles (**Tables 1** and **2**). After centrifugation, the total amounts of phenolic components and anthocyanins and antimicrobial and antioxidative activities of the supernatants were compared.

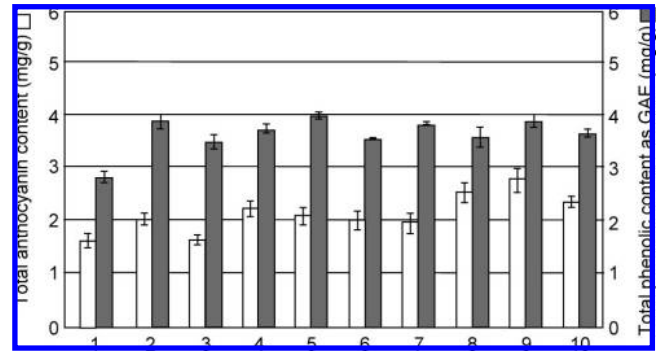

Figure 1. Amount of total phenolics and anthocyanins in enzyme-treated bilberry mash supernatant samples: (1) reference; (2) Pectinex Smash; (3) Pectinex BE 3-L; (4) Biopectinase CCM; (5) Pectinex Ultra SP-L; (6) Pectinex 3 XL; (7) Pectinex BE XXL; (8) Rohapect; (9) Pectinex Smash XXL; (10) Biopectinase Super 8X.

The effects of enzyme treatments on phenolic compounds were evaluated using the spectrophotometric Folin−Ciocalteu method. All enzyme treatments increased the total amount of phenolic compounds. The lowest increase was 0.6 mg/g and the highest increase, 1.2 mg/g; that is, the increase varied between 21 and 43% (**Figure 1**). The differences between the enzyme effects were rather small, although the lowest levels of phenolics were detected after Pectinex BE 3-L, Pectinex 3 XL, and Rohapect treatments. More pronounced effects were observed in the total anthocyanin levels when measured by the spectrophotometric method described in the European Pharmacopoeia. Rohapect and Pectinex Smash XXL treatments resulted in clearly higher anthocyanin contents (2.5–2.7 mg/g) as compared to the other treatments. Surprisingly, the anthocyanin content after the Pectinex BE 3-L treatment did not increase as compared to the reference. With other enzymes, an increase of 25−68% in anthocyanin content was observed (**Figure 1**).

The possible antimicrobial effect of the mash supernatant against *Salmonella* and *Staphylococcus* bacteria was measured in unadjusted pH conditions. Enzyme treatments as such did not have any significant effect on the pH of the berry mash, but all enzyme treatments increased the antimicrobial activity of mash supernatants against *Salmonella* and *Staphylococcus* bacteria (**Figures 2** and **3**). Inhibition profiles varied between different enzymes. Bilberry juices treated with Pectinex Ultra SP-L, Pectinex 3 XL, and Pectinex BE XXL were most efficient against *Salmonella* bacteria. The juices treated with Pectinex Smash, Pectinex BE 3-L, and Biopectinase CCM showed the strongest antimicrobial activity against *Staphylococcus* bacteria. Rohapect treatment resulted in the lowest effect against both bacteria.

To determine the pH dependence of antimicrobial activity, similar experiments were carried out in higher pH conditions. The pH of the growth medium containing berry material was adjusted to pH 6 (the pH of the control bacterial growth medium) at the beginning of cultivation. Neither enzyme-treated nor untreated bilberry samples showed any clear activity against *Salmonella* bacteria when the pH was adjusted to 6. Some activity was observed against *Staphylococcus* bacteria, but only at the end of cultivation (decrease in plate counts after 12 h of cultivation) (**Figures 4** and **5**).

