AN ABSTRACT OF THE THESIS OF

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A screening procedure combining HPLC and spectrophotometric analyses was developed to measure glycosidase activity, a side-activity that can destroy anthocyanins, in commercial pectolytic enzyme preparations used for juice and wine processing. Enzyme preparations were evaluated for ß-galactosidase and α-arabinosidase activities using cranberry juice as a substrate; and ß-glucosidase, ß-1,2-glucosidase and α-rhamnosidase, utilizing boysenberry juice. With cranberry juice, 24 out of 27 preparations exhibited significant ß-galactosidase activity which destroyed cyanidin-3-galactoside, the major pigment. No measurable amount of α-arabinosidase activity was detected. Cranberry juice processing demonstrated that some enzyme preparations could decompose anthocyanin pigments under processing conditions. Pigment losses were much greater when enzymes were used with juice than with crushed fruit. In the boysenberry juice system, enzyme preparations (26) were evaluated at two dosage rates. At the mean recommended dosage, one preparation produced a significant decrease in total monomeric anthocyanin and cyanidin-3-glucoside, relative to a control, indicating ß-glucosidase activity. At 0.1% dosage, four enzymes produced a significant decrease in cyanidin-3-sophoroside and two enzymes produced an increase in cyanidin-3-rutinoside, indicating ß-1,2-glucosidase activity. However, ß-glucosidase activity in juice processing enzymes was much lower and less prevalent than that found for ß-galactosidase. ß-galactosidase,

α-arabinosidase and ß-glucosidase activities were also determined in the preparations using standard procedures (nitrophenol-glycosides as substrates). Comparative results showed inconsistencies between the two procedures.

Pinot noir and Cabernet Sauvignon wines were made with commercial enzyme preparations along with controls. Total monomeric anthocyanin and individual pigments changes were monitored throughout winemaking and storage using spectrophotometric and HPLC methods. Three of the enzyme treated Pinot noir wines contained significantly less total monomeric anthocyanin than the control and a reduction in the major pigment, malvidin-3-glucoside. Although pigment changes were less pronounced in the Cabernet Sauvignon wines as compared to Pinot noir, two enzyme-treated Cabernet Sauvignon wines also showed significant degradation of malvidin-3-glucoside, 3-glucosylacetate and 3-glucosylcoumarate, in comparison to the control. Acylation did not appear to inhibit degradation of the anthocyanins. Preparations that resulted in the least amount of total monomeric anthocyanins also produced wines with lesser concentrations of quercetin glucuronide and higher quercetin aglycon, a larger ratio of caffeic to caftaric acid, and greater concentrations of *trans*-resveratrol.

Use of Anthocyanin Analyses to Determine Glycosidase Activity in Juice- and Wine-Processing Enzymes

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To Paulo Petry

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Dr. R.E. Wrolstad was involved in the experimental design, data analysis and writing of each chapter. Winemaking was done in the enology laboratory of B.T. Watson who was also involved in the experimental design of chapter 5. Dr. S.L. Price assisted in data collection, interpretation of data of chapter 5.

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CHAPTER 1. INTRODUCTION

Use of processing enzymes in fruit juice production has increased steadily since the introduction of commercial fungal pectinase enzymes in the early 1930's. While the first enzymes aided in clarification of juices which had previously been difficult to filter, later applications involved reducing juice viscosity to permit concentration to high Brix levels and pressing of soft fruits such as berries, ripe pears and cold-storage apples. More recently liquefaction enzymes have appeared on the market. These preparations include combinations of pectinases, pectic lyases, cellulases, and hemicellulases which digest cell walls, resulting in increased yields and Brix. In addition to these applications, pectic enzymes are also used to increase extraction of other components such as pigments, flavor and aroma compounds. One possible negative side effect with use of commercial enzymes results from the fact that they are typically derived from crude fungal enzyme preparations. Therefore, they may contain other compounds such as extraneous enzymes and proteins which may have deleterious effects on the quality of the final product.

The justification and need for this investigation became evident in 1990. Dr. Wrolstad's laboratory, which analyzes fruit juices for authenticity and quality, received a commercial cranberry juice concentrate which had an unusual pigment profile. It was depleted in ß–galactosides, although non-volatile acid and sugar profiles were typical of authentic cranberry juice. Since cranberries show a very consistent anthocyanin pattern with cyanidin and peonidin –3–galactoside and –3–arabinoside as the major pigments (Wrolstad et al., 1995), the sample was interpreted as being authentic but that the ß–galactoside pigments were degraded, possibly by exogenous enzymes used in processing. Dr. Wrolstad was subsequently informed that commercial enzymes used in processing had caused the pigment destruction. About this same time Jiang et al. (1990)

published a short communication reporting a commercial enzyme preparation which converted cyanidin–3–sophoroside to cyanidin–3–glucoside and cyanidin–3–glucosyl-rutinoside into cyanidin–3–rutinoside. These two cases showed that commercial enzyme preparations used in juice processing can alter anthocyanins. This is of major importance to juice color quality as well as to authenticity interpretations. These examples led to the concept that anthocyanin pigment analysis via high performance liquid chromatography (HPLC) might be a very useful method for measuring glycosidase activities of commercial enzyme preparations. Thus, the objectives for this research were to: devise an analytical method to measure glycosidase activity in commercial enzyme preparations, compare specific activities in preparations measured by the new method with standard colorimetric procedures, determine if pigment destruction occurred on a pilot plant scale under industry-recognized conditions, and to examine acylation of anthocyanins to determine if this had an inhibitory effect on enzymatic destruction of the pigments.

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CHAPTER 2. LITERATURE REVIEW

ENZYME USE IN JUICE AND WINE PROCESSING

Fruit Juice

Enzymes have been used in the fruit juice industry since the early 1930's, when the first pectolytic enzymes were introduced for clarification of apple juice by Kertesz (1930) and Willaman and Kertesz (1931) in the United States and by Mehlitz (1930) in Germany. Over the last 30 years juice enzyme technology has become quite sophisticated with the introduction of pectolytic preparations with specialized activities, clarifying and macerating enzymes, and juice liquefaction preparations. These enzymes are typically used in the treatment of crushed pulp or fruit and /or in the treatment of juice. General enzymes used in the fruit and citrus juice industries and their mode of action are listed in Table 2.1.

Clarification

The oldest and still the largest single use of commercial pectinases is in apple juice clarification (Rombouts and Pilnik, 1978). Endo (1965) and Yamasaki et al. (1967) thoroughly studied the mechanisms of apple juice clarification and found both pectin-esterase and endo-polygalacturonase were necessary to degrade the highly esterified pectin dissolved in apple juice as well as the pectin coating of the cloud particles. Cloud particles are positively charged protein complexes which are coated by negatively charged pectin. Pectinases remove or disrupt the pectin coating on the cloud particles thereby exposing the positively charged nuclei. The particles coagulate due to the reduction in electrostatic repulsion and precipitate out of solution (Endo, 1965; Yamasaki et al., 1967; Voragen and van den Broek, 1991). Other methods for cloud reduction include use of flocculating agents (usually gelatin or bentonite) or ultrafiltration with the latter also removing

Table 2.1 Enzymes used in the fruit juice and citrus juice industry[a]

Enzyme	Classification	Catalyzed reaction	Mechanism	Function
Pectinesterase (PE)	EC 3.1.1.11	Hydrolysis of ester bond	*Exo*-mechanism Random mechanism	Self-clarification of juices or with endo-PG
		Hydrolysis of α-1,4 bonds:		
*Endo*polymethylgalacturonase(*endo*-PMG)	EC 3.2.1.41	of pectin	Random mechanism	Pulp treatment before juice pressing
*Endo*polygalacturonase (*endo*-PG)	EC 3.2.1.15	of pectic acid	Random Mechanism	Juice clarification (i.e. apple) fruit/vegetable maceration, reduction in viscosity of juices to be concentrated
*Exo*polymethylgalacturase	EC (unclassified)	of pectin	*Exo*-mechanism	
*Exo*polygalacturonase (*exo*-PG)	EC 3.2.1.40	of pectic acid	*Exo*-mechanism	Pre-pressing pulp treatment
		Trans-elimination of α–1,4 bonds:		
*Endo*pectinlyase (*endo*-PL)	EC 4.2.2.10	of pectin	Random mechanism	Self-clarification of juices or with endo-PG/PE mixture
*Endo*polygalacturonatelyase (*endo*-PAL)	EC 4.2.2.2	of pectic acid	Random mechanism	Maceration of vegetables
*Exo*pectinlyase (*exo*-PL)	EC (unclassified)	of pectin	*Exo*-mechanism	
*Exo*polygalacturonatelyase (*exo*-PAL)	EC 4.2.2.9	of pectic acid	*Exo*-mechanism	
		Hydrolysis of ß-1,4 bonds:		
Endo-ß-(cellulase CX) glucanase	EC 3.2.1.4	of cellulose	Random mechanism	Pulp treatment before pressing and prediffusion
Exo-ß-(cellulase C1) glucanase	EC 3.2.1.91	of cellulose	*Exo*-mechanism	Total fluidification of fruits and vegetables, production of nectar concentrates
*Endo*galactanase	EC 3.2.1.89	of galactans	Random mechanism	elimination of sediments in clear concentrated juices
		Hydrolysis of α-1,5 bonds:		
*Endo*arabinase	EC (unclassified)	of arabinans	Random mechanism	Elimination of sediment in clear concentrated juices

Table 2.1 Continued

Enzyme	Classification	Catalzyed reaction	Mechanism	Function
		Hydrolysis of α-1,4 bonds:		
α-amylase	EC 3.2.1.1	of starch	Random mechnaism	Prevention of starch sedimentation in clear apple juice
ß-amylase	EC 3.2.1.2	of starch	*Exo*-mechanism	
Proteinase	-	Hydrolysis of proteins to peptides and aminoacids	-	Pre-pressing fruit pulp treatment, production of homogenized tropical fruits
Glucose-oxidase	EC 1.1.3.4	Oxidation of ß-glucose to gluconolactone	Redox with O_2 consumption	Anti-oxidant action in juices
α-L-rhamnooxidase	EC 3.2.1.40	Hydrolysis of naringin to prunin	*Exo*-mechanism	Reduction of bitter taste of grapefruit
ß-D-glucosidase	EC 3.2.1.21	Hydrolysis of prunin to naringenin		

[a] modification of Lanzarini and Pifferi, 1989

contaminating microorganisms. Ishii and Yokotsuka (1972) found that a single enzyme, pectin lyase, could also accomplish the same process as pectinesterase and endopolygalacturonase. Depectinization of apple juice is a prerequisite when it is going to be concentrated to a high soluble solids content without gelling, or developing haze or turbidity. Even with pectinase use, haze may develop in concentrates due to starch retrogradation; this may occur most noticeably in unripe apples which contain starch. To overcome this problem, amylase has been added to many pectinases which are recommended for apple juice clarification (Rombouts and Pilnik, 1978).

In addition to apple juice, the juices of many other deciduous fruits, such as berries, and grapes have traditionally been consumed as clear juices. Enzymes are added directly to juice to lower viscosity, to aid in clarification for clear juices to be concentrated, to increase filtering speed during finishing treatments of clear juices, to obtain chemico-physical stabilization, prevent precipitates and improve organoleptic features or prevent their alteration from present quality standards (Lanzarini and Pifferi, 1989).

Maceration

Macerating enzymes are used for two different purposes: when the final product is a clarified juice and when the final product is a fruit nectar. Maceration can be subdivided into two stages. First is the limited degradation of soluble pectin and middle lamella pectin by endopolygalacturonase and pectin lyase thus leading to tissue disintegration and formation of a cell suspension with a certain residual viscosity (Voragen and van den Broek, 1991). This is the basis of the maceration process. The next step is the extensive degradation of soluble, middle lamella and cell wall pectins by endopolygalacturonase and pectin esterase and/or pectin lyase which renders the gelled juice into a thin liquid. However, part of the cell wall remains and serves as a pressing aid to facilitate release of juice (Voragen and van den Broek, 1991). This is the basis of the pulp enzyming and juice

clarification. Adding macerating enzymes to crushed fruit is done to: increase juice yield, reduce processing time, improve extraction of particular compounds such as aroma and color, and to obtain partial or total liquefaction of the plant tissue (Lanzarini and Pifferi, 1989). These tasks are accomplished by degrading structural polysaccharides as well as skin tissue (Voragen and van den Broek, 1991).

Since the pulp of soft fruits, such as black currants (Koch, 1955), strawberries (Walker et al., 1954), raspberries, blueberries (Fuleki and Hope, 1964), prunes (Walker and Patterson, 1954) and plums (Vilenskaya, 1963) is not easy to press, use of commercial pectinase preparations has been common practice for a few decades. These fruits contain few pectolytic enzymes of their own, and thus the use of pectinase is necessary to facilitate pressing and to ensure high yields of juice and colored materials. These types of fruit also have high pectin contents and give very viscous juices. The juice may adhere to the pulp particles as a semi-gelled mass upon mechanical pulping, thus making it extremely difficult to extract the juice. When pectinases are added the gel structure collapses, the juice viscosity decreases and juice can be obtained easily and in high yields. At the same time the structure of the tissue is degraded and this allows the pigments, which are often enclosed in the skins of berries, to diffuse into the juice so that color extraction is also greatly enhanced. The amount of enzyme required for extraction varies directly with the pectin content in the fruit. Moreover in such fruits as berries, the high acid content and therefore low pH requires enzymes which have stable activity at low pH.

Enzymatic juice extraction has also become an established process in the production of apple juice. Some varieties of apples are harder to press than others (e.g. Golden Delicious) and prolonged storage increases the difficulty of processing, therefore use of macerating enzymes makes obtaining high yields possible (Lanzarini and Pifferi, 1989). Hemicellulases, more specifically endoarabinase, may also be added to crushed apple pulp in conjunction with pectinases to destroy haze polymers as soon as they are insolubilized.

The production of fruit nectars (comminuted fruit juices) requires enzymes that function differently than for juice clarification. Nectars are pulpy fruit drinks prepared from a variety of fruits such as pears, peaches, apricots, berries, guava, papaya and passion fruit, and which contain water, sugar and fruit acids (Rombouts and Pilnik, 1978, Voragen et al., 1986). They are usually prepared by grinding the fruits, adding water, acid and sugar and homogenizing. To prevent enzymatic damage of flavor and color consistency and cloud stability, blanching or pasteurization is often utilized. Preparations for this purpose predominately contain endopolygalacturonase, cellulases and proteases and only trace amounts of pectin lyase and pectinesterase. They are used to produce suspensions of loose cells from products like apples, apricots, potatoes, carrots, and tomatoes with the juice showing good cloud stability. This type of enzyme preparation also makes it possible to prepare nectar bases. The pulp is first separated from the juice, the juice is concentrated, and finally the pulp is added back to the concentrated juice. When reconstituted, this type of juice shows a stable cloud (Rombouts and Pilnik, 1978; Lanzarini and Pifferi, 1989).

