JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Substrate Specificities of Glycosidases from *Aspergillus* Species Pectinase Preparations on Elderberry Anthocyanins

SINA PRICELIUS,[†] MICHAEL MURKOVIC,[§] PHILIP SOUTER,[#] AND GEORG M. GUEBITZ*,[†]

Department of Environmental Biotechnology and Department of Food Chemistry and Technology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria, and Procter and Gamble Technology Center, Newcastle upon Tyne, United Kingdom

Attractive color is one of the most important sensory characteristics of fruit and berry products, and elderberry juice is widely used as natural colorant. When pectinase preparations were used in the production of elderberry juice for clarification, a concomitant decrease of anthocyanins and thus a color loss were observed. This paper demonstrates that this is due to side glycosidase activities contained in commercial pectinase preparations from *Aspergillus* sp. Using LC-MS, sequential deglycosylation of cyanidin-3-sambubioside, cy-3-glucoside, cy-3-sambubioside-5-glucoside, and cy-3,5-diglucoside was found to be catalyzed by specific glycosidases contained in the pectinase preparations. There was no big difference in the deglycosylation rate between monoglucosidic or diglucosidic anthocyanins. However, the degradation rate was decreased when rutinose was attached to cyanidin, whereas the structure of the aglycone itself had almost no influence. Pure β-glucosidases from *Agrobacterium* species and *Aspergillus niger* and the β-glucosidase N188 from *A. niger* did not show any conversion of anthocyanins, indicating the presence of specific glycosidases. Thus, an activity gel based assay was developed to detect anthocyanin-specific glycosidase activity in enzyme preparations, and according to LC-MS peptide mass mapping of digested bands, homologies to a β-glucosidase from *Aspergillus kawachii* were found.

KEYWORDS: Anthocyanins; pectinase; degradation mechanism; model elderberry juice solution

INTRODUCTION

It is generally accepted that anthocyanins, a subclass of flavonoids, are the most important water-soluble pigments in plant tissues, which are largely responsible and directly related to the attractive red and purple colors of many plants and fruits. The different color nuances are obtained through glycosylation, polyhydroxylation, and polymethoxylation of the derivates from 2-phenylbenzopyrylium cation, that is, the flavylium cation (*1*). Also, the extent of acylation of the sugar moieties with organic acids esterified to anthocyanin glycosyl units as well as the type of acids varies considerably (usually either aromatic phenolic acids or aliphatic dicarboxyl acid or a combination of both) (*2, 3*). The color and structure of anthocyanins change in response to pH and/or in the presence of copigments (*4*).

Anthocyanins are more stable at low pH, whereas color can be enhanced by self-association and copigmentation, when the anthocyanin molecule reacts with other natural plant components covalently or through weak interactions (*5*−*7*). Much work has concentrated on the stability of anthocyanins due to the growing interest in widespread use of anthocyanins as natural food colorants to develop safe, economical, and efficient food colors to replace banned coal tar or azo dyes (*8, 9*). However, anthocyanins tend to be decolorized or degraded during processing and storage of the commodity (*10, 11*). Enzymatic systems present in plant tissue play an important role in the degradation process. Anthocyanins are rapidly oxidized in the vacuole of plant cells in the presence of molecular oxygen by the action of tyrosinase or polyphenol oxidase and enzymatic hydrolysis of the glycosidic bonds to yield the much more unstable anthocyanidins, which are easily converted to polymeric brown or colorless compounds (*12*−*16*).

Consumers are always attracted to foods and drinks bearing pleasant colors. Enzymes, such as pectinases, have been used in juice and pulp treatment to increase anthocyanin content and color density and to improve clarification by breaking down the pectins that provide the firm structure of the berry; thus, polyphenols can be released in the juice (*17*). Several strains of *Aspergilllus niger* are in widespread use for the production of commercial pectinase preparations, and some formulations

* Corresponding author (e-mail guebitz@tugraz.at; telephone +433168738312; fax ++433168738815).

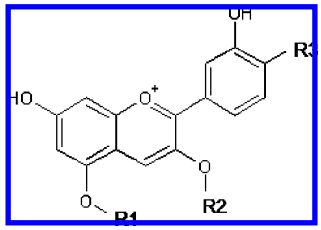
[†] Department of Environmental Biotechnology, Graz University of Technology.

[§] Department of Food Chemistry and Technology, Graz University of Technology.

[#] Procter and Gamble Technology Center.

10.1021/jf803131t CCC: $40.75
Published on Web 01/20/2009

Table 1. Anthocyanins Used in This Study



	R1	R2	R3
cyanin	glucose	glucose	OH
cyanidin	OH	OH	OH
kuromanin	OH	glucose	OH
peonin	OH	glucose	OCH_3
peonidin	OH	OH	OCH_3
keracyanin	OH	rutinose	OH

have been reported to degrade fruit anthocyanins (*4*). Commercial enzyme preparations are crude fungal preparations, containing impurities such as extraneous enzymes, proteins, mucilage, and melanoidins (*18*). Side activities can degrade anthocyanins and phenols that the pectinases were intended to extract (*19*).