The effects of the enzymatic treatments on antioxidant activity were measured as free radical scavenging activity. All pectinase treatments clearly increased the radical scavenging activity of bilberry juices as compared to the untreated reference sample (**Figure 6**). Radical scavenging activity increased about 30% after the enzymatic treatments. No major differences were

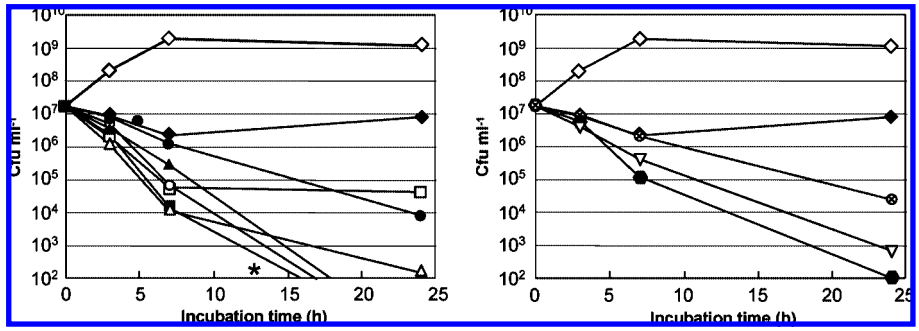

Figure 2. Effects of nine different pectinase preparations (100 nkat/g of berries) on antimicrobial activity of bilberry mash supernatant against *Salmonella* bacteria in unadjusted pH conditions: (◇) control bacterial growth; (◆) reference mash; (□) Pectinex Smash; (Δ) Pectinex BE 3-L; (○) Pectinex Ultra SP-L; (▲) Pectinex 3 XL; (■) Pectinex BE XXL; (●) Pectinex Smash XXL; (▽) Biopectinase CCM; (⊗) Rohapect; (⬢) Biopectinase Super 8X. The asterisk indicates 24 h plate count below the detection limit of 10^2 colony-forming units (cfu)/mL.

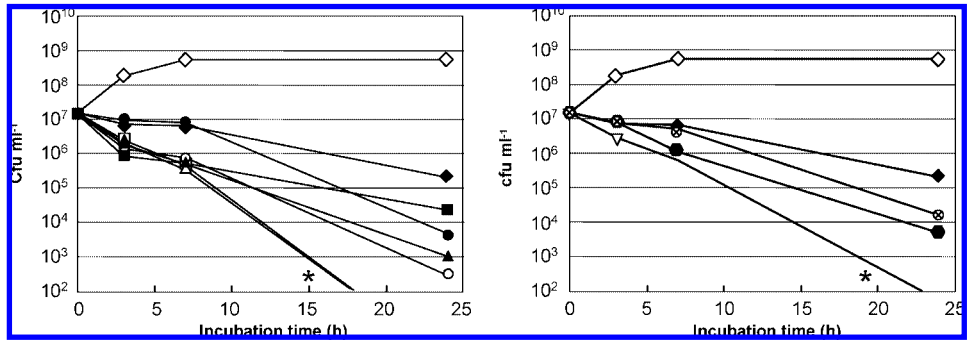

Figure 3. Effects of nine different pectinase preparations (100 nkat/g of berries) on antimicrobial activity of bilberry mash supernatant against *Staphylococcus* bacteria in unadjusted pH conditions: (◇) control bacterial growth (◆) reference mash; (□) Pectinex Smash; (Δ) Pectinex BE 3-L; (○) Pectinex Ultra SP-L; (▲) Pectinex 3 XL; (■) Pectinex BE XXL; (●) Pectinex Smash XXL; (▽) Biopectinase CCM; (⊗) Rohapect; (⬢) Biopectinase Super 8X. The asterisk indicates 24 h plate count below the detection limit of 10^2 cfu/mL.

observed when the effects of all the enzymatically treated supernatants were compared. However, the highest radical scavenging activity was associated with Pectinex Smash XXL enzyme and the lowest activity with Pectinex BE 3-L. These two enzymes also gave the highest and lowest amounts of anthocyanins, respectively.

Statistical analysis showed clear negative correlation between anthocyanin content and DPPH value ($r = -0.90$) in enzyme-treated samples. Thus, the higher the anthocyanin content, the higher was the radical scavenging activity (the lower the DPPH value). No other clear correlations were found in enzyme-treated samples.