Enzymatic maceration has some advantages of mechanical and/or thermal maceration in quality of the finished product. The enzymatically produced juice may be a very finely dispersed, cloud-stable product with increased content in soluble solids, pigments and vitamins. Oxidation and off-taste due to heating may also be minimalized.

Liquefaction

Liquefaction enzyme preparations usually contain a combination of cellulolytic, hemicellulytic and pectolytic enzymes (Rombouts and Pilnik, 1978; Voragen and van den Broek, 1991). Their goal is to not only macerate the fruit tissue but also digest the cell walls, thus increasing water and alcohol insoluble solids. Enzymatic liquefaction is used in tropical fruit processing, one reason being that the fruits themselves are often

inconvenient shapes or sizes and there may be no specific juice extraction machinery. Depending on the raw material, almost clear (black currants, sour cherry, passion fruit, papaya), cloudy (mangos, peaches) or pulpy juices (apples, apricots) may be obtained with juice yields of 90% or higher (Rombouts and Pilnik, 1978; Voragen et al., 1986; Voragen and van den Broek, 1991). After enzyme treatment, juices can be separated by centrifugation or filtering, and the use of expensive fruit presses can be avoided. The raw juices can also be clarified by the same methods as mentioned in previous sections. When cellulases are combined with liquefaction pectinases the viscosity of the pulp is drastically reduced during fluidification (Lanzarini and Pifferi, 1989).

Liquefaction enzymes can also be used in the treatment of apple pomace, which is the remaining material after pulped apples are pressed and the juice extracted. Pectinases and both endo- and exo-1,4-glucanases can be added to extract additional juice. However, quality changes must be monitored during this process. Potential quality defects include: changes in flavor due to the release of flavor precursors and flavor-releasing enzymes within the cell walls, increased liability to non-enzymatic browning when exposed to excessive heat treatment or inadequate storage, increased acid content, and increased susceptibility for haze formation (Voragen and van den Broek, 1991). Apple and pear concentrates produced by intensive enzyme mash treatments (such as liquefaction) or excessive heat may result in increased quantities of arabinans in juice. Normally the arabinans are soluble and thus do not form haze, but if appropriate enzymes are present during heating, the side chains from the branched arabinans can be removed. This results in the association of linear arabinan chains in concentrated juices which form insoluble crystals or haze.

Grape Juice and Wine

There are two main purposes for enzymes in grape juice and wine production: 1) enzyme treatment of the juice or wine in order to facilitate clarification, and 2) enzyme treatment of the crushed grapes in order to improve the yield and extraction of other components such as pigments, and flavor and aroma compounds (Felix and Villettaz, 1983). Another more recent use of enzymes is for use in white wines to release the glycosidically-bound flavor precursors. However, use of enzymes in the wine industry, other than pectolytic enzymes, remains extremely limited due to: traditionalism of the wine industry, which for the most part adheres to classical methods; narrow profits in the field cannot bear new technologies which would cause costs to increase; limitations of enzymatic activity connected with the composition of the must and the wine, such as low pH, ethanol concentration, presence of tannins, and fairly low temperatures; low-grade purity of enzyme preparations; and legal restrictions (Zamorani, 1989; Colagrande et al., 1994).

Clarification

The use of pectic enzymes in wine clarification was first suggested in 1941 by Cruess and Besone but the first commercial trial with enzymes was not reported until 1947 (Cruess and Kilbuck). After World War II, more work was done in this area. Initially, enzymes were only added to wines which did not clarify spontaneously by well-known methods such as bentonite and gelatin (Neubeck, 1975). Treatment with pectic enzymes after fermentation is successful, but larger quantities of enzymes and a longer processing time are required than when they are added to the must prior to fermentation, possibly due to alcohol inhibition (Neubeck, 1975). Later work indicated enzymes could be beneficial when added to the juice or must prior to fermentation or when added during or following crush.

Maceration

The amount of pectin within a grape depends on a number of factors: grape variety, degree of maturity, soil type, crop yield, and post harvest handling (Plank and Zent, 1993). In addition to pectin there are a number or other structural components present which would not be degraded by typical pectolytic preparations, such as colloidals composed of polysaccharides or hemicelluloses, proteins and polyphenolics. Therefore, macerating enzymes for grape juice and wine production must contain pectolytic, cellulytic and hemicellulytic activities. Macerating enzymes are used to improve processing, yield, and juice and wine quality.

Processing benefits include improved handling of 'hard to press' grapes, improved lees settling and clarification rates (and thus faster racking), and reduced viscosity for downstream processing (Plank and Zent, 1993). In the United States, extraction of red grape juice from the Concord variety of *Vitis labrusca* has been routinely carried out with enzymes since this fruit has a slimy consistency after crushing (Reed, 1966; Rombouts and Pilnik, 1978). Wine grapes such as Muscat, which have slip-skins, are also difficult to press due to "sliminess" and enzymes are utilized to break down the pectins and hemicelluloses responsible for this phenomenon (Plank and Zent, 1993).

Pectolytic enzyme addition to a 'classical fermentation on skins' has been reported to reduce the lag time prior to fermentation as well as shorten the fermentation time period by some researchers (Felix and Villettaz, 1983), while others have reported enzyme use slowed fermentation (Ough et al., 1975). Adding enzymes to must after crush has also been shown to increase free run juice and press juice, particularly if the must was treated at higher temperatures to favor the extraction of color from the grape skins (Reed, 1966). While the increase in free run was reported by many authors, their findings varied widely, from a 10% increase with Concord grapes (Yang and Wiegand, 1950) to 0% increase in white Riesling, French Colombard, and Muscat grapes (Berg and Akiyoshi, 1962) and

Semillon grapes (Blouin and Barthe, 1963). An increase in yield of 10% in free run translates to an increase of approximately 16 gallons of juice per ton of grapes in the usual winery operation (Neubeck, 1975). Although various researchers reported an increase in free run, the increase in total must or wine yield (free run + pressed material) was less dramatic (Neubeck, 1975). In conjunction with greater juice yield, musts produced with enzymes yields a larger quantity of dry lees, which confirms the enzyme intervention on the colloid complex compounds bound to cell structures (Zamorani, 1989). Commercial pectolytic and macerating enzyme preparations have been reported to promote color extraction in the processing of red grapes or wine as well (Cruess et al., 1955; Ough and Berg, 1974; Ough et al., 1975; Shoseyov et al., 1990; Zent and Inama, 1992; Plank and Zent, 1993). Haight and Gump (1994) reported "perceived differences" in color extraction when comparing Rubired grape juice made from commercial enzymes versus a control with no enzyme, enzyme-treated juices were higher, but no statistically significant difference could be proven.

After a review of literature concerning the advantages and disadvantages of hydrolytic enzymes (especially pectolytic) in wine making, Zamorani (1989) suggested that these enzymes should only be used on must and pressed grapes when the advantage derived from must fluidification is greater than the probable disadvantages in the color and aromatic complex. He also stated that these preparations should be used only when undesirable changes can be prevented by suitable operations such as ready centrifugation, use of low temperatures and sulphur dioxide. Therefore, in selecting the proper enzyme preparation, one must consider parameters such as the type of wine that is being produced, processing conditions, economic considerations and the desired outcome (Plank and Zent, 1993).

Release of Aroma and Flavor Precursors

In early studies with commercial pectic enzymes, researchers claimed an increase in the fruity or aromatic aroma of the wine (Cruess and Kilbuck, 1947; Yang and Wiegand, 1950; Cruess et al., 1955; Hennig, 1956; Endo, 1961), but there was no understanding of the mechanisms involved to support the claims. Complex reactions involved in red wine color fading and white wine browning could have had a significant effect on flavor as well as modification of the ester profile. As early as 1969, Luthi and Jakob reported that gas chromatograms of volatile substances recovered from apple juice treated with a high level of commercial pectinase showed a marked change in ester profile as compared with that of the untreated apple juice. In the mid-1980's research on wine flavor development finally elucidated the reaction mechanisms involved in flavor development and aroma modification (Plank and Zent, 1993) and a number of reviews have been published on this subject (Williams et al., 1989,1992; Williams, 1993). Recently in studies involving glycosidically-bound flavor and aroma compounds, researchers have used commercial pectinases but at very high dosage rates. Work in this area has been reported with wine grapes (Gunata et al., 1988; Williams et al., 1989; Llaubères, 1990; Francis et al., 1992; Caldini et al., 1994; Naiker and Cabalda-Crane, 1994; Sefton et al., 1994).

Fruit juice processors have also observed flavor increases while using pectolytic enzymes during processing. Advances in flavor chemistry have determined that many of the non-volatile flavor precursors are predominately glycosides. Fruits in which glycosidic precursors have been identified include: apples (Schwab and Schreier, 1988; Roberts et al., 1993); apricots (Salles et al., 1991; Crouzet, 1993); blackberry (Humpf and Schreier, 1991); lulo fruit (*Solanum vestissmum*) (Suárez et al., 1991); passion fruit (Shoseyov et al., 1990); pineapple (Wu et al., 1991); various *Prunus* species (Krammer et al., 1991); quince fruit (Winterhalter et al., 1991); raspberry (Pabst et al., 1991); sour cherries (Schwab et al., 1990); strawberries (Sanz et al., 1993); and tomato (Marlatt et al., 1992).

Citrus Juice

Enzymes have been used in the citrus industry for over 30 years (see review article by Rombouts and Pilnik, 1978). There are two main types of enzymes used in the citrus juice industry: pectinases and enzymes used in debittering. Currently, Florida regulations do not permit the use of commercial enzymes in orange juice processing, but processors in other regions may legally add pectolytic and macerating enzymes during production (Wrolstad et al., 1994). Another enzyme that is used in the citrus industry is glucose oxidase which oxidizes glucose to gluconic acid with oxygen consumption. This enzyme has been proposed for the stabilization of odor and color of juices due to oxygen elimination, reduction of glucose susceptible to the Maillard reaction, and the prevention of oxidation of limonene from which off-flavors would develop (Lanzarini and Pifferi, 1989).

Depectinization

There are four main applications of pectinases in the citrus industry: 1) in the preparation of natural cloudifying agents, 2) in pulp wash recovery, 3) to lower the viscosity of orange juice, and 4) for the clarification of lemon juice (Janda, 1983). Commercial pectinases may be added to juice to aid in cloud preservation (Mizarhi and Berk, 1970; Baker and Bruenner, 1972). This effect is caused by a rapid depolymerization of the low methoxyl pectin or pectate so that calcium pectate coagulation can not occur (Rombouts and Pilnik, 1978). Pectinases are used to aid in the recovery of natural colorings (carotenoids) and cloudifying agents from the citrus peels. In this application peels may be boiled to soften the tissue, ground and mixed with pectinase. After incubation the material can be pressed and the cloudy liquid centrifuged, concentrated and pasteurized (Wieland, 1972). In pulp wash recovery, pectinases may be added to increase amounts of soluble solids and reduce viscosity so that a concentrate of up to 65-70°Brix

can be obtained without risk of gelation, as well as allowing most of the juice from finisher pulp to be released by pressing. For this application the pectinases used must not contain pectinesterases. Concentrating orange juice is facilitated by adding pectinase, which lowers the viscosity, without causing turbidity problems when the orange juice is rediluted and significantly improves settlement stability (Braddock and Kesterson, 1979; Braddock, 1981). Pectinesterases which are active at very low pH's (2.2 to 2.8) are available which modify the pectin in lemon juice, within 3 hours at ambient temperature, to such an extent that the cloud can be coagulated and precipitated with an agent. This enzymatic method shortens the clarifying process from traditional methods which may take 4 to 16 weeks (Janda, 1983). Pectinases can also be utilized during the recovery of citrus oils from the peels. This involves separation of the oils expressed in the presence of water. Pectinases are added to break the gels to give improved yields of citrus oils (Neubeck, 1975; Braddock and Kesterson, 1979).

Debittering

There are two chemically different bitter compounds found in citrus fruits: flavonoids which occur in all parts of the fruit and are in highest concentration in the albedo, and limonoids which occur in the albedo and in the center bundle and segment covers. Grapefruit and naval orange products are known to have a particularly bitter taste.

The main bitter citrus flavonoid is naringin [α-L-rhamnosyl-(1,2)-ß-D-glucosyl-(1,7)-naringenin] which may occur in large quantities in grapefruit (Ting, 1958; Griffiths and Lime, 1959). Fungal naringinase preparations contain α-rhamnosidase and ß-glucosidase which hydrolyze naringin to prunin [ß-D-glucosyl-(1,7)-naringenin] and rhamnose. Further hydrolysis yields naringenin and glucose (Thomas et al., 1958). Naringenin is a non-bitter product and prunin is less bitter than naringin. Naringinase preparations need to be free of pectinase contaminants because they may adversely affect

the juice cloud (Neubeck, 1975; Rombouts and Pilnik, 1978). Rouseff (1993) advised against the use of enzymes containing ß-glucosidase activity . He found that ß-glucosidase can liberate ferulic acid from feruloylglucoside. Free ferulic acid is a precursor to *p*-vinylguaiacol in orange juice (Naim et al., 1992), which has a flavor threshold of 50 ppb and has been described as a "rotten fruit" odor (Tatum et al., 1975).

Limonoid bitterness is caused by limonin, a cyclic terpene with two δ-lactone rings and a furan ring substituent. While this compound is not present in the intact fruit, it is slowly formed in the acidic juice out of the A-ring monolactone, its non-bitter precursor. Therefore, this type of bitterness is often referred to as delayed bitterness. Instead of using enzymes to reduce the limonin content, biological methods have been proposed (Lanzarini and Pifferi, 1989) and microorganisms such as: *Pseudomonas*, *Arthrobacter globiformis*, *Acinetobacter* sp., and *Corynebacterium fascians*, have been examined. Enzymes extracted from these microorganisms do not provide an effective degradation of limonoids at the natural pH of juice, due to their high optimum pH, about 8.0 (Lanzarini and Pifferi, 1989).

ENZYME SUBSTRATES AND MECHANISMS

Plant Cells and Cell Walls

There are three main compartments or structures in a plant cell: 1) the cell wall which contains cellulose, hemicelluloses and pectic materials; 2) the cytoplasm, which is stratified on the cell wall and contains the nucleus, plastids, enzymatic proteins and growth factors; and 3) the vacuole, in which sugars, acids, salts, polyphenols, and pigments are dissolved (Goodwin and Mercer, 1983; Lanzarini and Pifferi, 1989). There are three layers in the plant cell wall: middle lamella, primary wall and secondary wall. In the parenchyma tissue, which forms the major part of the tissue of fruit, the middle lamella

acts as an intercellular binding substance and is composed primarily of pectic material. Secondary walls contain less pectin and some lignin. The primary cell wall consist of microfibrils (cellulose fibers) embedded in a matrix of pectins, hemicelluloses and proteins.