When pectinase preparations are used in the production of elderberry juice for clarification, a concomitant decrease of anthocyanins and thus a color loss were observed. The aim of this study was to investigate the role of enzyme side activities in pectinase preparations and to study the rate and mechanism of color loss and anthocyanin breakdown that take place during elderberry juice processing. Although anthocyanin degradation was previously ascribed to glycosidase impurities (e.g., β -glucosidases) (*4, 13, 20*); we show here that pure β -glucosidases do not show this activity. Consequently model substrates and elderberry juice were used to elucidate the degradation mechanism and to identify the enzyme responsible for anthocyanin degradation.

MATERIALS AND METHODS

Chemicals and Enzymes. Methanol and acetonitrile used were of HPLC grade quality and purchased from Roth (Carl Roth GmbH, Karlsruhe, Germany) and VWR Prolabo, respectively. All other chemicals were of analytical grade from Sigma-Aldrich Chemie (Steinheim, Germany). The flavonoids cyanidin-3,5-di-*O*-glucoside (cyanin), cyanidin, cyanidin 3-*O*-glucoside (kuromanin), peonidin 3-*O*-glucoside (peonin), peonidin, cyanidin-3-*O*-rutinoside (keracyanin), quercetin 3-β-D-glucoside, and quercetin were all purchased from Sigma-Aldrich Chemie. For chemical structures, see **Table 1**.

Purified Enzymes. All purified enzymes appeard as single bands on electrophoresis gels (*21, 22*). Polygalacturonase (38 kDa) from *Sclerotium rolfsii* (*21*) and pectate lyase from *Bacillus pumilus* (*22*) were produced as previously described. Pectate lyase E-PECLY from *Aspergillus* sp., β-glucosidase E-BGOSAG from *Agrobacterium* sp. (AB), β-glucosidase E-BGLUC from *A. niger* (AN), and β-galactosidase E-BGLAN from *A. niger* were purchased from Megazyme (Wicklow, Ireland) (see **Table 2**).

Enzyme Preparations. P5146 and P4716 polygalacturonases from *A. niger* and P2401 and P4300 polygalacturonases from *Rhizopus* sp. were purchased from Sigma-Aldrich Chemie. P2736 Pectinex 3XL (contains mainly pectintranseliminase, polygalacturonase, and pectinesterase from *A/ niger*) and P2611 Pectinex Ultra SPL from *Aspergillus aculeatus*, products from Novozymes for food processing, were also purchased from Sigma-Aldrich Chemie. Novozyme 188, a commercial β-glucosidase from *A. niger*, was purchased from Novozymes, USA (see **Table 2**).

Enzyme Assays. *β-Glucosidase Activity.* β-Glucosidase activity was measured according to the method of Schewale et al. (*23*) at 37 °C, pH 4.0, with *p*-nitrophenyl-glucopyranoside as substrate. Enzymes hydrolyze the substrate into the corresponding sugar and *p*-nitrophenol, which is then measured photometrically at 405 nm and the concentration determined by using $\varepsilon_{405nm} = 18.3\ mM^{-1}\ cm^{-1}$.

Table 2. Enzymes Used for Anthocyanin Degradation[a]

enzyme	organism	abbreviation	pectinase activity (U/mL)	β-glucosidase activity (U/mL)
polygalacturonase	*Sclerotium rolfsii*		193	nd
pectate lyase	*Bacillus pumilus*		nt	nd
pectate lyase	*Aspergillus* sp		nt	nd
β-glucosidase	*Agrobacterium* sp.	AB	nt	282
β-glucosidase	*Aspergillus niger*	AN	nt	113.1
β-galactosidase	*Aspergillus niger*		175	0.1
polygalacturonases	*Aspergillus niger*	P5146	199	102.9
polygalacturonases	*Aspergillus niger*	P4716	5214	122.1
Pectinex 3XL	*Aspergillus niger*	P2736	3793	nt
Pectinex Ultra SPL	*Aspergillus aculeatus*	P2611	>10000	13.1
polygalacturonase	*Rhizopus* sp.	P2401	503	10.7
polygalacturonase	*Rhizopus* sp.	P4300	196	8.6
β-glucosidase	*Aspergillus niger*	Novozyme 188	nt	71.6

[a] nd, not detected; nt, not tested.

Glucose Assay. The release of glucose from cyanin was determined by incubating 1 mg mL^{-1} of this anthocyanin with 10 U of β-glucosidase activity (calculated as mentioned above) from each enzyme preparation at pH 4.0 for 1 h at 37 °C, 550 rpm (thermomixer comfort, Eppendorf). Released glucose was quantified using a glucose kit purchased from Megazyme (D-Glucose-HK/G6P-DH format), following the instructions of the supplier for preparation of reagents/samples and procedure of the measurement.