Antimicrobial Properties of Bilberry Juices and Press Cakes after Enzymatic Treatments. Bilberry juices were pressed from mash treated with Pectinase BE XXL and Pectinex BE 3-L from harvesting year 2003 berries, and the amount of total phenolics and the antimicrobial properties of the juices and the residual press cakes were determined. The phenolics contents of reference juice and press cake (no enzyme treatment) were 3.3 g/L and 7.8 g/kg, respectively. Enzyme treatment clearly increased the amount of phenolic compounds in juices as well as in press cakes. The highest increases in phenolic compounds were 30 and 37% in juices and press cakes, respectively. In press cakes, the amount of phenolic compounds increased when the enzyme concentration was increased from 10 to 50 nkat/g. Corresponding increases were less clearly observed in juices. The highest concentration of phenolics measured in enzyme-treated juices was 4.3 g/L, and that in press cakes was 10.7 g/kg. The most efficient enzymes were Pectinex BE XXL (10 nkat/g) and Pectinex BE 3-L (50 nkat/g) for juices and press cakes, respectively.

Enzyme treatments clearly increased the antimicrobial activity of bilberry juice and press cake against *Salmonella* and *Staphylococcus* bacteria in unadjusted pH conditions (acidic conditions, pH around 4.5) (**Figures 7** and **8**). Antibacterial effects were stronger against *Salmonella* than against *Staphylococcus* bacteria. In general, increases in activities of 3–5 logarithmic units and 1–2 logarithmic units [in colony-foming units (cfu) per milliliter] were observed against *Salmonella* and *Staphylococcus* bacteria, respectively. It was also observed that the press cakes possessed higher antimicrobial activity than the juices. Antimicrobial activity of the press cakes also increased when the enzyme concentration was increased from 10 to 50 nkat/g. Major differences were not observed between the effects of Pectinex BE XXL and Pectinex BE 3-L.

Enzymes as such did not have any antimicrobial activity against *Salmonella* or *Staphylococcus* bacteria. Furthermore, when the berry material was treated with heat-inactivated enzymes, antimicrobial effects were comparable to the effects of control press cake and juice. The growth of *Lactobacillus rhamnosus* was not affected by the control juice or press cake or by samples treated with enzymes.

DISCUSSION

Berry juices are known to have some antibacterial activity against various bacteria due to organic acids and phenolic compounds present in the berry fruits. Transportation and storage of berry juice concentrates at low temperatures prior to final packaging is a common practice in the juice industry and introduces a potential risk for postconcentration contamination with microbes, possibly including human pathogens. Increased antimicrobial activity of berry products is therefore desired by the berry-processing industry.

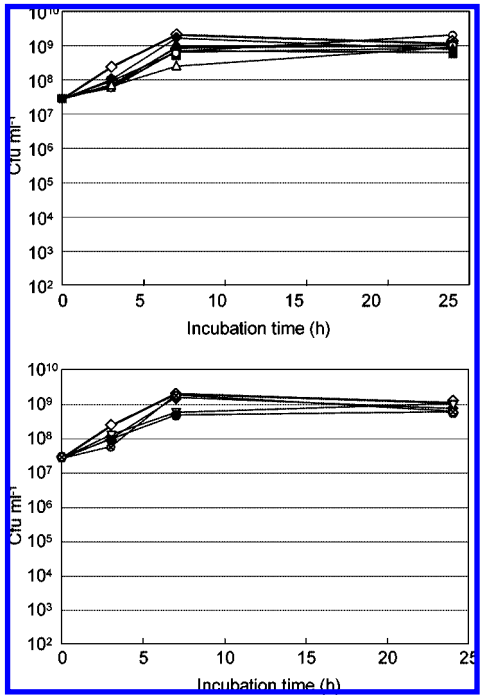

Figure 4. Effects of nine different pectinase preparations (100 nkat/g of berries) on antimicrobial activity of bilberry mash supernatant against *Salmonella* bacteria. pH was adjusted to 6 at the beginning of cultivation: (◇) control bacterial growth; (◆) reference mash; (□) Pectinex Smash; (Δ) Pectinex BE 3-L; (○) Pectinex Ultra SP-L; (▲) Pectinex 3 XL; (■) Pectinex BE XXL; (●) Pectinex Smash XXL; (▽) Biopectinase CCM; (⊗) Rohapect; (⬢) Biopectinase Super 8X.