The action of enzymes in degrading cell walls is influenced particularly by temperature, pH and the presence of inhibitors. In addition, the physical structure of the wall such as fragment size, degree of crystallinity of cellulose and degree of enmeshment of the various constituents, will influence the accessibility of the substrates to the enzymes. The chemical structure of the wall, including degree of methylation, degree of branching and composition of side chains, will also strongly influence degradation. These physical and chemical properties of fruits vary not only from species to species, but also with the maturity of the fruit (Voragen and van den Broek, 1991).

Pectin and Pectolytic Enzymes

Pectic substances are natural polysaccharides occurring mainly in the middle lamella and the primary cell wall of higher plants. It is responsible for the integrity and coherence of plant tissues. Even though it occurs in fruits and vegetables in low quantities, usually less than 1% w/w, pectin has a large impact on the consistency of homogenized fruits and on the cloudy appearance and the cloud stability of juices (Rombouts and Pilnik, 1978). Pectins are unique among common carbohydrates because their main component is a sugar acid, galacturonic acid, instead of a simple sugar. The main chains of pectic carbohydrates are composed primarily of linear polymers of galacturonic acid residues linked by α-1,4 bonds. The methyl ester of pectic acid and rhamnose may be found in the main chain as well. Pectin is pectic acid esterified with methyl alcohol. The extent of esterification varies between fruits (43-65% in grapes to 85-95% for apples) and with the age of the fruit, usually decreasing with increasing maturity

(Lanzarini and Pifferi, 1989). Free carboxyl groups in the pectic acid chain can be acylated and these may be the branch points for side chains of neutral sugars such as galactose, arabinose and xylose. The branches are α-1,3 and α-1,6 bonds. In juice processing, pectins may cause a variety of problems: insoluble pectin will trap juice in the presscake during pressing thus reducing yield; soluble pectin can go into solution increasing viscosity and thus hindering filtration when manufacturing a clear juice or causing difficulties when producing a concentrate; and soluble pectin may form stable particles, which could cause cloud formation in the juice after filtering (Boyce, 1986).

Two main groups of enzymes are used to degrade pectin: pectin esterases which remove the methyl alcohol group changing pectin to pectic acid, and pectin depolymerases which split the pectic acid chain (Figure 2.1). The esterase group is comprised of pectinesterases which are specific for the methyl ester of galacturonic acid polymers. They remove the methyl ester group from the pectin and thus change the molecular charge; a neutral ester group is replaced by an ionizable acid group. While pectin esterases of plant origin proceed along the polygalacturonan backbone in a sequential fashion, pectin esterases of fungal origin proceed in a random mode and have the strongest affinity for high methoxyl pectin (Voragen and van den Broek, 1991). The pectic acid chain can be cleaved by two mechanisms: hydrolysis and ß-elimination. Endo- and exogalacturonases hydrolyze pectic acid by random cleavage of interior glycoside bonds and systematic cleavage of saccharides from the reducing end, respectively. Endogalacturonase rapidly causes depolymerization and thus reduces viscosity. Both enzymes prefer substrates with high molecular weight. Lyases randomly cleave pectic acid and pectin via ß-elimination, splitting the glycoside bond without the addition of water. Pectin lyase is an endolyase which acts on glycosidic bonds between methylated galacturonic acid molecules. It is only found in microorganisms and is the only enzyme that will cleave fully-esterified pectin. The use of pectin lyase is preferable in fruit juice processing as it permits the use of only a single enzyme and avoids the reduction of fruit juice stability due to the coagulation of

pectin partially de-esterified with endogenous Ca^{2+} and the release of methanol (Alana et al., 1989). Pectate lyase is an exopectate lyase which breaks glycosidic linkages between non-methylated galacturonic acid molecules, and is only found in microorganisms as well. Pectate lyases have an absolute requirement for calcium ions for their activity (Rombouts and Pilnik, 1978) and a pH optimum of about 8.5 - 9.5 (Voragen and van den Broek, 1991), and thus have very limited activity in most fruit juices.

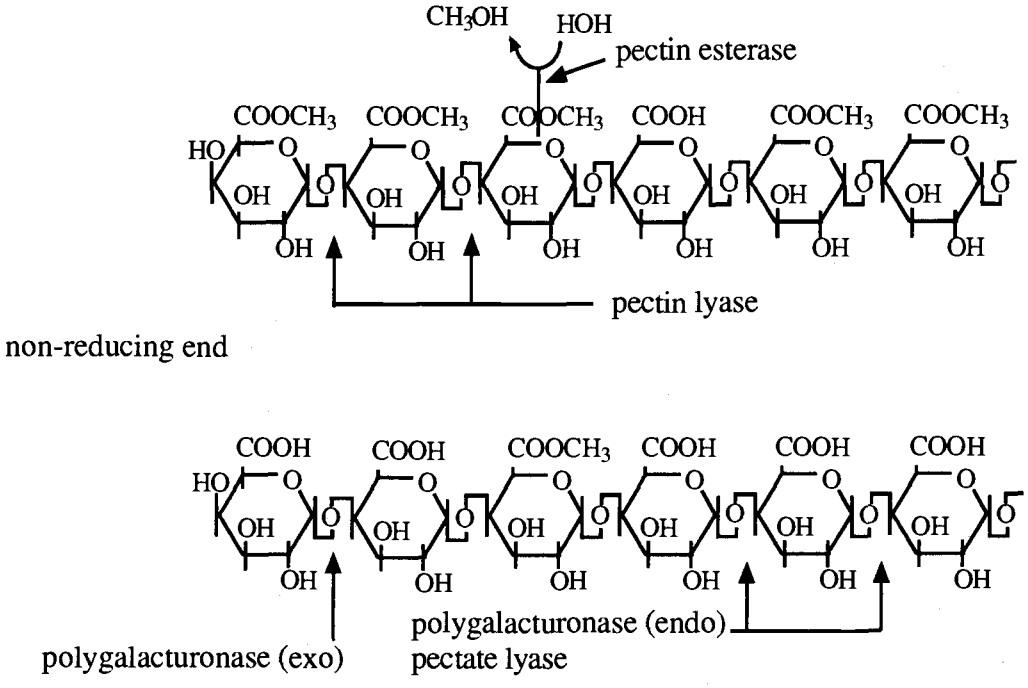


Figure 2.1 Schematic diagram of pectin and pectin degrading enzymes. (Adapted from Rombouts and Pilnik, 1978; Voragen and van den Broek, 1991.)

Hemicellulose and Hemicellulases

Hemicelluloses are polysaccharides that are extracted from plant cells by strong alkali. They consist of branched chain heteropolysaccharides with the main chain being composed of xylose, mannose and galactose linked with ß-1,4 bonds (Goodwin and Mercer, 1983). Hemicelluloses are usually named according to the predominant sugar in

the main chain, e.g. xylans, mannans, glucomannans, and galactoglucomannans (Robinson, 1991). Side chains of methyl glucuronic acid, arabinose, glucose and galactose are attached by various linkages, including α-1,2; ß-1,3; ß-1,4; and ß-1,6 bonds.

Hemicelluloses are degraded by two types of enzymes, endo- and exo-hemicellulases. Endohemicellulases randomly cleave interior bonds and many types exist which are specific for different sugar backbones, such as arabinases and galactanases. Exohemicellulases systematically hydrolyze units from the nonreducing end of a chain.

Cellulose and Cellulases

Cellulose is the most well known of all plant cell wall polysaccharides. Cell walls of land plants consist of large amounts of cellulose microfibrils embedded in a continuous phase. Cellulose is a linear glucose polymer coupled by ß-1,4 bonds (Goodwin and Mercer, 1983). The major structural variability in celluloses is their degree of polymerization, or number of glucose units in a chain. In the secondary wall the average polymerization is 14,000, which is why this wall contains a high degree of crystallinity, thus explaining its mechanical strength. In the primary wall polymerization appears to fall into two groups: approximately 500 or between 2500 and 4500. The cellulose in primary cell walls tends to have a lower degree of crystallinity, thus making it more susceptible to enzymatic degradation (Voragen and van den Broek, 1991).

Cellulase is a multiple enzyme system composed of endoglucanases, exoglucanases and cellobiase and it is only produced by microorganisms. Endoglucanases randomly hydrolyze the ß-1,4 linked glucan chain of cellulose. Exoglucanases break the bonds at the non-reducing end of the chain, producing glucose or cellobiose. Cellobiases (ß-glucosidase) split cellobiose into its two component glucose molecules (Voragen and van den Broek, 1991).

COMMERCIAL ENZYME PREPARATIONS

Commercial enzyme preparations are fungal enzymes typically derived from food-grade strains of *Aspergillus niger* and/or *Trichoderma longibrachiatum* (formerly *T. reesei*). Traditionally these preparations are rather crude and often contain varying degrees of pectinesterases, polygalacturonases and pectin lyases, as well as other enzymes such as cellulases, xylanases, arabanases, galactanases, glycosidases, proteases, esterases and oxidoreductases. In addition to the various enzymes they may also contain impurities such as extraneous enzymes, proteins, mucilage and melanoidins (Martino et al., 1994). Commercial enzyme preparations vary widely between manufacturers and it is very difficult to compare enzyme activities since a variety of substrates and preparation media are used by the manufacturers. During the 1971-72 season, Neubeck (1975) examined five commercial pectinases from four manufacturers for use in apple juice clarification, grape juice and wine treatment. He found that the 12 measured parameters (pectic activity as well as other specific enzyme activities such as ß-glucosidase, α-galactosidase, protease and amylase) differed widely among the preparations. He also has noted that different lots of the same pectinase product from a manufacturer may show some deviation in the nonpectic enzyme activities, but the pectic activities would be expected to follow a characteristic pattern for each preparation.

In the United States, use of pectinases and cellulases from *Aspergillus niger* are acknowledged by the Food and Drug Administration as GRAS (generally recognized as safe), if prepared in accordance with "good manufacturing practice" as defined by the FDA (Neubeck, 1975; Denner, 1983). The European Economic Community (EEC) classifies enzyme products currently used by the food industry as processing aids and as such they are exempt from the European Directive for Food Additives (Dir. 89/107/EEC) (Moll, 1994). As of 1991, the annual market value of pectinases used in fruit and vegetable processing worldwide was US $16 million (Voragen and van den Broek, 1991).

Pectic Enzyme Production

Commercial pectic enzyme preparations in the United States usually consist of extracellular material from *Aspergillus niger* cultures and contain pectinase and pectase activity (Fogarty and Kelly, 1983; Pilnik and Rombouts, 1983). First a strain must be selected that produces the desired enzyme in good quantity. Rigid maintenance of culture purity and freedom from any variation whatsoever are essential for microbial use in enzyme production (Beckhorn et al., 1965). As of 1988 (Bigelis and Das), the secretion mechanisms of fungal pectic enzymes were still unknown, and no fungal genes related to pectin degradation had been cloned.

While the procedures adopted by various manufacturers vary to a degree, there are only two general methods used in enzyme production, surface (solid-substrate) and submerged culture (Figure 2.2) (Beckhorn et al., 1965; Underkofler, 1966a,b; Fogarty and Kelly, 1983). Surface fermentation was the original method used to obtain enzymes, while submerged culture techniques became more widely used only since World War II (Underkofler, 1966b). Surface fermentation involves the cultivation of mold on moist wheat bran (Beckhorn et al., 1965) or other milled cereal products such as grits (Fogarty and Kelly, 1983). Additional materials are added, such as other nutrients containing carbohydrates, proteins, salts, and acid or buffer to regulate the pH, and the bran is steam-sterilized, cooled and inoculated with spores. The bran is then incubated in chambers where temperature and humidity are controlled by circulating cool moist air from above. The extent of fungal growth is monitored by heat evolution and by periodical sampling and the batch is harvested when the enzyme level has reached its maximum. Enzymes are extracted from the mold by either batch extraction with water or buffer followed by filtration, or by percolation of water or buffer through the mold bran (Underkofler, 1966a,b). Submerged culture methodology for mold is also well known.

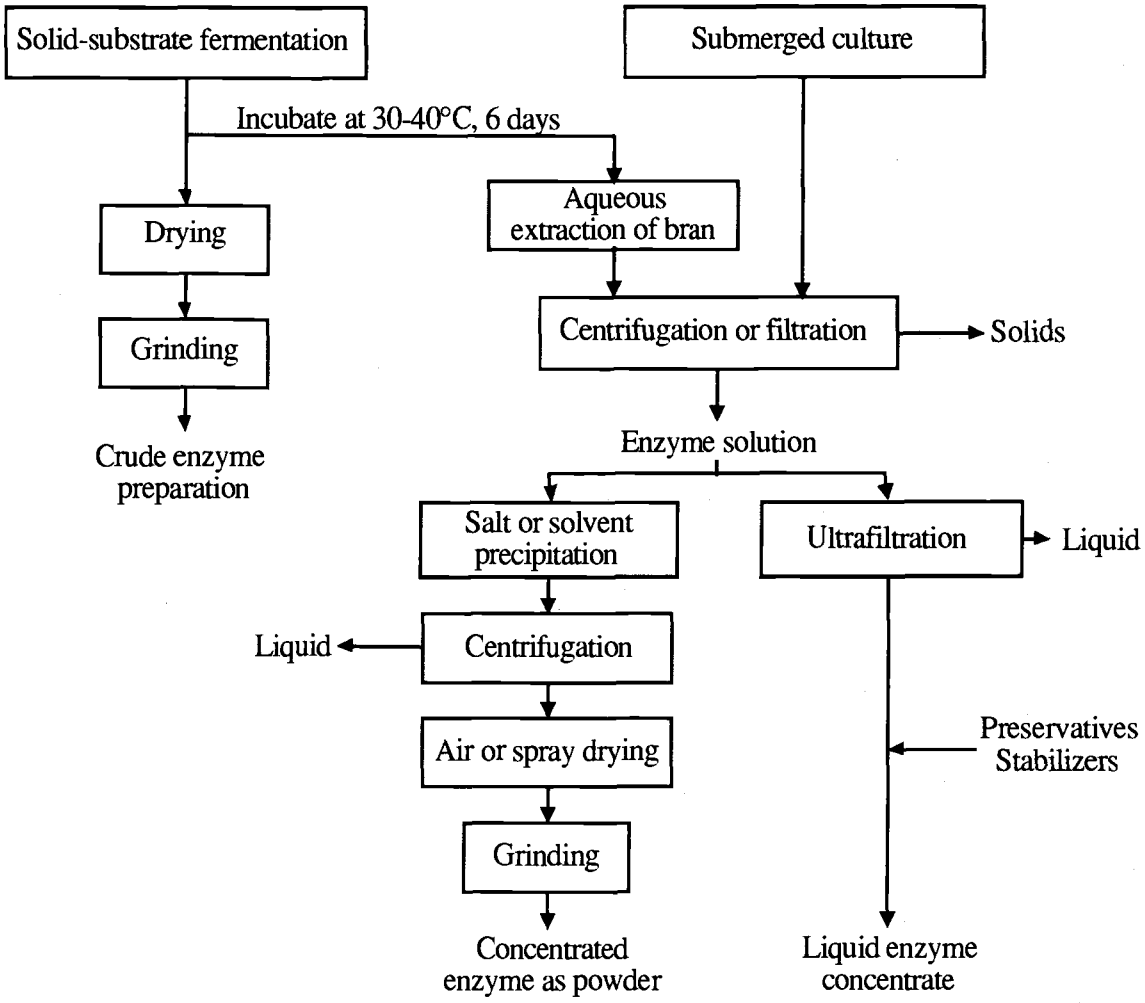


Figure 2.2 Production of fungal pectinases (Voragen and van den Broek, 1991).