Phenol Oxidase Activity. Phenol oxidase activity of enzyme preparations was determined in 100 mM sodium citrate buffer, pH 4.0, with 200 mg L^{-1} (+)-catechin as a substrate at 37 °C. The reaction was followed at 420 nm in a spectrophotometer to detect browning (*24*).

Pectinase Activity Assay. For the determination of polygalacturonase activity the reduced sugars were measured with sodium polygalacturonic acid (PGA) by a dinitrosalicylic acid (DNS) reagent (*25*). The reaction mixture contained 450 μL of 0.25% PGA solution in pH 4.0 buffer; 50 μL of appropriately diluted enzyme solution was incubated at 37 °C for 5 min, and the reaction was stopped by addition of 750 μL of DNS solution. After 5 min of boiling, subsequent cooling, and centrifugation, the absorbance was read at 540 nm. The reducing sugars formed were quantified using D-galacturonic acid as a standard.

Anthocyanin Transformation Studies. *Model Substrates.* For degradation of the model substrates 445 μL of 100 mM sodium citrate buffer, pH 4.0, and amounts of different enzyme samples equivalent to 10 U of β-glucosidase activity were added to 20 μL of 0.25 mg of cyanin dissolved in DMSO. The reaction mixture was incubated from 1 to 12 h at 37 °C, 550 rpm (thermomixer comfort, Eppendorf). The reaction was stopped by adding 10 μL of concentrated sulfuric acid. Samples were kept at room temperature for 15 min and then centrifuged at 16000*g* (Hereaus, Biofuge primo) for 20 min, filtered with syringe filter (13 mm, with 0.2 μm pore size), and directly measured with HPLC-UVD. Controls were run using the identical conditions, and all experiments were performed in three replicates. One unit of specific glycosidase activity was defined as the amount of enzyme that converts 1 μmol of anthocyanin per minute.

Elderberry Juice. Elderberry concentrate (*Sambucus nigra*) was purchased from Iprona AG, LANA, and stored at −20 °C. According to Murkovic et al. (*26*) this elderberry juice contains two main anthocyanins in significant quantities: cyanidin-3-glucoside (42%) and cyanidin-3-sambubioside (42%). Besides the two main anthocyanins two additional diglycosides can be found in minor amounts, cy-3,5-diglucoside (12%) and cy-3-sambubioside-5-glucoside (4%). The juice was thawed, and 75 μL of elderberry juice was diluted with 900 μL of 100 mM sodium citrate buffer, pH 4.0. The enzyme preparations were dosed on the basis of 15 U of β-glycosidase activity. Degradation of anthocyanins was started by adding the enzyme sample, and the mixture was incubated at 37 °C, 550 rpm (thermomixer comfort, Eppendorf) for various time intervals as indicated below. The reaction was stopped by adding 10 μL of concentrated sulfuric acid, centrifuged at 16000*g* for 20 min, and directly measured with HPLC. Controls were run using the same conditions.

In parallel, the decolorization was followed spectrophotometrically at 515 nm.

HPLC Analysis. Anthocyanins and conversion products were separated by an HPLC-UVD system equipped with a reverse phase column (RP-18 250 mm × 3 mm 5 μm) with eluent A [water/aqueous formic acid/acetonitrile (87:10:3 v/v)] and eluent B [water/formic acid/acetonitrile (40:10:50 v:v)] as the mobile phase at a flow rate of 1 mL min^{-1} at 25 °C. A Dionex HPLC system (Dionex Corp., Sunnyvale, CA) consisting of a P580 pump, an ASI-100 automated sample injector, and a PDA-100 photodiode array detector was used with the following operating conditions: injection volume, 10 μL; column temperature, 25 °C; detection wavelengths, 512, 500, 330, 254 nm. The mobile phase was a degassed mixture of A and B under multistep conditions: 6% B from 00 to 01 min, 6−20% B from 1 to 25 min, 20−100% B from 25 to 31 min, 100−6% B from 31to 38 min, continuing at the initial conditions for the next 2 min before the next injection.

Samples treated with pectinase P5146 and pectinase P4716 were further analyzed with HPLC-MS. The MS spectra were acquired with an Agilent Ion Trap SL with electrospray ionization with direct infusion (flow = 5 μL min^{-1}) as well as coupled to the Dionex HPLC-UVD system (flow = 1 mL min^{-1}). Degradation products were measured in positive ion mode, and the electrospray voltage was set to +3500 V, respectively. Dry gas (5 L min^{-1}) temperature was set to 350 °C and nebulizer to 15 psi. Maximal accumulation time was fixed to 300 ms, and the loading of the trap was controlled by the instrument with an ion count current (ICC) of 30000.