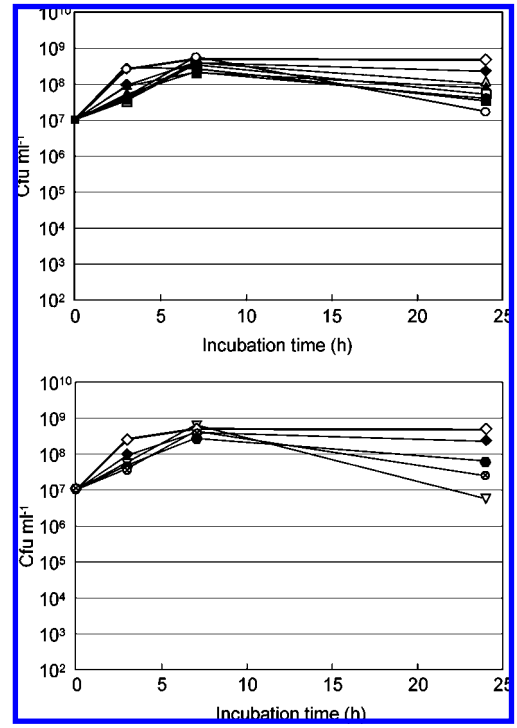

Figure 5. Effects of nine different pectinase preparations (100 nkat/g of berries) on antimicrobial activity of bilberry mash supernatant against *Staphylococcus* bacteria. pH was adjusted to 6 at the beginning of cultivation: (◇) control bacterial growth; (◆) reference mash; (□) Pectinex Smash; (Δ) Pectinex BE 3-L; (○) Pectinex Ultra SP-L; (▲) Pectinex 3 XL; (■) Pectinex BE XXL; (●) Pectinex Smash XXL; (▽) Biopectinase CCM; (⊗) Rohapect; (⬢) Biopectinase Super 8X.

Especially, natural preservatives or antimicrobial agents have attracted much interest in recent years. There is also a current demand for functional foods stabilizing gut microbiota and promoting gut health. In our earlier studies (*5*–*7*), phenolic berry extracts of common Nordic berries selectively inhibited the growth of harmful bacteria and human intestinal pathogens, without affecting the growth of beneficial lactic acid bacteria. Bilberry has been shown to be effective against *Bacillus cereus*, *Salmonella enterica* sv. Typhimurium and sv. Infantis, *Staphylococcus aureus*, *Clostridium perfringens*, and *Helicobacter pylori*. *Campylobacter jejuni* and *Candida albicans* were inhibited with phenolic extracts of cloudberry, raspberry, and strawberry, which were all rich in ellagitannins (*5*). *Salmonella* and *Staphylococcus* were the most sensitive bacteria and were therefore used as test organisms in the present study.

Bilberry mash was treated with nine commercial cell wall-degrading enzyme preparations. Dosing of the enzymes was based on their polygalacturonase (PG) activity, which resulted in the presence of variable amounts of other enzyme activities. Enzyme treatment significantly increased the antimicrobial activity of bilberry against two severe human pathogens, *Salmonella* and *Staphylococcus* bacteria. Increased antimicrobial activity was most probably due to increased amounts of phenolic compounds and also due to changes in phenolic profiles. To the best of our knowledge, the impact of commercial enzyme preparations on antimicrobial activity of berry products has not previously been investigated. Vattem et al. (*9, 19*) and Vattem and Shetty (*20*) showed that solid-state bioprocessing of cranberry pomace, using the food-grade fungus *Rhozopus oligosporus* or *Lentinus edode* resulted in enrichment of total phenolics and improved antimicrobial activities against important foodborne pathogens such as *Listeria monocytogenes*, *Vibrio parahemolyticus*, and *Escherichia coli* O157:H7.