Commercially, submerged cultures are grown in deep tanks which are equipped for introduction of sterile air and for vigorous agitation, since these organisms are aerobic. The amount of air, degree of air dispersion, and amount of agitation are interdependent variables and the appropriate conditions for maximum enzyme production must be determined for each individual fermentation process. Once all of the parameters have been optimized, strict control is exercised in order to obtain consistently high enzyme yields (Underkofler, 1966b). Once enzyme has been produced, crude enzyme liquors are obtained by separating the cellular material and insoluble medium ingredients from the

liquid containing dissolved materials. One method used on a commercial scale is filtration. Concentration of the crude liquor is usually conducted by vacuum evaporation at low temperatures. Obtaining a dry enzyme preparation usually involves precipitation by salts ($(NH_4)_2SO_4$, Na_2SO_4, NaCl, or Na_2HPO_4) or solvents (isopropanol, acetone, ethyl acetate, methanol, or ethanol), followed by drying (Fogarty and Kelly, 1983).

The submerged culture process is by far the preferred method: it is more flexible; easier to control with respect to pH, aeration, and temperature; and sterile operating conditions are more easily maintained (Fogarty and Kelly, 1983). It is generally accepted that commercial enzymes produced from the two commercial processing methods contain a number of different pectolytic enzymes. It is interesting to note that pectinesterase has been reported to be more readily produced by the surface culture technique (Ishii et al., 1980).

Future Trends

The methodology described above has been in commercial use for several decades. With increasing knowledge of the enzyme-based processes, we are in need of better formulated enzyme preparations containing specific, technologically-relevant enzymes, void of undesirable side activities. Researchers with the 'Eureka Project on Fruit Juice Enzymes', (which pools knowledge between companies and research institutes in Western Europe, Russia, Hungary and Turkey) are working on a project that they hope will lead to commercial 'tailor-made enzyme preparations' (Grassin et al., 1994). They are looking at the structure of polymers in stone and berry fruits which cause processing problems or are not degraded and then they will select enzymes which hydrolyze these molecules while still obtaining a quality finished product. Novel purification techniques to remove the undesirable activities from enzyme preparations are also being developed. Martino et al. (1994) are working with bentonite to separate desirable enzymes in a commercial

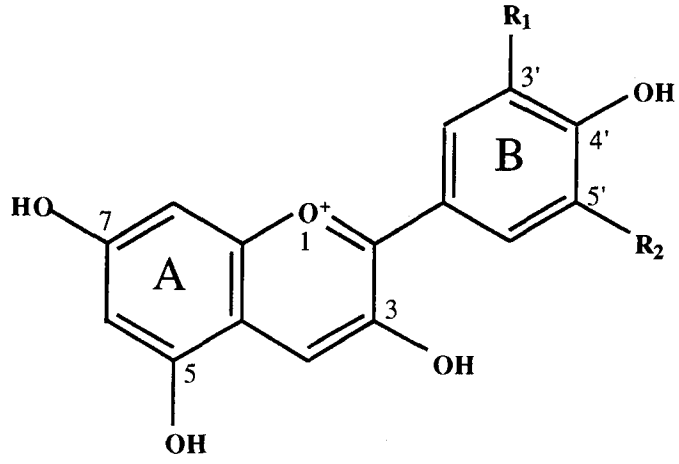
preparation from undesirable ones. Another approach for purified enzymes is to boost the production of desired enzymes by genetic manipulation or recombinant DNA technology, and to improve their properties by site-directed mutagenesis and express the modified genes in already tailored food-grade microorganisms (Voragen and van den Broek, 1991).

ANTHOCYANINS

Anthocyanins are water-soluble flavonoids, found mainly in the vacuoles of plant cells (Goodwin and Mercer, 1983), although they may sometimes be located in spherical vesicles called "anthocyanoplasts" (Strack and Wray, 1994). This group of pigments is responsible for giving many fruits, flowers, and leaves their attractive red, violet and blue colors. There have been a number of good reviews published on anthocyanins in general (Hayashi, 1962; Harborne, 1967; Timberlake and Bridle, 1975; Hrazdina, 1982; Iacobucci and Sweeny, 1983) and anthocyanins in fruits and vegetables (Timberlake, 1981; Macheix et al., 1990; Mazza and Miniati, 1993). Anthocyanins have many functions: the ability to impart color to plant or plant products (one of the most significant functions), giving way for aesthetic value as well a means for identification; in flowers and leaves - to ensure fertilizations and seed dispersal by animals; in leaves - to act as a UV-light screen; as well as being associated with resistance to pathogens, and enhancers and/or regulators of photosynthesis in some plants (Mazza and Miniati, 1993). Recent findings also suggest that anthocyanins may have pharmacological properties, including reducing capillary permeability and fragility, antiulcer activity and providing UV-radiation protection (Harborne and Grayer, 1988; Mazza and Miniati, 1993).

Structures

Anthocyanins are glycosides of anthocyanidins, which are hydroxylated and methoxylated derivatives of phenyl-2-benzopyrylium (flavylium salt structure) (Figure 2.3). They are unique in that they have a positive charge associated with the heterocyclic ring, which is delocalized over the whole structure (Timberlake and Bridle, 1975). Sugars can combine with the anthocyanidin (often referred to as the aglycon) to form monoglycosides, diglycosides, and triglycosides. In fruits as well as other parts of plants, glycosylation of anthocyanidins almost always occurs in the 3-position and there are five sugars that are involved in the formation of 3-monoglycosides: glucose, arabinose, galactose, xylose and rhamnose (Robinson, 1991). Glucose, galactose and xylose are linked to the anthocyanidin by a ß-bond while arabinose and rhamnose are joined by α-bonds (Goodwin and Mercer, 1983). Glucose, galactose and arabinose have been found linked to all six of the common aglycons while xylose has only been found in connection with cyanidin, and rhamnose with pelargonidin (Macheix et al., 1990). Diglycosides can be found in two different forms. Two sugar molecules may be linked to two different hydroxyls (usually glucose and most commonly at the 3- and 5-positions) or only to position 3. The former are called 3,5-diglucosides and the latter, 3-diglycosides. The most common diholosides in fruits are: rutinose (6-*O*-α-L-rhamnosyl-D-glucose), sambubiose (2-*O*-ß-D-xylosyl-D-glucose), sophorose (2-*O*-ß-D-glucosyl-D-glucose) and gentiobiose (6-*O*-ß-D-glucosyl-D-glucose). Triglycosides are not very widespread in fruits. There are two types as well: 3-diglycosides to which an extra glucose molecule is bound in position 5, or three sugar molecules are linked to the C-3 ring thus forming a triholoside.



$R_1 = H$	$R_2 = H$	Pelargonidin (pgd)
$R_1 = OH$	$R_2 = H$	Cyanidin (cyd)
$R_1 = OH$	$R_2 = OH$	Delphinidin (dpn)
$R_1 = OCH_3$	$R_2 = H$	Peonidin (pnd)
$R_1 = OCH_3$	$R_2 = OH$	Petunidin (ptd)
$R_1 = OCH_3$	$R_2 = OCH_3$	Malvidin (mvd)

Figure 2.3 Chemical structures of anthocyanidins present in fruits.

Phenolic acids, as well as dicarboxylic aliphatic acids, may be attached to the sugar groups to form acylated anthocyanins. These are fairly common in fruits and the existence of the acylated group appears to stabilize the anthocyanins in the acid environment of cell sap (Macheix et al., 1990). Common acylating acids include: *p*-coumaric, ferulic, caffeic, sinapic, *p*-hydroxybenzoic, malic, succinic, malonic, oxalic, succinic and acetic (Mazza and Miniati, 1993).

Occurrence

There are 18 anthocyanidins known today, but only six are widespread in plants (Strack and Wray, 1994). Hydroxylated derivatives, at the 3, 5, 7 and 4' positions, of these six anthocyanidins are found in fruits. Macheix et al. (1990) conducted a literature

search of 40 fleshy fruits and found that cyanidin was the most common (in 90% of examined fruits) followed by delphinidin (35%), peonidin (30%), pelargonidin (20%) and petunidin and malvidin (15% each). The six most common anthocyanins found in fruits are (in decreasing order): cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside and peonidin-3-glucoside (Macheix et al., 1990). Every anthocyanin-containing fruit can be characterized by its anthocyanidin glycosides, or its anthocyanin fingerprint, which includes the number and relative quantity of its anthocyanins. The number of anthocyanins within a fruit can vary from only one, which is fairly rare (mango, certain red-skinned peach cultivars, and *Viburnum lantana* blackberries), to 16 or more glycosides as found in *Vaccinium* sp., *Empetrum nigrum* and *Vitis vinifera* cv. Cabernet Sauvignon.

Stability

Anthocyanins are notoriously unstable under neutral or basic conditions. At pH's below 2, the anthocyanin exists primarily in the red flavylium cation form (AH^+). As the pH increases, the proton is quickly lost and the molecule shifts to a quinoidal base. This form usually exists as a mixture, as the pK_a's of the 4', 7 and (if present) 5-OH groups are very similar (Iacobucci and Sweeny, 1983). Between pH 4 and 5, the pseudo bases are predominate and solutions are virtually colorless. As the pH increases the anhydro bases become progressively more stable and are increasingly found. Between pH 5 and 6 there is little color left since a very small amount of the blue quinoidal base is the only colored form present. Ionization and stability of the anhydro bases increases until pH 12, when ring fission occurs with formation of slightly yellow, ionized chalcones (Timberlake and Bridle, 1975). If an anthocyanin is kept at an adequate acidic pH (typically 2-3), it should be quite stable at room temperature. The anthocyanin is much more stable than its parent anthocyanidin. Sweeny and Iacobucci (1983) found that cyanidin-3-rutinoside held at

room temperature, pH 2.8, had a half-life of 65 days; while the corresponding free anthocyanidin (cyanidin) had a half-life of only 12 hours.

Anthocyanin ring constituents have a profound affect on color intensity and anthocyanin stability. The degree of hydroxylation is important; anthocyanins which contain more hydroxyls are less stable. Conversely, a higher degree of methoxylation increases stability (Francis, 1989). Rommel et al. (1992) found that cyanidin-3-glucoside, which has two hydroxy groups, is the most labile pigment in blackberry juice and wine. At a given pH, 3-glycosides are more colored than 3,5- and 5-glucosides, yet diglucosides in wine are more stable to decolorization than are their corresponding monoglucoside. On the other hand, the diglucoside pigments contribute more to browning, presumably because of the extra sugar available for Maillard type reactions (Francis, 1989). The type of sugar group may also affect stability. Starr and Francis (1968) found the galactosides of cyanidin and peonidin were more stable that the arabinosides in cranberry juice. Anthocyanins with two or more acylating groups are stable in neutral or weakly acidic media, possibly due to hydrogen bonding between phenolic hydroxyl groups in the anthocyanidins and aromatic acids (Mazza and Miniati, 1993).

When anthocyanins are to be used as colorants in food products, one must remember that they are sensitive to light, temperature, pH, ascorbic acid, oxygen and sulphur dioxide (which is often employed as a preservative). To avoid light instability anthocyanins can be reacted with carbonyl compounds, such as acetaldehyde, to stabilize them. Two patents in 1980 claimed that pigment degradation in soft drinks induced by light can be minimalized by the addition of one or more of gallic acid, gallic acid dimer, tannic acid, or rutin; or the addition of flavones and/or flavonols, such as the use of enocyanin in propylene glycol containing 25% rutin (Francis, 1989). Acylated anthocyanins are much more stable to light than are monoglycosides (Harborne and Grayer, 1988). Studies have conclusively shown a rapid decrease in pigment at high temperatures and thus high-temperature-short-time (HTST) treatments are recommended

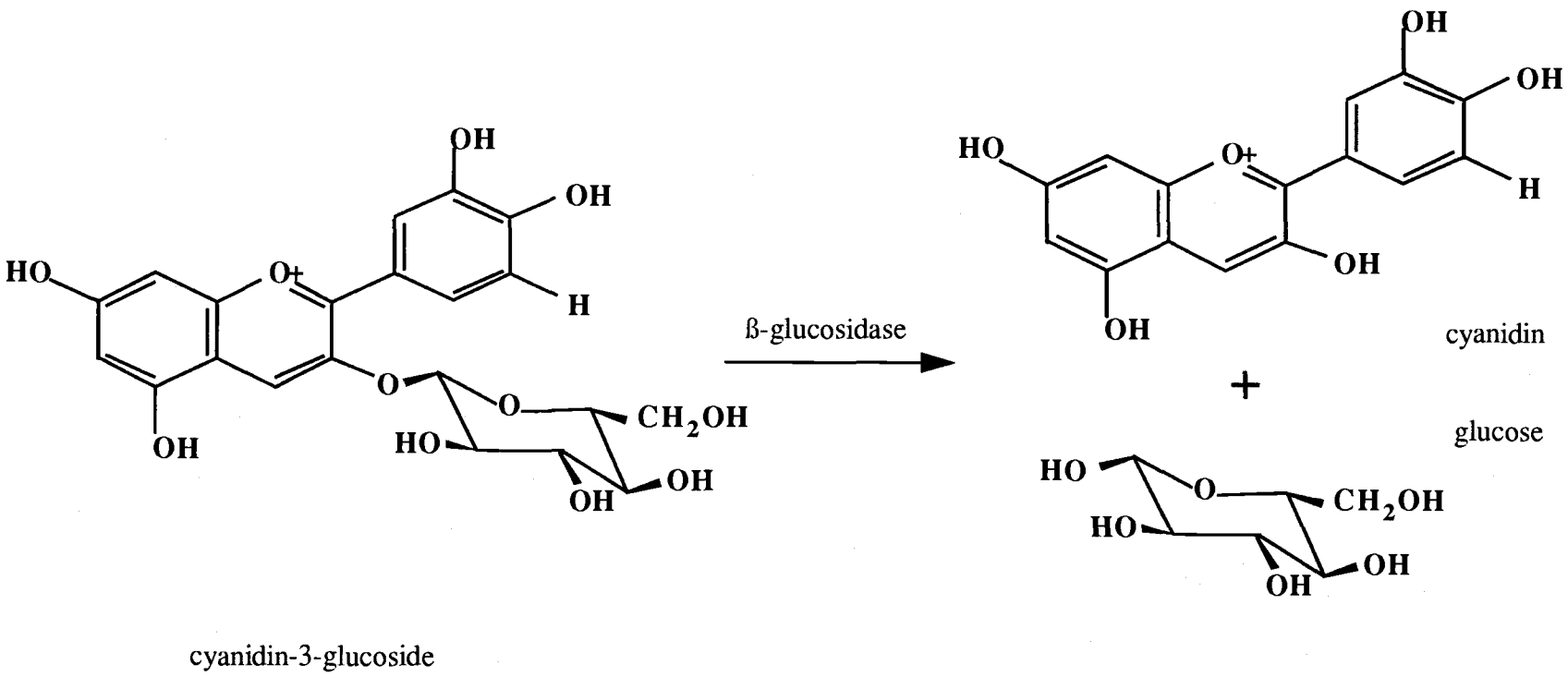
(Francis, 1989). The exact mechanism for destruction is not known, but theories include sugar hydrolysis to form unstable anthocyanidins and formation of chalcones at high temperatures (Broulliard, 1982). Ascorbic acid and oxygen both cause anthocyanin degradation and should be considered accordingly in product formulation and packaging (Markakis, 1982; Francis, 1989).