Cyanidin and kuromanin as reference compounds as well as retention times and UV−vis spectra provided by HPLC-DAD were used to verify the identity of the degradation products. All other compounds were for the time being identified on the basis of software library spectra.

Activity Gel. Enzyme samples were desalted by first passing the enzymes through a HiTrap 1.6/2.5 desalting column (Amersham-Pharmacia Biotech) connected to an ÄKTA system using 100 mM citrate buffer, pH 4.0. Polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (*27*), with some modifications; that is, 2-mercaptoethanol (β-mercaptoethanol) was omitted for the sample buffer, and the samples were heated only up to 40 °C for 5 min.

Ready Gel Tris-HCl gels, 4−15% linear (Bio-Rad), were used, and broad-range molecular weight markers from Pharmacia were used as standards. The specific glycosidase active bands were detected after incubation of the gel in 100 mM sodium citrate buffer, pH 4.0, for 30 min and reaction with cyanin (0.2 mg/mL in sodium citrate buffer, pH 4.0). By decreasing the pH with concentrated HCl the specific glycosidase active bands were identified due to the disappearance of the color.

Protein Digestion. Total proteins were stained using Coomassie blue G-250 (CBB) (Roth, Karlsruhe, Germany). To characterize the enzyme, protein bands of interest were cut from polyacrylamide gels and digested overnight using trypsin (Sigma) as described elsewhere (*28, 29*). The resulting peptides were eluted, concentrated by vacuum centrifugation, and separated by RP nano-LC (LC1100 series, Agilent Technologies, Palo Alto, CA; column, Zorbax 300SB-C18, 3.5 μm, 150 mm × 0.075 mm; eluate, 0.1% formic acid in 0−60% acetonitrile). The mobile phase was a degassed mixture of eluent A [water/acetonitrile/formic acid (96.09: 3: 0.01)] and eluent B [acetonitrile/water/formic acid (89.09: 10 0.01)] under multistep conditions: 1% B from 00 to 05 min, 1−60% B from 5 to 75 min, 60% B from 75 to 90 min, 90 min continuing at the initial conditions, 1% B, before the next injection (flow = 300 nL/min). The peptides were analyzed by online MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies).

Thereafter, a database search was conducted using the MS/MS ion search (MASCOT, http://www.matrixscience.com) against all entries of NCBInr (GenBank; http://www.ncbi.nlm.nih.gov/index.html) with subsequent parameters: trypsin digestion; up to one missed cleavage site; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide total, ±1.2 Da; MS/MS total, ± 0.6 Da; peptide charge, +1, +2, and +3.

RESULTS

The activity profile of the enzyme preparations is shown in **Table 2**. High β-glucosidase activity was found in the pectinase preparations P5146 and P4716, whereas pectinase P4300, the polygalacturonase from *S. rolfsii*, and the pectate lyases showed almost no β-glucosidase activity.

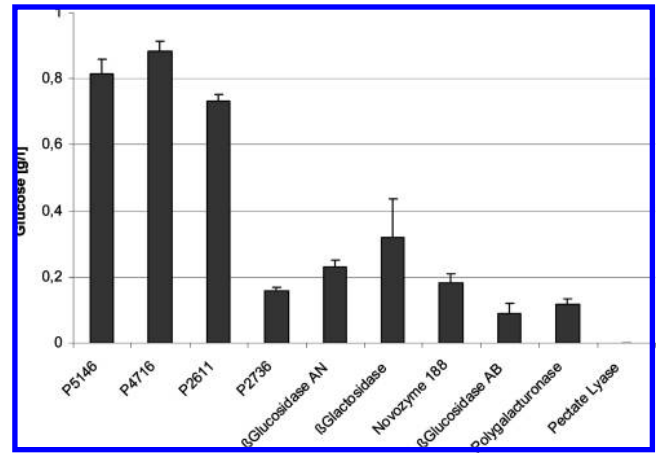


Figure 1. Release of glucose from cyanin using pectinases (crude enzyme mixtures) and pure β-glucosidases, pure β-galactosidase, pure pectate lyases, and pure polygalacturonases. All enzymes were dosed on the basis of 10 U of β-glucosidase activity.

None of the enzymes caused a change in absorbance at 420 nm when incubated with (+)-catechin. Thus, there was no browning due to quinine forming, suggesting that none of the enzymes contained phenol oxidase activity.

When dosed at the same β-glucosidase activity of 10 U, the highest activity for the hydrolysis of cyanin into cyanidin and glucose was found for pectinase P5146 and pectinase P4716. Only a low release of glucose was measured after incubation with pure β-glucosidase, β-galactosidases, pectate lyases, and the pure polygalacturonase from *S. rolfsii* (**Figure 1**).