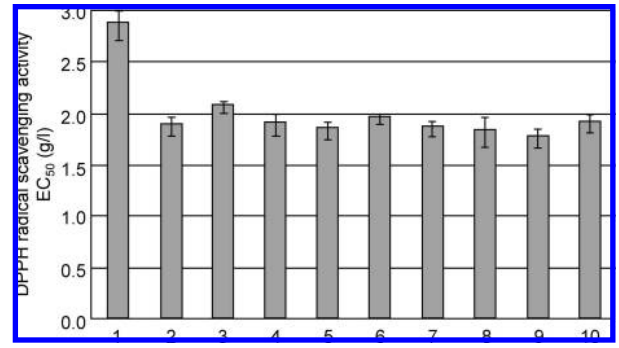

Figure 6. Effects of various pectinase enzymes on DPPH radical scavenging activity of bilberry mash supernatant: (1) reference; (2) Pectinex Smash; (3) Pectinex BE 3-L; (4) Biopectinase CCM; (5) Pectinex Ultra SP-L; (6) Pectinex 3 XL; (7) Pectinex BE XXL; (8) Rohapect; (9) Pectinex Smash XXL; (10) Biopectinase Super 8X.

Our screening methods showed that the amounts of phenolics and anthocyanins were remarkably increased by enzyme treatments. The ability of enzyme treatments to improve the extractability of the juice with concomitant enhancement of the release of phenolic compounds from the cell walls was studied by Buchert et al. (*15*). These experiments showed that Pectinex Ultra SP-L, Pectinex Smash, Pectinex BE 3-L, and Biopectinase CCM increased the total content of anthocyanins by 13–41% as measured by a quantitative HPLC method. Our results agree with the results of Buchert et al. (*15*). In our work, Pectinex Smash XXL was the most efficient enzyme in the release of anthocyanins from bilberries. Recently, Koponen et al. (*21*) compared the effects of several pectinolytic enzyme preparations on extractability of anthocyanins into bilberry juices, and they

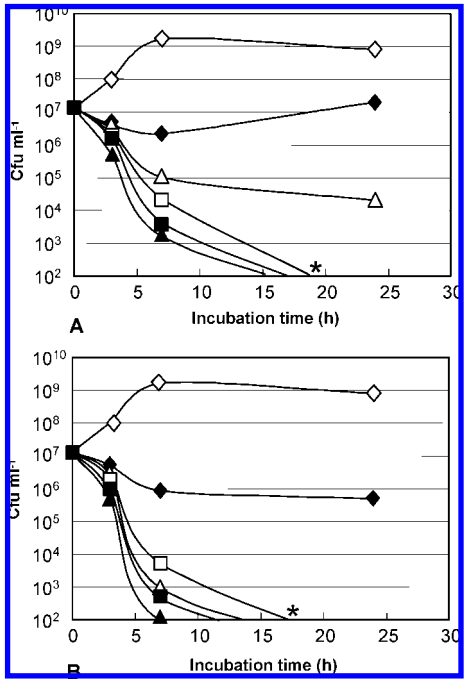

Figure 7. Effects of enzyme treatments on antimicrobial activity of bilberry juice (**A**) and press cake (**B**) against *Salmonella* bacteria: (◇) control bacterial growth; (◆) reference juice/press cake; (Δ) Pectinex BE 3-L, 10 nkat/g; (▲) Pectinex BE 3-L, 50 nkat/g; (□) Pectinex BE XXL, 10 nkat/g; (■) Pectinex BE XXL, 50 nkat/g. The asterisk indicates 24 h plate count below the detection limit of 10^2 cfu/mL.