ANTHOCYANIN DESTRUCTION BY ENZYMES

Commercial Enzymes

Juice

Under certain processing conditions some enzyme preparations may degrade pigments in fruit juice by hydrolytically removing the glycosidic substituents of the anthocyanins. Anthocyanin decomposition is an undesirable side-activity in fungal enzyme preparations that has been reported in: raspberry juice (Jiang et al., 1990), raspberry wine (Withy et al., 1993), strawberry juice (Tanchev et al., 1969; Blom, 1983), cherry nectar (Blom and Juul, 1982), blackberry wine, jellies, and jams (Yang and Steele, 1958), and chrysanthemin, isolated from blackberry (Huang 1955; 1956). Blom (1983) reported the presence of ß-glucosidase in a commercial enzyme preparation when he noted a decrease in pelargonidin-3-glucoside in strawberry juice with a subsequent increase in the unstable pelargonidin aglycon (Figure 2.4). After isolation, purification and characterization he reported it as anthocyanin-ß-glycosidase (Blom, 1983; Blom and Thomassen, 1985). Jiang et al. (1990) reported changes in individual pigments in addition to gross changes in total anthocyanin content in raspberry juice.



cyanidin-3-glucoside

Figure 2.4 Enzymatic destruction of cyanidin-3-glucoside yielding cyanidin aglycon and glucose.

They described the conversion of cyanidin-3-sophoroside to cyanidin-3-glucoside and cyanidin-3-glucosylrutinoside to cyanidin-3-rutinoside by a commercial enzyme preparation and described it as ß-1,2-glucosidase activity (Figure 2.5). Wrolstad et al. (1994, 1995) reported a case in which they analyzed a commercial cranberry juice concentrate sample that had an unusual anthocyanin profile; the ß-galactoside pigments were depleted, but its nonvolatile acid and sugar profiles were typical of authentic cranberry juice. They interpreted the sample as authentic, only that the ß-galactoside pigments were degraded. They were subsequently informed that the pigment destruction was due to commercial enzymes used during processing. Researchers have isolated ß-glucosidase (McCleary and Harrington, 1988; Watanabe et al. 1992; Unno et al., 1993; Martino et. al., 1994), ß-galactosidase (Bahl and Agrawal, 1969) and α-L-arabinosidase (Lerouge et al., 1993) from commercial enzyme preparations and all have the potential to destroy anthocyanins.

While anthocyanin destruction by ß-glucosidase is considered detrimental in many cases, there are instances where ß-glucosidase activity may be advantageous. One example is in the Italian citrus industry during processing of blood oranges 'Sanguinello' (Lanzarini and Pifferi, 1989; Mian et al., 1992; Martino et al., 1994). Degradation of the anthocyanins by ß-glucosidase would help prevent the discoloration due to anthocyanin browning during thermal treatment of the juices. ß-glucosidase activity may also be beneficial in processing rosé wines from red grape varieties as well as removing excess pigment in blackberry wine (Yang and Steele, 1958).

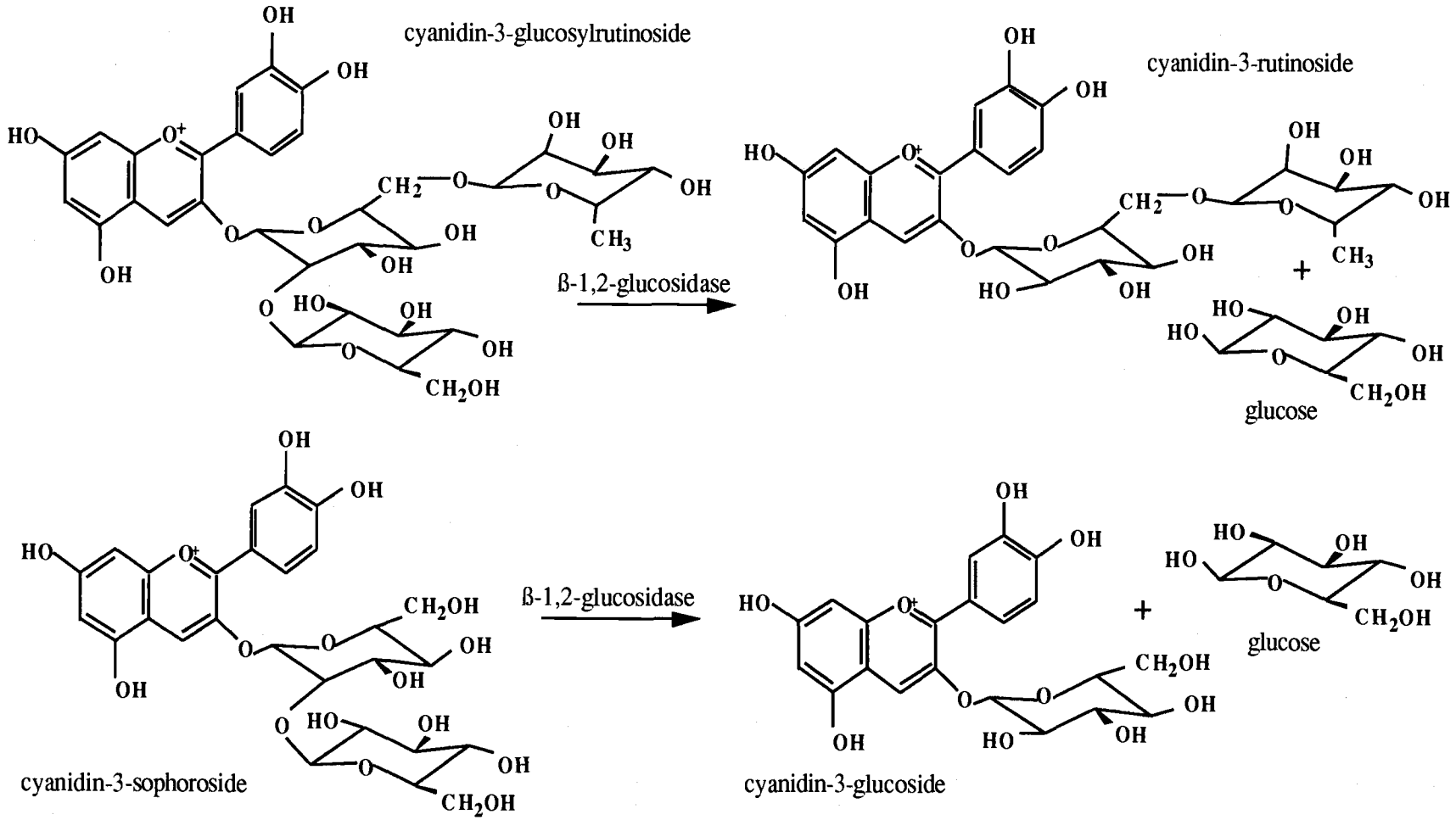


Figure 2.5 Destruction of cyanidin-3-glucosylrutinoside and cyanidin-3-sophoroside by ß-1,2-glucosidase.

Wine

As early as the 1950's and 60's there were contradictory findings as to the effect of pectic enzymes on color intensity of wines. Due to differences in winemaking procedures it is very difficult to compare results between researchers and therefore the use of controls with no enzyme addition are essential. Reports of increased color intensity due to enzyme use were discussed previously. In 1963, Blouin and Barthe found evidence of an increase in juice yield, yet a slight decreasing trend in color intensity of 3-month-old Malbec wine made from must preheated to 80°C, in relation to an increase in pectic enzyme addition. Berg and Marsh (1956) reported the effect of pectic enzyme use with heat extraction of Zinfandel, Alicante, Carignane, and Grenache grapes. They found that while the enzymes were very important for clarification there were no differences in color intensity after 29 months of storage. However, they did note that after 54 months of storage the enzyme-treated wines had lost somewhat more color than the controls. This group also used pectic enzymes in port production and found that one lot increased in color intensity due to enzyme treatment while one lot lost color. At that time, they suggested that the fungal enzymes that were used may have contained ß-glucosidase activity, in reference to Huang's (1955) work with blackberry pigments. Samples of Bulgarian wine prepared from pectic enzyme-treated Zaitchine grapes had almost 18% more color than did a control wine when 0.3% enzyme was used, but 60% less color than control when 0.7% of the same enzyme was used (Neubeck, 1975). Montedoro and Bertuccioli (1976) produced Sangiovese wine with the addition of cellulases, pectinases and proteases in various concentrations. They found that the anthocyanin content in the musts of each treatment were higher than a control with no enzyme addition, except the resulting wine made with pectinase was 20% lower in anthocyanin content than the control. Wines made with cellulase and protease had higher anthocyanin contents than the control. Working with aromatic red rice wine, which has a characteristic red color like that of grape wine, Saigusa

et al. (1994) isolated ß-glucosidase from a commercial saccharifying agent produced from *Aspergillus niger*, which had destroyed the pigments in the wine.

Economic Value

The loss of pigment in fruit juices and concentrates due to enzymatic degradation may have great economic consequences. Other than the cranberry concentrate (Wrolstad et al., 1994, 1995), very few industrial or commercial incidents have been reported in which commercial enzyme preparations were positively identified as the cause of anthocyanin destruction. One case where ß-glucoside enzymes possibly resulted in anthocyanin destruction was recently reported in a Concord grape juice concentrate (Wrolstad et al., 1994, 1995). Concord grapes contain the -3-ß-D-glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin along with *p*-coumaric-acylated derivatives and 3,5-diglucosides of delphinidin and cyanidin (Hong and Wrolstad, 1990). During the 1992 production season, the processor had made a number of changes in processing operations, including additional high-temperature-short-time (HTST) treatments and a change in enzyme suppliers. Although the cause of the anthocyanin destruction has not been proven, ß-glucosidase activity from pre-press or clarification enzymes is a possible cause. Due to the poor quality of this concentrate the price was subsequently reduced, resulting in an estimated economic loss of about US $700,000 for this firm.

Mold

Mold-contamination (mainly *Botrytis cinerea*) of fruit also degrades anthocyanins in strawberry wine (Pilando et al., 1985), strawberry juice (Rwabahizi and Wrolstad, 1988), red raspberry juice (Boyles and Wrolstad, 1993), and red wines (Cassignard et al., 1977), with increasing levels of mold contamination in the fruit accelerating color degradation. Pilando et al. (1985) suggested that the decrease in anthocyanin was due to

ß-glucosidase activity in the mold. Pitt (1968) found ß-galactosidase activity in *Botrytis cinerea*, and this may have the potential to destroy galactoside pigments. Uchiyama (1969) screened a number of strains of *Aspergillus*, *Mucor* and *Penicillium* for an "anthocyanin-decoloring enzyme" using cyanidin-3-glucoside from reddish white peaches. He found that most of the strains tested had some anthocyanin degrading ability, but one strain of *Aspergillus niger* isolated from soils contained the most activity.

When working with wines and *Botrytis cinerea*, many researchers refer to laccase activity from the mold as the cause of pigment degradation, rather than glycosidase activity. Dubernet et al. (1977) found that a crude preparation of laccase from *Botrytis cinerea* had a high relative activity for anthocyanins and the optimum pH for this activity was between pH 3-4, the same as for grapes and berries. He did not report any ß-glucosidase activity, although it might have also been in the crude extract.

Anthocyanin degradation by mold is a substantial problem for juice and wine processors that needs to be examined more closely. One area to pursue is the actual origin of the problem; whether it is due to ß-glycosidase and/or laccase produced by the mold. Generally, mold is inherent when dealing with fruit crops. Therefore a possible threshold for the extent of mold presence without noticeable anthocyanin degradation would also be helpful to growers and processors.

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CHAPTER 3

ANTHOCYANIN ANALYSIS AS A MEASURE OF GLYCOSIDASE ACTIVITY IN ENZYMES FOR JUICE PROCESSING

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ABSTRACT

A screening procedure combining HPLC and spectrophotometric analyses was developed to measure glycosidase activity of enzyme preparations used for juice processing. Enzyme preparations (27) were evaluated; several contained ß–galactosidase activity which can decompose cranberry juice pigments. ß–galactosidase and α–arabinosidase activities were also determined using standard procedures (nitrophenol-glycosides as substrates). Comparative results showed inconsistencies between the two procedures. Cranberry juice processing demonstrated that some enzyme preparations could decompose anthocyanin pigments under processing conditions. Pigment loss was much higher when enzymes were used with juice than with crushed fruit.