Model Substrates. Differences between nonenzymatic (control) and enzyme treatments in the anthocyanin content clearly showed a degradation of anthocyanins through enzyme treatment. Only the crude enzyme preparations were able to hydrolyze cyanin to a certain degree within 1 h of incubation, whereas the pure β-glycosidases, the pure pectate lyases, and the pure polygalacturonases did not show activity. Variations were observed in the degradation of the pure anthocyanins with different pectinases. The yield of degradation increased with increasing incubation time and increasing enzyme concentration (data not shown). Only pectinase P5146 and pectinase P4716 completely hydrolyzed the model substrates cyanin and peonin in <20 min. The resulting aglycones cyanidin and peonidin were identified by using HPLC. The amounts of these aglycones decreased with time due to further degradation (data not shown). There was no significant influence of the chemical structure of the aglycone on degradation rates. Consequently, peonin, cyanin, and even quercetin 3-β-D-glucoside were degraded with similar velocities by pectinase P5146 and pectinase P4716. However, when the sugar residue was changed to a rutinose, using the model substrate keracyanin, the degradation rate decreased significantly (**Figure 2**). None of the tested enzymes were able to degrade quercetin aglycone (data not shown).

Degradation of Elderberry Juice. According to UV−vis spectra, retention times, and literature data (*26, 30*), elderberry juice contained four anthocyanins, which were identified in order of elution as cy-3-sambubioside-5-glucoside (rt 7.2 min), cy-3,5-diglucoside (rt 7.7 min), cyanidin-3-sambubioside (rt 11.33 min), and cyanidin-3-glucoside (rt 11.89 min).

Incubations with pectinase P5146 solutions were followed spectrophotometrically; the degradation of anthocyanins was accompanied by a complete color loss. According to HPLC

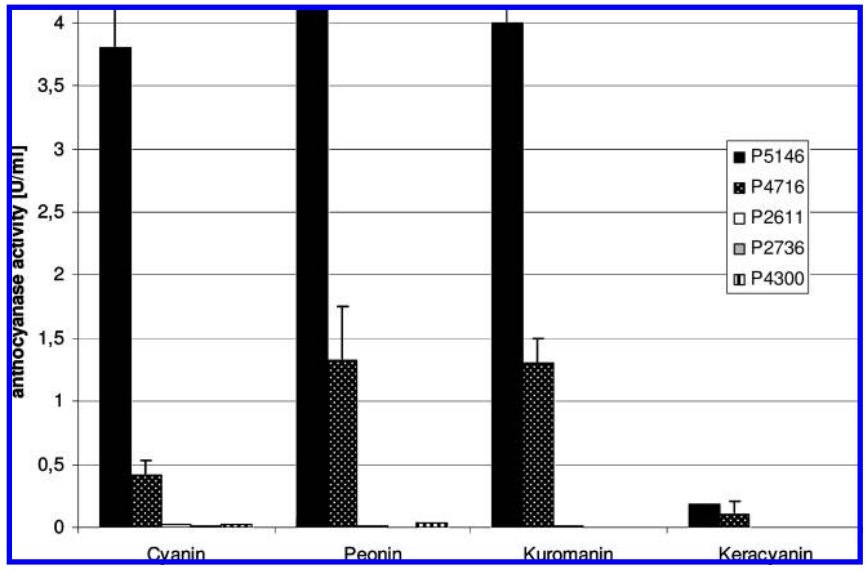


Figure 2. Treatment of model anthocyanins with pectinases.

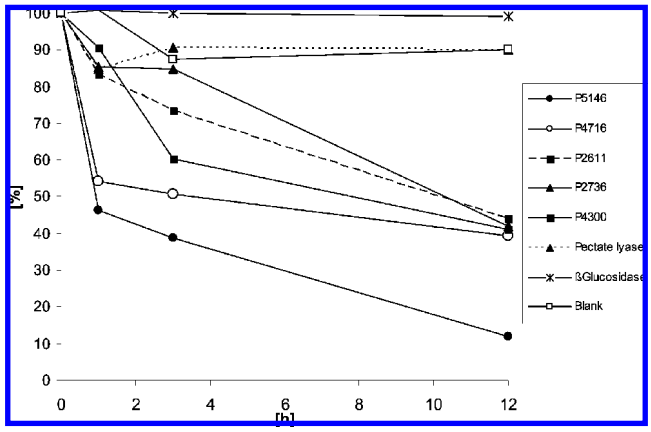


Figure 3. Enzymatic reduction of the total anthocyanin content in elderberry juice by pectinase preparations, pectate lyase from *Aspergillus* sp., and β-glucosidase from *Agrobacterium* sp. All enzymes were dosed on the basis of 15 U of β-glucosidase activity.