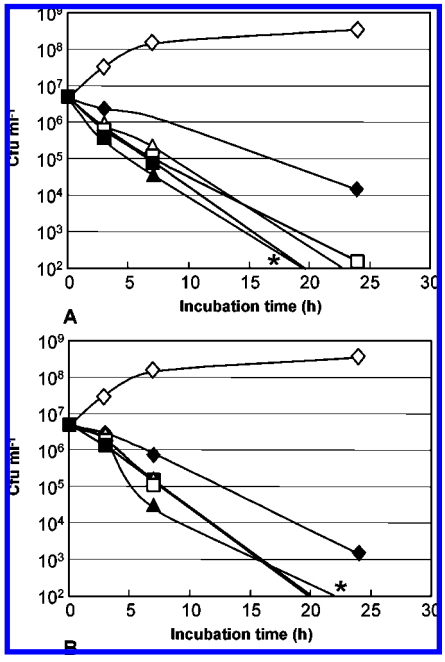

Figure 8. Effects of enzyme treatments on antimicrobial activity of bilberry juice (**A**) and press cake (**B**) against *Staphylococcus* bacteria: (◇) control bacterial growth; (◆) reference juice/press cake; (Δ) Pectinex BE 3-L, 10 nkat/g; (▲) Pectinex BE 3-L, 50 nkat/g; (□) Pectinex BE XXL, 10 nkat/g; (■) Pectinex BE XXL, 50 nkat/g. The asterisk indicates 24 h plate count below the detection limit of 10^2 cfu/mL.

also found that Pectinex Smash XXL was the most efficient enzyme. This enzyme preparation contains the highest pectic lyase activity, resulting in total pectin depolymerization and thus very efficient release of phenolic compounds from the cell wall matrix (*21*).

The majority of bilberry phenolics are anthocyanins, which are composed of 3-*O*-galactosides, 3-*O*-glucosides, and 3-*O*-arabinosides of five aglycones, delphinidin, cyanidin, petunidin, peonidin, and malvidin. In addition, minor amounts of hydroxycinnamic acids, flavonols, and catechins are found in bilberry (*22*). Differences in the antimicrobial properties found in our study may also partly be due to the different levels of deglycosylating enzyme activities present in the enzymes (**Table 2**), resulting in different phenolic profiles. These different enzyme activities can hydrolyze the anthocyanins to corresponding anthocyanidins, resulting in less stable structures and also possibly altered antimicrobial properties. Pectinex BE 3-L, Pectinex BE XXL, and Pectinex 3 XL are especially rich in α-arabinosidase and β-galactosidase activities, whereas Pectinex Smash XXL is devoid of these activities. The lack of sugar side-chain cleaving activity may also explain the highest anthocyanin contents of Pectinex Smash XXL treated samples. Pectinex Ultra SP-L, Pectinex Smash, Rohapect, and Biopectinase CCM contain rather low quantities of the deglycosylating activities. It has previously been found that especially galactoside linkages of anthocyanins are easily hydrolyzed by commercial pectinase preparations containing β-galactosidase activities, which affects the profile of the extracted anthocyanins (*15*). We found that the lowest increase in phenolic compounds and anthocyanins was associated with Pectinex BE 3-L enzyme, which has a rather high sugar side-chain cleaving activity and may thus affect the stability of extracted anthocyanins. Thus, tailored changes in the chemical composition of the solubilized anthocyanins can either decrease or increase the antimicrobial properties.

Bacterial growth is highly dependent on the pH of the growth medium. In our experiments, a high increase in antimicrobial activity of enzyme-treated bilberry samples was observed only in low-pH conditions, which is also the natural pH of berry products, such as juices. The pH is known to affect the antimicrobial activity of phenolic compounds. Friedman and Jürgens (*23*) demonstrated that caffeic, chlorogenic, and gallic acids are not stable at high pH and that the pH- and time-dependent spectral transformations are not reversible. Pure phenolic acids, such as hydroxycinnamic acids, have recently exhibited both bactericidal and bacteriostatic activity against several strains of *L. monocytogenes*. The nature of the antimicrobial effect was dependent on medium pH. For example, all of the hydroxycinnamic acids were bactericidal at pH 4.5 but only bacteriostatic at higher pH. By contrast, chlorogenic acid inhibited the growth of *L. monocytogenes* only at pH 6.5 (*24*). Medium pH also affects the antimicrobial activity of caffeic, *p*-coumaric, and ferulic acids toward *E. coli* and *S. aureus*. Inhibition of both species increased as the pH of the bacterial growth medium decreased from 7.0 to 5.0 (*25*). In our earlier studies we have also shown that cloudberry and raspberry phenolics and ellagitannin fractions are very efficient against *S. aureus* at pH 6 (*7*). Anthocyanins, which are the main flavonoids in bilberry, are known to be exceptionally sensitive to pH changes. They are more stable in acidic pH than in neutral or alkaline pH (*26*). This is also one possible explanation why berries rich in these compounds were not active in our experiments when the pH was increased. There appear to be complex interactions between the pH of the growth media and antimicrobial activity, varying in different bacteria and berry species. Thus, when the antimicrobial efficacy of berry phenolic compounds is evaluated either in food matrices or in the human body, pH is a very important parameter to be considered.