INTRODUCTION

Macerating enzymes are used to facilitate pressing of fruit by reducing viscosity, to improve juice yields by degrading structural polysaccharides, and to improve color extraction by degradation of skin tissue (Voragen and van den Broek, 1991). Since most such enzymes are relatively crude preparations, they may also impart undesirable activities. Anthocyanin decomposition is a side-activity in fungal enzyme preparations that has been reported with raspberry (Jiang et al., 1990) and strawberry juices (Tanchev et al., 1969), cherry nectar (Blom and Juul, 1982), blackberry wine, jellies, and jams (Yang and Steele, 1958), and chrysanthemin, isolated from blackberry (Huang 1955; 1956). Jiang et al. (1990) reported changes in individual pigments in addition to gross changes in total anthocyanin content in fruit juices. They reported the conversion of cyanidin-3-sophoroside to cyanidin-3-glucoside and cyanidin-3-glucosylrutinoside to cyanidin-3-rutinoside by a commercial enzyme preparation.

Cranberries (*Vaccinium macrocarpon*) contain 4 major pigments, cyanidin-3-galactoside, peonidin-3-galactoside, cyanidin-3-arabinoside and peonidin-3-arabinoside, and two minor pigments, cyanidin- and peonidin-3-glucosides (Macheix et al., 1990). Cranberries are unusual in having galactosides as the major anthocyanins; glucosides usually predominate in most anthocyanin-pigmented fruits. Cranberry juice provides an appropriate system for examining ß-galactosidase and α-arabinosidase activities of enzyme preparations. Such activities may have undesirable effects on color quality as the unstable aglycons would be destroyed. Anthocyanin pigment content is an important quality factor in cranberry juice.

Our objective was to develop an analytical method for measuring glycosidase activity of enzyme preparations which may be used in fruit juice processing. In addition we determined the effects of such activity on pigment stability and color quality of cranberry juice under typical processing conditions.

MATERIALS AND METHODS

Juice and Enzyme Samples

For an enzyme screening assay and for anthocyanidin identification, cranberry juice concentrate (50°Brix) was obtained from Kerr Concentrates, Inc. (Salem, OR) and diluted to 7.5°Brix with distilled water prior to analysis. The following enzymes were evaluated in the screening assay: Cellulase AC, Cellulase Tr Concentrate, Cellulase TRL, Clarex® L, Clarex® ML, Hemicellulase, Pearex® L, Pectinase AT, Spark-L HPG® (Solvay Enzymes, Inc., Elkhart, IN); Biopectinase 150L (Quest International, Sarasota, FL); Pectinex® BE 3XL, Pectinex™ Ultra SP-L, Pectinex™ 1XL (Novo Nordisk Ferment, Ltd., Dittingen, Switzerland); Rohapect® B1L, Rohapect® D5L, Rohapect® MB, Rohapect® TL, Rohapect® VR concentrate, Rohapect® 7104, research grade enzymes: EL 65-92, EL 66-

92, EL 67-92, EL 68-92 (Rohm, Darmstadt, Germany); Cytolase® CL, Cytolase® M102, Cytolase® M103M, Cytolase® PCL5, and Cytolase® 219 (Gist-brocades, Inc., King of Prussia, PA; formerly Genencor International, Inc.). For pilot plant processing, frozen whole cranberries were obtained from Ocean Spray Cranberries, Inc. (Markam, WA) in approximately 27 kg bags. Whole frozen cranberries and cranberry juice concentrate (50°Brix) were obtained from Clermont, Inc. (Hillsboro, OR), for the berry vs. juice trials. The concentrate was diluted to 7.5°Brix with distilled water prior to analysis.

Enzyme Screening

Enzymes were diluted, 10-fold (liquid) and 25-fold (granular), with distilled water and added at 0.1% by weight to 10 mL cranberry juice in 125 mL Erlenmeyer flasks. The flasks were heated in a waterbath (50°C, 150 rpm) for 2 hr. A control sample was heated without enzyme addition. After incubation, approximately 5 mL of juice was passed through an activated Sep-Pak (Water Associates, Milford, MA) and prepared for HPLC analysis. An additional 1 mL aliquot was added to 1 mL of cold methanol to inactivate the enzyme, vortexed and held on ice for 0.25-1 hr before analysis for total monomeric anthocyanin.

Juice Processing

A process typical of commercial practice (Figure 3.1) was used to produce juices in the pilot plant of the Department of Food Science, Oregon State University, from batches of berries ranging from 20-30 kg. Partially frozen berries were ground in a hammer mill (Model D Comminuting Machine, W.J. Fitzpatrick Co., Chicago, IL) equipped with 1.27 cm dia. circular pore mesh and knives operating at 480 rpm, which produced mainly 1/4 berries. The mash was heated in a steam jacketed kettle to 50°C.

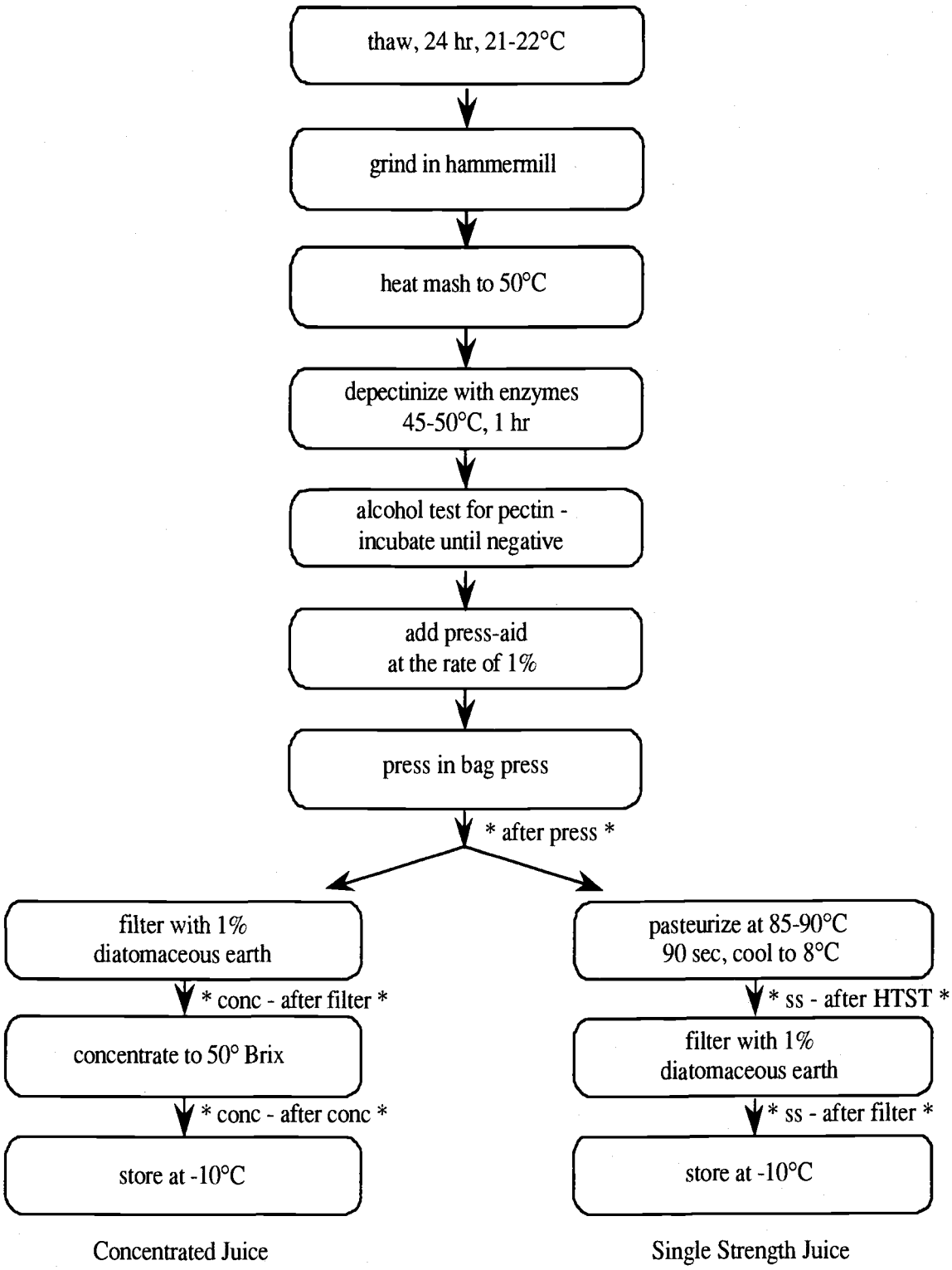

Figure 3.1 Cranberry juice processing unit operation. Sample times are designated by (*name*).

Enzymes were used within recommended levels, 0.07% (w/w) for Pectinase AT and 0.025% (w/w) for Pectinex® BE 3XL. A negative alcohol precipitation test (1:1 juice:95% ethanol + 1% HCl) was used to monitor completion of depectinization. One percent Silvacel® Wood Fiber Press Aid (Weyerhaeuser Co., Tacoma, WA) was used as press aid. Pressing was done in a Willmes bag press (Type 60, Moffet Co., San Jose, CA) using the following pressure program: 0 bar for 5 min, followed by 1 bar for 5 min, 0 bar for 5 min, 2 bar for 5 min, 0 bar for 5 min, and 4 bar for 5 min. The juice was divided equally with half to be concentrated and the remainder left as single strength juice. The single strength juice was pasteurized using an APV-Crepaco high temperature short time (HTST) unit (type "junior", APV-Crepaco, Inc., Tonawanda, NY). Filtering was done with the addition of 1% Filter-Cel®, a celite diatomite product (Johns-Manville Sales Corp., Lompoc, CA) using a multi-pad filtration unit (Hermann Strassburger KG, Westhofen bei Worms, Germany) and SWK-Supra 2600 filters (Seitz-Filter-Werke, Theo & Geo Seitz GmbH und Co., Bad Kreuznach, West Germany). Half of each batch was concentrated to 50°Brix using a Centri-Therm centrifugal evaporator (Model CT-1B, Alfa-Laval Inc., Newburyport, MA). Control lots were run without enzyme or heating. All treatments were processed in duplicate. Duplicate 50 mL samples were taken at 5 times during each process, designated by (* sample name *, Figure 3.1) and stored at -10°C until analyzed. Juice samples were thawed for 45 min in a 30°C waterbath immediately prior to analysis. Brix was measured on each sample and concentrated samples were diluted to 7.5°Brix with distilled water before further analysis. Total monomeric anthocyanins were measured and HPLC analyses of anthocyanins were performed. Duplicate measurements were done on each treatment combination.

Berry-Enzyme Trials

Frozen whole cranberries (250 g) were thawed overnight at 4°C. Berries were crushed in a stainless steel Waring blender (Waring Products Co., Winsted, CT) for 2 sec, transferred to a 600 mL Erlenmeyer flask and heated to 50°C. Enzyme was added at 0.07% (w/w) for Pectinase AT and 0.06% (w/w) for Pectinex BE 3XL and mixed well. The flasks were held at 50°C in an orbital waterbath (150 rpm). At 0, 15, 30, 60, 90, 120, and 180 min, the berry mashes were mixed thoroughly and 25 g samples were removed, weighed and frozen in liquid nitrogen. Aqueous anthocyanin extracts were prepared as described by Wrolstad et al. (1990). A control was run at 50°C without enzyme addition. Treatments were performed in triplicate. Samples were analyzed for total monomeric anthocyanin and by HPLC for individual anthocyanins.

Juice-Enzyme Trials

Cranberry juice (100 mL) was heated to 50°C in a water bath. Enzymes were diluted 1:10 (by weight) with distilled water and added at the same dosage as above. Flasks were mixed well, covered, and held in a 50°C waterbath. Samples (4 mL) were taken at 0, 15, 30, 60, 90, 120, and 180 min. From the 4 mL, two 0.50 mL aliquots were each mixed separately with 0.50 mL cold methanol to inactivate the enzyme, vortexed, covered and held on ice until analyzed for total monomeric anthocyanin. The remaining 3 mL was passed through an activated Sep-pak and prepared for HPLC analysis. A control was run at 50°C without enzyme addition. Treatments were performed in triplicate.

Total Anthocyanins

Monomeric anthocyanin pigment content was determined by the pH differential method of Wrolstad (1976) using a Shimadzu UV160U double beam spectrophotometer

(Shimadzu Scientific Instruments, Kyoto, Japan). Anthocyanin content was calculated as mg cyanidin-3-galactoside/100 mL juice using a molar absorptivity of 30,200 and a molecular weight of 445. All samples were analyzed in duplicate.

HPLC of Anthocyanins

The sample cleanup procedure I of Hong and Wrolstad (1990) was used to prepare anthocyanins for analysis. A Perkin-Elmer Series 400 Liquid Chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with a Hewlett-Packard diode array detector, Model 1040A, a Hewlett-Packard Series 9000 computer (Hewlett-Packard Co., Palo Alto, CA) and a Beckman Autosampler, Model 501 (Beckman Instruments, Inc., San Ramon, CA) were used. Conditions for enzyme screening assay and juice processing trials: column LC-18, 25 cm x 4.6 mm, 5 μm particle size (Supelco, Inc., Bellefont, PA) with a C-18 guard column (Micro-Guard® ODS-10, 30 x 4.6 mm, Bio-Rad Laboratories, Richmond, CA) attached before the column, isocratic mobil phase 7.85% acetonitrile, 9.7% acetic acid and 0.97% phosphoric acid in aqueous solution, 1.0 mL/min flow rate, and 50 μL injection volume. Anthocyanin detection was at 520 nm, 20 nm band-width. Conditions for berry vs. juice time trials: column ODS (C-18), 25 cm x 4.6 mm, 5 μm particle size, 12 nm pore (PolyLC Inc., Columbia, MD), and isocratic mobile phase, 10.7% acetonitrile, 9.4% acetic acid, and 0.94% phosphoric acid in aqueous solution. All samples were analyzed in duplicate.

HPLC Analysis of Anthocyanidins

The same HPLC and detector/integrator as used for anthocyanin analyses were used. The hydrolysis and concentration procedures described by Hong and Wrolstad (1986) were used with an initial sample of 2 mL cranberry juice directly adsorbed onto a Sep-Pak without NaCl addition. The same column and conditions as described for HPLC

analysis of anthocyanins in the enzyme screening assay were used. UV-Vis spectra were taken directly from chromatographic runs.