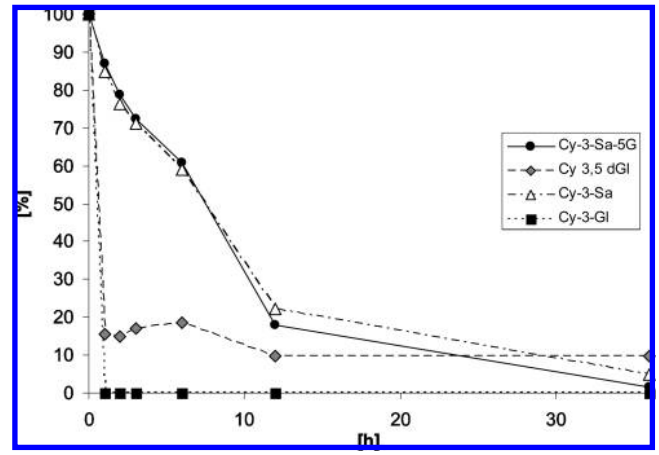


Figure 4. Treatment of anthocyanins from elderberry juice with pectinase P5146 from *Aspergillus niger*.

analysis, the enzyme treatments reduced the total anthocyanin content of elderberry juice to a distinct extent within 12 h (**Figure 3**), although enzymes were dosed with the same β-glucosidase activity of 15 U. The different anthocyanins were not degraded in a similar velocity. Anthocyanins with glucose as the sugar moiety were degraded, whereas the anthocyanins with sambioside residues were almost resistant to enzymatic attack. Controls did not show significant changes in proportions of the individual anthocyanins. Only one enzyme preparation, namely, P5146, caused a significant decrease in all monomeric anthocyanins, as compared to control treatment (**Figure 4**).

Degradation Mechanism. According to LC-MS analysis, pectinase sample P5146 cleaved glucose of the anthocyanin to form kuromanin and glucose, followed by a second hydrolysis step to form cyanidin, which was then transformed to the colorless chalcone form and further degradation products (**Figures 5** and **6**).

Protein Digestion. Activity gels were found to be a suitable tool to detect specific glycosidase activity in enzyme preparations (**Figure 7**). Band intensity decreased over time. Sharp white bands in the gel stained with cyanin indicated specific glycosidase activity. The corresponding protein bands in gels stained with Coomassie blue were cut out and digested. When the specific glycosidase in pectinase sample of P5146 was analyzed, a mass of 93646 with a score of 884 could be found. Twenty-three peptides matched to conserved regions of a β-glucosidase from *Aspergillus kawachii*. The sample of pectinase P4716 had a mass 93687 and a score of 749; 17 peptides indicated as well a β-glucosidase from *A. kawachii*. The "Probability based Mowse Score" was identified with Mascot. Values against NCBI and individual ions scores of >57 indicate identity or extensive homology.

DISCUSSION

Attractive color is one of the most important sensory characteristics of fruit and berry products. However, anthocyanins are unstable plant pigments and susceptible to degradation via chemical and enzymatic processes (*19*, *31*). On the other hand, enzymes such as pectinases are routinely used in fruit processing. Commercial pectic enzyme preparations were found to cause color changes when incubated with juices, that is, elderberry juice. However, until now this activity was not assigned to specific enzymes. Here we demonstrate that this is due to anthocyanin-specific glycosidase side activities contained in commercial pectinase preparations.

Wightman and Wrolstadt (*13*) found that enzyme preparations with high β-glucosidase activity measured by the *p*-nitrophenyl-glucopyranoside did not necessarily show anthocyanin-degrading