All enzyme treatments clearly increased the antioxidant activity of bilberry samples as a consequence of increased amounts of anthocyanins. The antioxidant activity of anthocyanins depends on aglycone structure and glycosylation pattern in a complex manner. In many studies, antioxidant potentials of anthocyanin aglycones were generally higher than those of the corresponding glycosides (*28–30*). Kähkönen et al. (*30, 31*) extensively studied antioxidant activities of berry anthocyanins and also compared antioxidant activities of anthocyanins and their aglycones. According to their results, bilberry anthocyanins were highly active radical scavengers in the DPPH test. Among the aglycones tested, they found that delphinidin possessed the highest activity, followed by cyanidin and peonidin, malvidin, and petunidin. The monoglucosides of cyanidin, delphinidin, and malvinidin were almost as active as their aglycones, whereas the activity of peonidin 3-glucoside was lower than that of the corresponding aglycone. Cyanidin, peonidin, and malvidin galactosides were weaker scavengers than the corresponding glucosides, the difference being 15–23%. In our study Pectinex Smash XXL treatment resulted in the highest antioxidant activity and Pectinex BE 3-L, the lowest. It can therefore be hypothesized that anthocyanin glycosides play a more important role than aglycones in the antioxidant activity of bilberry. If radical scavenging activity of the berry products can be increased using enzymes, it may be reflected as beneficial health effects in humans. Products that increase antioxidant status may have many applications in the future as components of functional foods. However, clinical trials are needed to verify the increased antioxidant capacity of enzyme- treated berry products.

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LITERATURE CITED

(1) Häkkinen, S. H.; Kärenlampi, S. O.; Heinonen, M. I.; Mykkänen, H. M.; Törrönen, R. A. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 2274–2279.

(2) Häkkinen, S.; Kärenlampi, S.; Mykkänen, H.; Heinonen, M.; Törrönen, R. Ellagic acid-content in berries: influence of domestic processing and storage. *Eur. Food Res. Technol.* **2000**, *212*, 75–80.

(3) Cavanagh, H. M.; Hipwell, M.; Wilkinson, J. M. Antibacterial activity of berry fruits used for culinary purposes. *J. Med. Foods* **2003**, *1*, 57–61.

(4) Kontiokari, T.; Laitinen, J.; Järvi, L.; Pokka, T.; Sundqvist, K.; Uhari, M. Dietary factors protecting women from urinary tract infection. *Am. J. Clin. Nutr.* **2003**, *77*, 600–604.

(5) Nohynek, L.; Alakomi, H.-L.; Kähkönen, M.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K.-M.; Puupponen-Pimiä, R. Berry phenolics − antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer* **2006**, *54*, 18–32.

(6) Puupponen-Pimiä, R.; Nohynek, L.; Meier, C.; Kähkönen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K.-M. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* **2001**, *90*, 494–507.

(7) Puupponen-Pimiä, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kähkönen, M.; Heinonen, M.; Määttä-Riihinen, K.; Oksman-Caldentey, K.-M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991–1000.

(8) Rauha, J.-P.; Remes, S.; Heinonen, M.; Hopia, A.; Kähkönen, M.; Kujala, T.; Pihlaja, K.; Vuorela, H.; Vuorela, P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* **2000**, *56*, 3–12.