Specific Enzyme Determination

ß-galactosidase, ß-glucosidase and α-arabinosidase activities of enzyme preparations were measured using nitrophenol-glycoside substrates. ß-galactosidase was measured based on an assay by Solvay Enzyme, Inc. (1991). It was performed with 3 mL of enzyme sample (diluted 100-fold in 0.1 M KH_2PO_4-NaOH buffer containing 10^{-3} M $MnCl_2$, pH 6.5) and a 2.2 mM *o*-nitrophenol-ß-D-galactopyranoside substrate solution to a final concentration of 0.55 mM in a final volume of 4 mL. Incubation was at 37°C for 1 min and the reaction was stopped by adding 4 mL of 0.2 M Na_2CO_3. Free *o*-nitrophenol was determined by absorbance at 400 nm. ß-glucosidase and α-arabinosidase were measured by the method of Dopico et al. (1989) at 34°C, pH 4.5 with *o*-nitrophenol-ß-D-glucopyranoside and *p*-nitrophenol-α-L-arabinopyranoside, respectively, as substrates. One unit of enzyme was defined as that amount which liberated 1 µmol of *o*-nitrophenol or *p*-nitrophenol/min under specified conditions and with extinction coefficients of 21,300 and 18,300, respectively (Dawson et al., 1986). The specific activities of enzymes were expressed as units/mg of protein in the preparation. Protein was determined by Kjeldahl method (AOAC, 1984). Specific activities and protein determinations were performed in duplicate.

Experimental Design and Statistical Analysis

Experimental designs for the enzyme screening assay, the specific enzyme activity determinations and the protein measurements were completely randomized designs with enzymes as treatments. A two factor factorial experimental design (treatment x time) was

used for the juice processing trial (completely randomized design) and for the berry vs. juice trials (randomized complete block design). All data sets were analyzed by a general linear models procedure with SAS® (Proprietary Software Release 6.04 Copyright 1985, 86, 87 SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Enzyme Screening

Cranberries show a very consistent anthocyanin pigment pattern, with -3-galactosides and -3-arabinosides of cyanidin and peonidin the major pigments (Wrolstad et al., 1995). Therefore they provided an effective model to measure undesirable galactosidase and arabinosidase activities. The commercial enzymes analyzed were fungal enzymes derived from strains of *Aspergillus niger* and/or *Trichoderma longibrachiatum* (formerly *T. reesei*). They are marketed for pectinase, cellulase and hemicellulase activities to decompose cell walls, allowing higher juice yields and pigment extractions, and reducing viscosity. Glycosidase activities could decompose anthocyanins in juice by hydrolyzing the glycosidic substituents from the pigment, producing the aglycon, which is unstable. Enzymes recommended for use in juice and wine processing were tested. A few of the enzymes had been specifically marketed for use in cranberry juice production, but a wide variety of enzymes were needed to develop and test our analytical method. An enzyme/juice reaction time of 2 hr was used to reflect commercial processing practices and a 0.1% dosage rate was used to standardize the assay since recommended dosage rates ranged from 0.001 to 1.0%. These dosage amounts were well below those used by Tanchev et al. (1969) and Jiang et al. (1990), and more closely reflected true commercial practices. Jiang et al. used up to 2.0% enzyme for 4 hr at 45°C when they observed the decomposition of cyanidin-3-sophoroside to cyanidin-3-glucoside and cyanidin-3-

glucosylrutinoside to cyanidin-3-rutinoside, in red raspberry juice using a commercial enzyme preparation.

The galactoside pigments were the predominant ones decomposed by the enzyme treatments, with cyanidin-3-galactoside destroyed to a greater extent than peonidin-3-galactoside. No significant destruction of glucosides or arabinosides ($p>0.05$) was observed. Total monomeric anthocyanin content and percent of individual pigments were compared (Table 3.1) relative to the control after 2 hr reaction time among the different enzyme treatments. Individual pigment quantities were estimated by the following equation: (percent peak area from HPLC chromatogram) x (total monomeric anthocyanin determined spectrophotometrically). Two enzyme preparations showed ≥ 90% reduction in cyanidin-3-galactoside, 7 exhibited a 40-90% reduction and 8 showed 20-40% reduction. The reduction of total anthocyanin by 21 of the enzymes confirmed observations in previous reports on raspberry (Jiang et al., 1990) and strawberry juices (Tanchev et al., 1969), and cherry nectar (Blom and Juul, 1982). The destruction most likely involved two steps. First an enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar occurred, and then a spontaneous transformation of the aglycon pigment into colorless forms (Huang, 1956). In further support of this hypothesis, Blom (1983) found and partially purified an anthocyanin-ß-glycosidase from a commercial pectolytic enzyme preparation which catalyzed a lytic reaction on the ß-glycosidic bond of anthocyanins in strawberry juice.

The amounts of protein and specific activities of ß-galactosidase, ß-glucosidase and α-arabinosidase were compared in the enzyme preparations (Table 3.2). Amounts of cyanidin-3-galactoside destroyed in the juice were compared (Figure 3.2) as measured by HPLC, with ß-galactosidase activity for the enzyme preparations determined spectrophotometrically using *o*-nitrophenol-ß-galactoside as substrate. Some inconsistencies were apparent between the methods.

Table 3.1 Total monomeric anthocyanin and percent of pigments[a] in cranberry juice after heating with 0.1% enzyme for 2 hr at 50°C

Enzyme	Cyd-3-gal	Pnd-3-gal	Pnd-3-arab	Total Monomeric Anthocyanin (mg/100 mL)[b]
Control	100	100	100	18.8
Biopectinase 150L	77 *	81 *	103	16.8 *
Cellulase AC	51 *	55 *	105	13.9 *
Cellulase TRconc	99	100	110	19.1
Cellulase TRL	100	99	108	18.9
Clarex L	91	91 *	100	18.0 *
Clarex ML	81 *	86 *	103	17.1 *
Cytolase 219	69 *	76 *	111 *	17.6 *
Cytolase CL	92 *	100	110	18.6
Cytolase M102	73 *	80 *	113 *	15.9 *
Cytolase M103M	70 *	77 *	109	16.2 *
Cytolase PCL5	56 *	65 *	111	14.8 *
EL 65-92	2 *	2 *	118 *	8.6 *
EL 66-92	95	98	109	18.6
EL 67-92	59 *	69 *	106	15.0 *
EL 68-92	89 *	98	108	18.3
Hemicellulase	70 *	73 *	107	15.8 *
Pearex L	54 *	60 *	106	14.3 *
Pectinase AT	47 *	54 *	107	13.9 *
Pectinex Ultra SP-L	64 *	69 *	107	15.2 *
Pectinex 1XL	75 *	77 *	111	15.6 *
Pectinex BE 3XL	84 *	85 *	110	17.5 *
Rohapect 7104	10 *	11 *	116 *	9.7 *
Rohapect B1L	84 *	86 *	107	17.3 *
Rohapect D5L	58 *	61 *	112	14.8 *
Rohapect MB	92 *	94	108	18.3
Rohapect TL	63 *	69 *	110	15.3 *
Rohapect VR	56 *	65 *	107	14.7 *
Spark L	93 *	93	110	18.6

[a] Percentage based on control as 100%.

[b] Calculated as mg cyanidin-3-galactoside/100 ml juice, with the MW = 445 and molar abs = 30,200; std error for total monomeric anthocyanin is 0.298.

* significantly different from control $p<0.05$

Table 3.2 Total protein and specific enzyme activities in commercial enzyme preparations

		UNITS/ µg protein		
Enzyme	Protein (mg/g)[a]	ß-galactosidase[b]	ß-glucosidase[c]	α-arabinosidase[d]
Biopectinase 150L	25	19	14	9
Cellulase AC	294	2	73	16
Cellulase TRconc	255	4	50	5
Cellulase TRL	95	tr[e]	27	36
Clarex L	7	30	58	21
Clarex ML	31	14	48	6
Cytolase 219	42	5	35	15
Cytolase CL	115	3	17	30
Cytolase M102	69	8	86	15
Cytolase M103M	160	1	77	14
Cytolase PCL5	41	11	108	31
EL 65-92	67	48	25	51
EL 66-92	15	2	1	tr
EL 67-92	13	27	100	144
EL 68-92	7	nd[f]	1	1
Hemicellulase	257	1	88	12
Pearex L	20	29	77	23
Pectinase AT	43	26	12	12
Pectinex 1XL	9	55	27	28
Pectinex BE 3XL	16	32	10	12
Pectinex Ultra SP-L	66	45	6	8
Rohapect 7104	29	82	65	135
Rohapect B1L	24	8	66	42
Rohapect D5L	26	16	59	72
Rohapect MB	12	14	11	9
Rohapect TL	31	10	39	48
Rohapect VR	26	16	41	83
Spark L	29	7	30	35

[a] Total protein determined by Kjeldahl, using 6.25 as the multiplication factor.

[b] One unit will hydrolyze 1.0 µmol *o*-nitrophenyl-ß-D-galactopyranoside to *o*-nitrophenol and galactose per minute at pH 6.5 at 37°C. Calculated with millimolar extinction coefficient of *o*-nitrophenol as 21.3 (Dawson et al., 1986).

[c] One unit will hydrolyze 1.0 µmol *o*-nitrophenyl-ß-D-glucopyranoside to *o*-nitrophenol and glucose per minute at pH 4.5 at 34°C. Calculated with a millimolar extinction coefficient of *o*-nitrophenol as 21.3 (Dawson et al., 1986).

[d] One unit will hydrolyze 1.0 µmol *p*-nitrophenyl-α-L-arabinopyranoside to *p*-nitrophenol and arabinose per minute at pH 4.5 at 34°C. Calculated with a millimolar extinction coefficient of *p*-nitrophenol as 18.3 (Dawson et al., 1986).

[e] trace

[f] not detectable <0.1 Units/µg protein

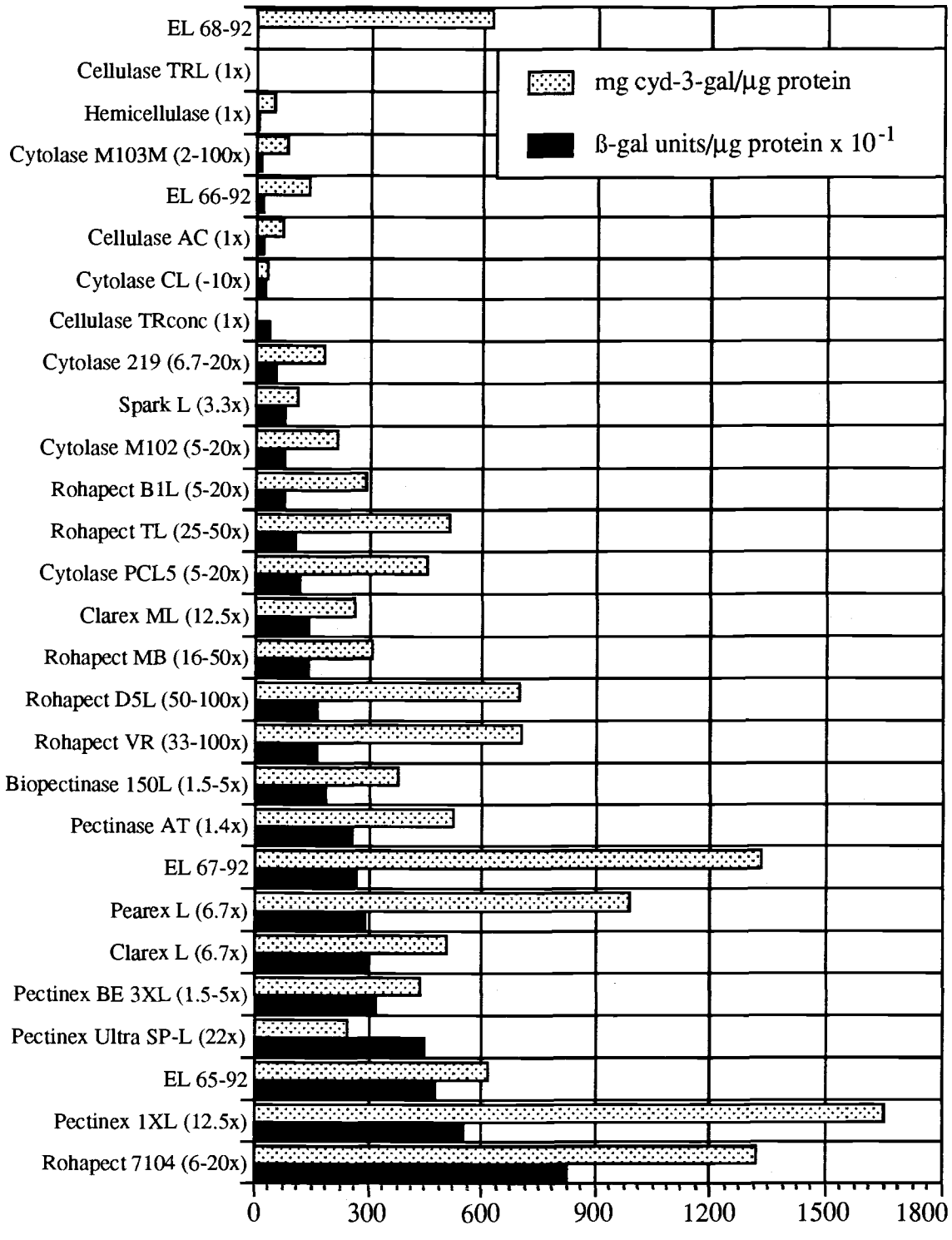

Figure 3.2 Cyanidin-3-galactoside destroyed in cranberry juice in 2 hr at 50°C versus specific activity of ß-galactosidase (assayed using *o*-nitrophenol-ß-galactosidase as substrate). Ratio of experimental enzyme dosage to manufacturer's recommended dosage is given parenthetically following enzyme name. Enzymes are ordered by increasing specific activity of ß–galactosidase.

Higher activities were measured by the HPLC assay than the standard colorimetric procedure except for Cellulase TRconc and Pectinex Ultra SP. We believe the HPLC assay procedure is a more reliable index of anthocyanin pigment stability in juice processing since it measures the true pigment changes. ß-glucosidase and α-arabinosidase activities were also measured. Again, agreement was not clear between HPLC and standard assays. Cyanidin-3-glucoside and peonidin-3-glucoside are minor pigments in cranberry juice (1% and 4%, respectively). Limitations would be inherent when basing an assay for ß-glucosidase on changes in concentrations of trace constituents. Therefore cranberry juice would not be a good system for measuring ß-glucosidase activity.

Cranberry Juice Processing Trial

The screening assay measured ß-galactosidase and α-arabinosidase activity of enzyme preparations, but was not intended to accurately predict amounts of pigments lost during juice processing. Many enzyme preparations would increase pigment yield during juice extraction; this would tend to counteract the effects of anthocyanin destruction from anthocyanin glycoside hydrolysis. Pilot-plant processing trials were conducted to determine the extent of pigment destruction (if any) when juice was processed using typical conditions. Two enzymes selected, Pectinex BE 3XL and Pectinase AT, were recommended for cranberry juice production and showed a moderate cyanidin-3-galactoside destruction in initial enzyme screening.