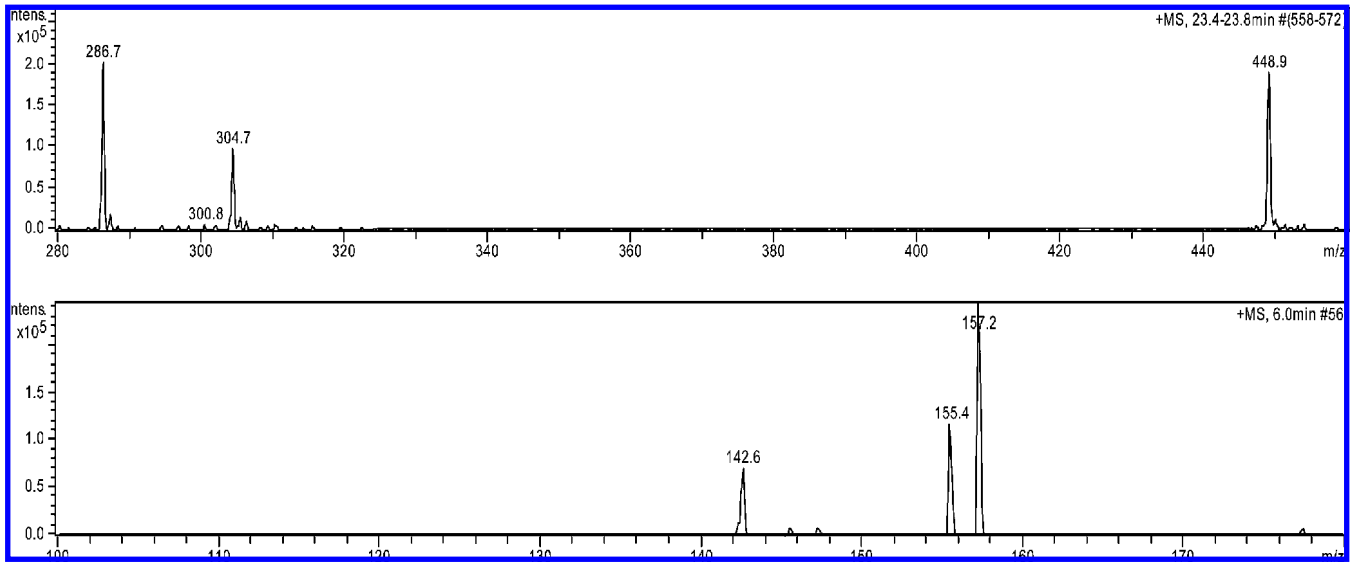


Figure 5. Mass spectrometry data (after HPLC separation) of cyanin incubated with *Aspergillus niger* pectinase P5164 for 2 h.

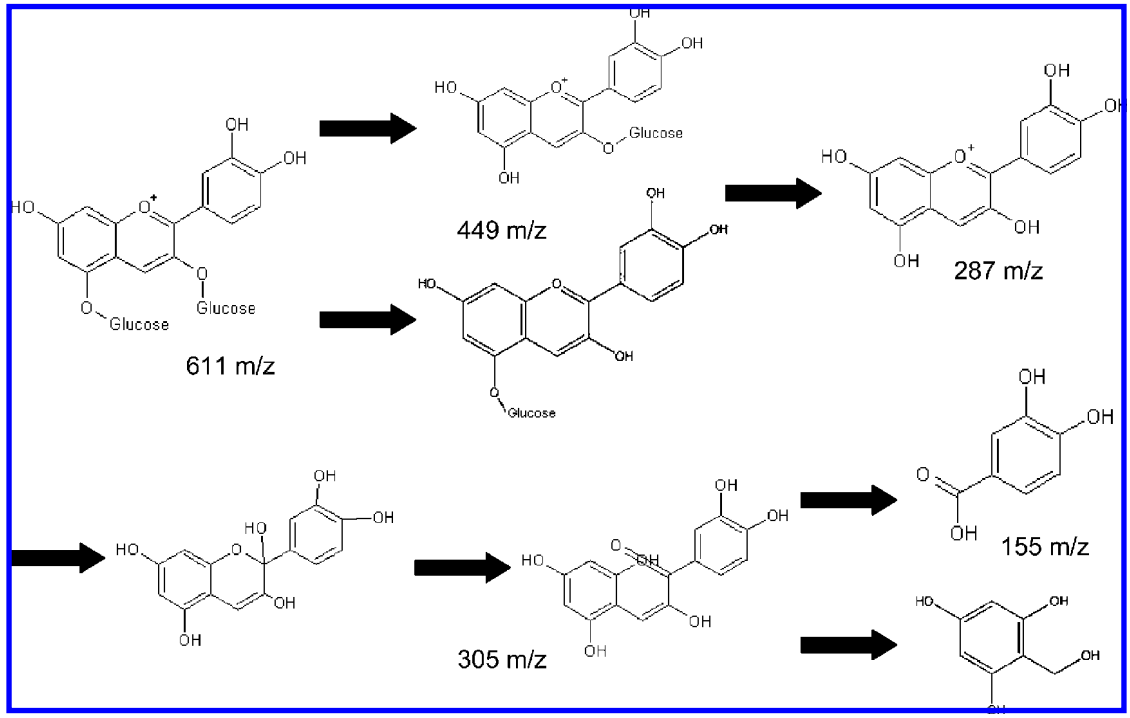


Figure 6. Hypothetical degradation pathway of cyanin with pectinase P5146 from *Aspergillus niger.*

activity. The present results support this fact; here we show that various commercial pectinases, dosed on the basis of the same β-glucosidase activity, did not have similar hydrolysis activity on cyanin to cyanidin as determined by HPLC and via the amount of glucose released. Pure β-glucosidases from *Agrobacterium* sp. and from *A. niger* did not have any significant specific glycosidase activity. Pectinase P5146 and pectinase P4716 were able to decolorize cyanin significantly more rapidly than the other pectinase preparations tested in this study.

To investigate the influence of the structure of the aglycones and/or the type of sugar attached, enzymatic hydrolysis with pectinase P5146 and pectinase P4716 of structurally different anthocyanins was carried out. Interestingly, there was no big difference in the degradation rate between the conversion of monoglucosidic or diglucosidic cyanidin or peonin. Both pectinase preparations P5146 and P4716 hydrolyzed the β-glycosidic bond to form the aglycone. Surprisingly, all of the β-glucosidases tested converted the anthocyanins only at a much reduced extent. Like Keppler and Humpf (*32*), we observed a decrease in degradation rate when rutinose was attached to cyanidin. Thus, it can be concluded that the kind of sugar moiety is the restrictive part in the enzymatic degradation of anthocyanins and that the structure of the aglycone apparently has almost no influence. On the other hand, the presence of the anthocyane aglycon type disturbs the action of the pure β-glucosidases, suggesting the existence two major enzymes types with different substrate specificities.