(9) Vattem, D. A.; Lin, Y.-T.; Labbe, R. G.; Shetty, K. Phenolic antioxidant mobilization in cranberry pomace by solid-state bioprocessing using food grade fungus *Lentinus edodes* and effect on antimicrobial activity against select food borne pathogens. *Innovative Food Sci. Emerging Technol.* **2004**, *5*, 81–91.

(10) Kähkönen, M. P.; Hopia, A. I.; Heinonen, M. Berry phenolics and their antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 4076–4082.

(11) Mullen, W.; McGinn, J.; Lean, M. E. J.; MacLean, M. R.; Duthie, G. G.; Yokota, T.; Crozier, A. Ellagitannins, flavonoids and other phenolics: their contribution to the antioxidant capacity anf vasorelaxation properties of red raspberries. *J. Agric. Food Chem.* **2002**, *50*, 5191–5196.

(12) Wang, S. Y.; Jiao, H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *J. Agric. Food Chem.* **2000**, *48*, 5672–5676.

(13) Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686–2693.

(14) Versari, A.; Biesenbruch, S.; Barbanti, D.; Farnell, P. J.; Galassi, S. Effect of pectolytic enzymes on selected phenolic compounds in strawberry and raspberry juices. *Food Res. Int.* **1997**, *30*, 811–817.

(15) Buchert, J.; Koponen, J.; Suutarinen, M.; Mustranta, A.; Lille, M.; Törrönen, R.; Poutanen, K. Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and black currant juices. *J. Sci. Food Agric.* **2005**, *85*, 2548–2556.

(16) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(17) *European Pharmacopoeia*, 4th ed.; Monograph 01/2002:1602 (Bilberry fruit, fresh; Council of Europe: Strasbourg Cedex, France, 2001; p 739, ISBN 92-871-4587-3.

(18) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25–30.

(19) Vattem, D. A.; Lin, Y.-T.; Labbe, R. G.; Shetty, K. Antimicrobial activity against select food-borne pathogens by phenolic antioxidants enriched in cranberry pomace by solid-state bioprocessing using the food grade fungus *Rhizopus oligosporus*. *Process Biochem.* **2003**, *39*, 1939–1946.

(20) Vattem, D. A.; Shetty, K. Ellagic acid production and phenolic antioxidant activity in cranberry pomace (*Vaccinium macrocarpon*) mediated by *Lentinus edodes* using a solid-state system. *Process Biochem.* **2003**, *39*, 367–379.

(21) Koponen, J. M.; Buchert, J.; Poutanen, K. S.; Törrönen, A. R. Effect of pectinolytic juice production on the extractability and fate of bilberry and black currant anthocyanins. *Eur. Food Res. Technol.* 2007, in press.

(22) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; González-Paramás, A. M.; Törrönen, A. R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477–4486.

(23) Friedman, M.; Jürgens, H. S. Effect of pH on the stability of plant phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 2101–2110.

(24) Wen, A.; Delaquis, P.; Stanich, K.; Toivonen, P. Antilisterial activity of selected phenolic acids. *Food Microbiol.* **2003**, *20*, 305–311.

(25) Herald, P. J.; Davidson, P. M. Antibacterial activity of selected hydroxycinnamic acids. *J. Food Sci.* **1983**, *48*, 1378–1379.

(26) Revilla, E.; Ryan, J. M.; Martin-Ortega, G. Comparison of several procedures used for the extraction of anthocyanins from red grapes. *J. Agric. Food Chem.* **1998**, *46*, 4592–4597.

(27) Satué-Gracia, M. T.; Heinonen, M.; Frankel, E. N. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin−liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362–3367.

(28) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304–309.

(29) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.

(30) Kähkönen, M. P.; Heinämäki, J.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **2003**, *51*, 628–633.

(31) Kähkönen, M. P.; Heinämäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: isolation, identification and antioxidant activities. *J. Sci. Food Agric.* **2003**, *83*, 1403–1411.

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