Elderberry juice has been reported to contain significant quantities of four cyanidin glycosides. They have three types of linkages: β-glucosides, β-1,2-glucoside, and sambioside (*26*). Therefore, it is a good model system to test enzyme preparations not only for glycosidase but also for sambiosidase activity. The results indicate that the glucoside moieties are not equally accessible to the action

Figure 7. Activity gel of pectinases P5146 and P4716 from *Aspergillus niger* stained with cyanin.

of the different enzymes. This is probably due to the steric hindrance because of the bulkier sambubioside.

Only pectinase sample P5146 was able to degrade anthocyanins with sugar moieties other than glucose even though the degradation rate decreased. This supports the fact that some glycosidases are more specific than others or that in sample P5146 there are several glycosidases present specific to different anthocyanins. The mechanism of enzymatic hydrolysis of cyanin was investigated using HPLC-MS.

In the literature two major pathways for the degradation of anthocyanins have been described (*33*). The first pathway proceeds through the carbinol pseudobase to give the chalcone and coumarin glycosides. The second route involves hydrolysis of the glycosidic bond as the first step in anthocyanin degradation to form the anthocyanidin. The aglycon, which is less stable than its glycoside, is converted to a highly unstable α-diketone intermediate to finally form aldehydes and benzoic acid derivates. We have observed the latter route of anthocyanidin degradation in our studies. The measured degradation products confirmed that the overall process of the degradation of cyanin involves first the hydrolytic removal of glycoside substituents by enzymes to yield the unstable cyanidin and then, second, a ring opening, formation of the chalcone, and finally degradation to phenolic acids. Like Seeram et al. (*10*), we identified the final degradation products as protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid. These compounds are plausible degradation fragments of flavylium cation. Degradation products such as aglycones and phenolic acids have been confirmed by comparing their retention times to authentic standards and/or by determination of their molecular masses.

These compounds were also detected when pure cyanidin aglycone was incubated with pectinase preparations under the same conditions, but were undetectable in the control and in the β-glucosidase samples studied. We also noted that the degradation rate of pure cyanidin aglycone was slower than that of the aglycone produced from cyanin via enzymatic hydrolysis. This agrees with the observation published by Piffaut et al. (*36*).

The result that commercial pectolytic enzymes are responsible for a loss of anthocyanins supports the observation in previous studies on raspberry, bilberry, black currant, cranberry, asparagus, strawberry juices, and cherry nectars (*10, 11, 20, 31, 34*−*37*). It is still poorly understood which enzyme is responsible for the color loss because pure β-glucosidase showed only weak activity. It would be reasonable to assume that the impurity in the pectinase sample, which is responsible for decolorization, is an anthocyanin-β-glucosidase. Already various papers have described β-glucosidases with degradation mechanisms similar to those in our studies (*4, 13, 20*), although the pure β-glucosidase of *Agrobacterium* sp. and from *A. niger* and the β-glucosidase N188 from *A. niger* we tested did not show a significant conversion of the model substrates.

To identify the enzyme that degrades anthocyanins in pectinase samples P5146 and P4716, we used a combination of gel electrophoresis and highly sensitive nano-LC-MS. We observed only one sharp white band in the gel stained with anthocyanin, indicating that there is only one enzyme that has specific glycosidase activity in the crude enzyme preparations.

The proteins of interest were tryptically digested, and an aliquot of the obtained peptide mixture was analyzed by mass spectrometric technique. The obtained peptide mass fingerprints were subsequently compared to "virtual" fingerprints stored in databases. Surprisingly, specific glycosidases from the pectinase preparations P5146 and P4716 and an enzyme preparation from *A. niger* showed close homology to a β-glucosidase from *A. kawachii*, although pure β-glucosidase from *A. niger* did not show significant specific glycosidase activity. We conclude that this indicates the presence of highly specific anthocyanin-β-glucosidase in the tested pectinase preparations. Bifunctional glucosidases have been reported (*38*), whereas the β-glucosidase of *A. kawachii* shows close similarities to a tannase of *A. niger* (*39*). Summarizing our results, we clearly demonstrated that specific glycosidases are present as side activities in commercial pectinase preparations and that this activity does not result from β-glucosidase impurities. These results combined with a more detailed biochemical characterization of specific glycosidases should allow screening for glycosidase-free pectinase producing organisms.

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Received for review May 28, 2008. Revised manuscript received December 15, 2008. Accepted December 15, 2008.

JF803131T