In the first juice lot with Pectinex BE 3XL (I), the berries were not totally depectinized before pressing and additional enzyme was necessary to finish depectinizing the juice. In the second lot with Pectinex BE 3XL (II), the same total amount of enzyme was added and it was fully depectinized before pressing. Thus, the two lots of Pectinex BE 3XL were not true replicates and were therefore statistically analyzed separately. The total enzyme contact time for Pectinex BE 3XL was 6.4 hr in both (I) and (II). In (I) the

mash was pressed after 1 hr and enzyme was added to the juice for an additional 5.4 hr to achieve complete depectinization. In (II), with the same total dosage of enzyme, complete depectinization took 6.4 hr, before pressing. Total enzyme contact time before pressing for Pectinase AT was 1-1.5 hr.

Enzymes are used in fruit juice production to increase juice yields (Janda, 1983). These ranged from 67 to 80% and differences between treatments and controls were not significant ($p<0.05$). There was a difference ($p<0.05$) in total monomeric anthocyanins at each sampling time for the Pectinex BE 3XL (I) relative to the control (Figure 3.3). New filter pads were used for each run to avoid cross contamination. Use of unconditioned pads (and perhaps excess filter pads) resulted in large (ca. 50%) losses of anthocyanin pigments through adsorption.

Individual pigment quantities were estimated by the equation: (percent individual peak area by HPLC) x (total monomeric anthocyanin, spectrophotometrically). A difference was found in total monomeric anthocyanin content between Pectinex BE 3XL (I) and the control after pressing ($p<0.05$). For comparative purposes, individual pigment quantities were normalized to "after press" values to compensate for filtration losses: (mg individual pigment at sample point) x (total anthocyanin "after press") / (total monomeric anthocyanin at sample point). Pectinex BE 3XL (I) was different from control in the amount of cyanidin-3-galactoside, at each sampling point, and Pectinase AT was different from control ($p<0.05$) at each sampling point in peonidin-3-galactoside content (Figure 3.4). No differences occurred in amounts of arabinoside and glucoside pigments between controls and any of the enzyme treatments.

In HPLC analyses, an additional peak was detected in some Pectinex BE 3XL (I) samples which eluted approximately 2 min after peonidin-3-arabinoside. This peak was determined to be the aglycon, cyanidin, by comparing retention time and spectra to cyanidin obtained by acid hydrolysis of cranberry juice. Retention times were confirmed by augmenting the original sample with anthocyanidin hydrolysate. This confirmed

Huang's (1956) proposal that the initial step in anthocyanin degradation is hydrolysis to the unstable aglycon.

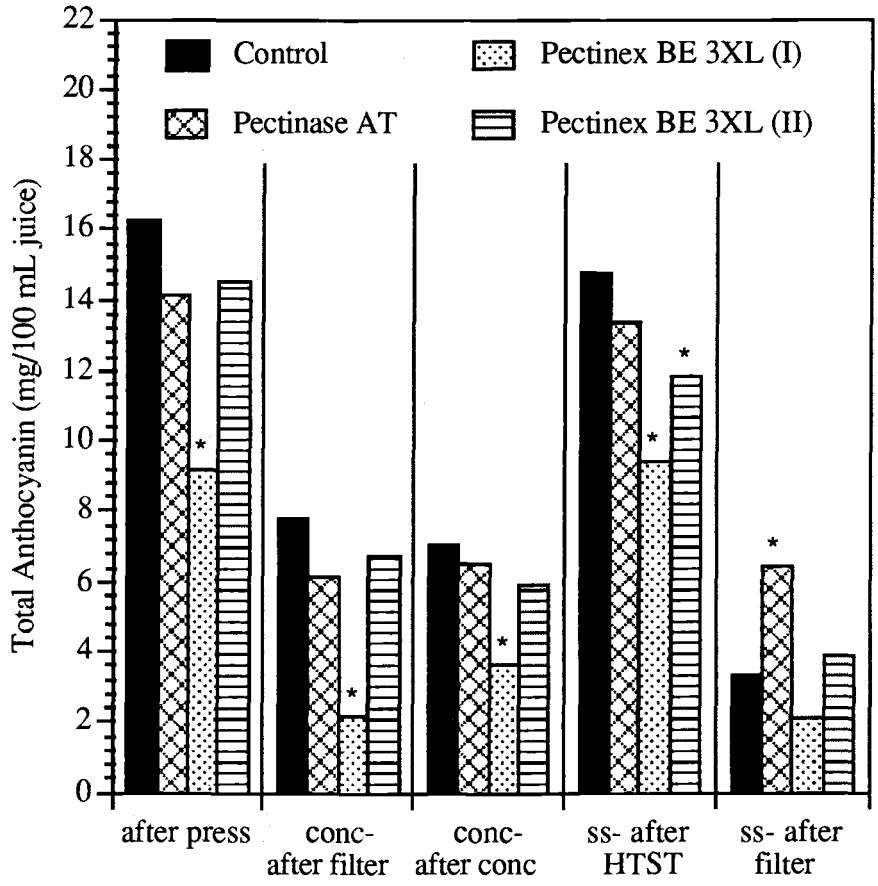

Figure 3.3 Total monomeric anthocyanin at various stages during processing of cranberry juice. * designates significantly different from control $p<0.05$.

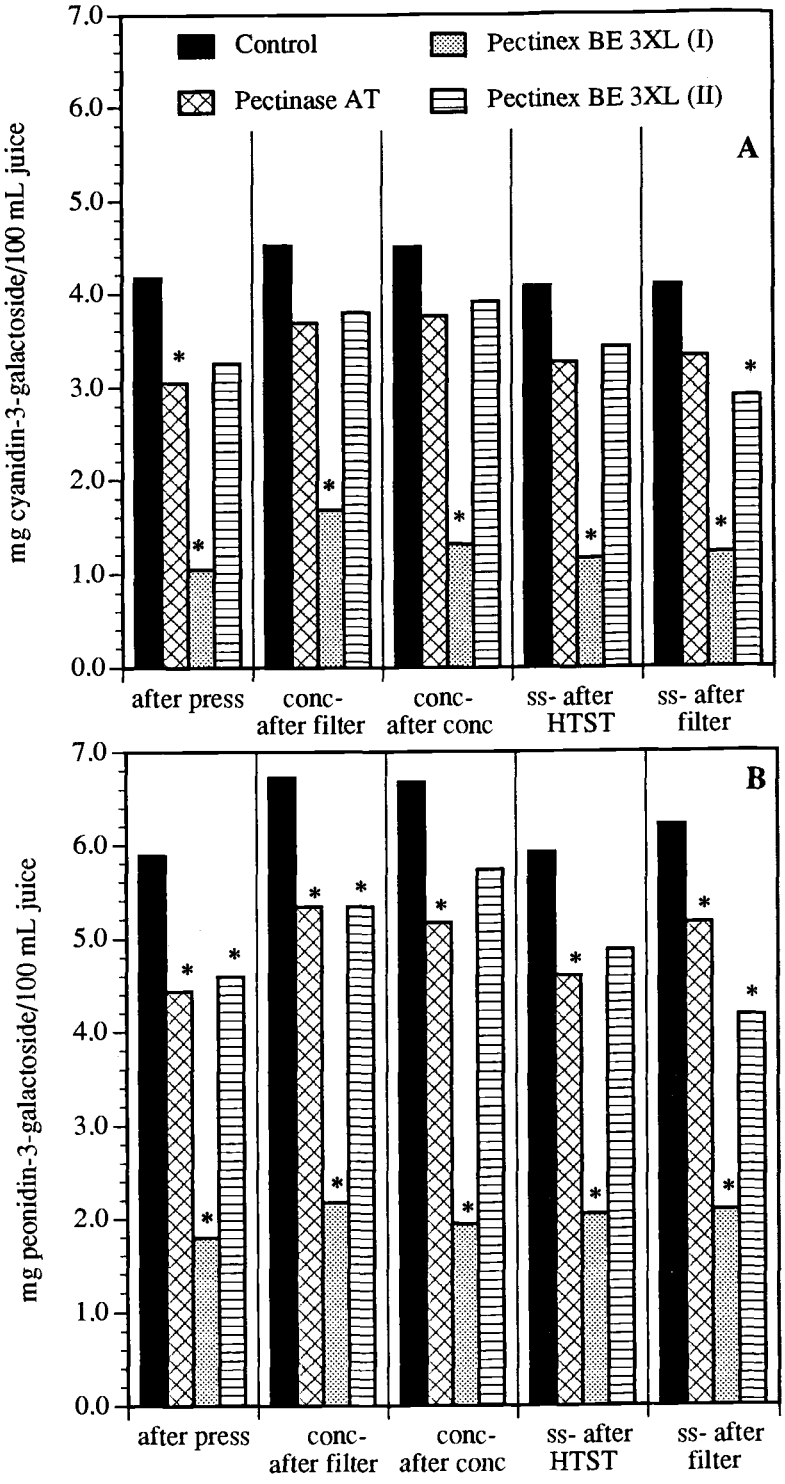

Figure 3.4 Cyanidin-3-galactoside (A) and peonidin-3-galactoside (B) concentrations at different stages during cranberry juice processing. * designates significantly different from control $p<0.05$.

Enzyme Action on Fruit vs. Juice

This experiment was designed to test for differences in pigment degradation when enzyme preparations acted on crushed fruit or clarified juice. In addition we examined anthocyanin degradation with time at recommended enzyme dosage levels. Total monomeric anthocyanin (Figure 3.5) and cyanidin-3-galactoside (Figure 3.6) losses were compared over time in berries and juice. Results for peonidin-3-galactoside were similar to cyanidin-3-galactoside. Both treatments showed pigment destruction ($p<0.05$) for clarified juice, with Pectinex BE 3XL having greater activity. The amount of Pectinex BE 3XL was increased to 0.06% to ensure that the depectinization step would be complete in 1 hr, as it was with Pectinase AT. This was within the manufacturer's recommended range. No difference ($p>0.05$) occurred, however, between enzyme treatments and the control when crushed berries were the starting material. Pigment measurements for the berry trial showed much larger standard deviations than for the clarified juice trial, probably because of analytical limitations in analyzing heterogeneous berries. The higher variability may have obscured enzyme-induced pigment destruction in the crushed fruit trial; nevertheless pigment destruction was evidently much more pronounced with clarified juice. Possibly competitive inhibition may retard anthocyanin hydrolysis in the crushed fruit system. These results could account for the greater anthocyanin destruction which occurred in the pilot plant juice processing trial with Pectinex BE 3XL (I), where enzyme was added to crushed fruit, and again to pressed juice. Much less pigment destruction occurred in trial (II) where the same enzyme quantity was added to fruit. These results imply that pigment destruction could be minimal during early stages of juice processing, but become substantial during later stages of clarification. Inactivation of enzymes with high-temperature-short-time (HTST) treatment may be appropriate for some situations.

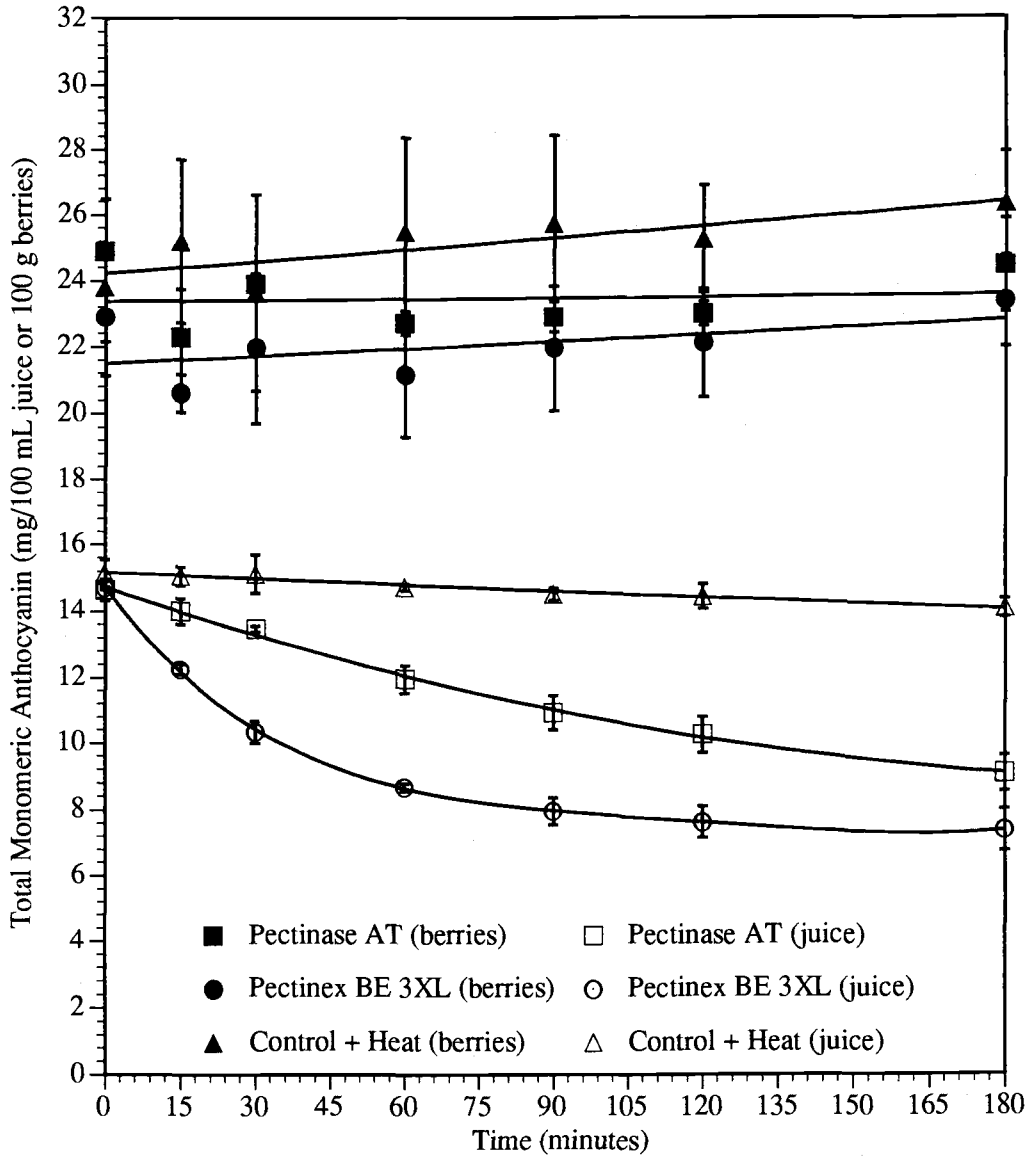

Figure 3.5 Changes in total monomeric anthocyanin content with time for crushed cranberries and cranberry juice treated with Pectinase AT and Pectinex BE 3XL at 50°C.

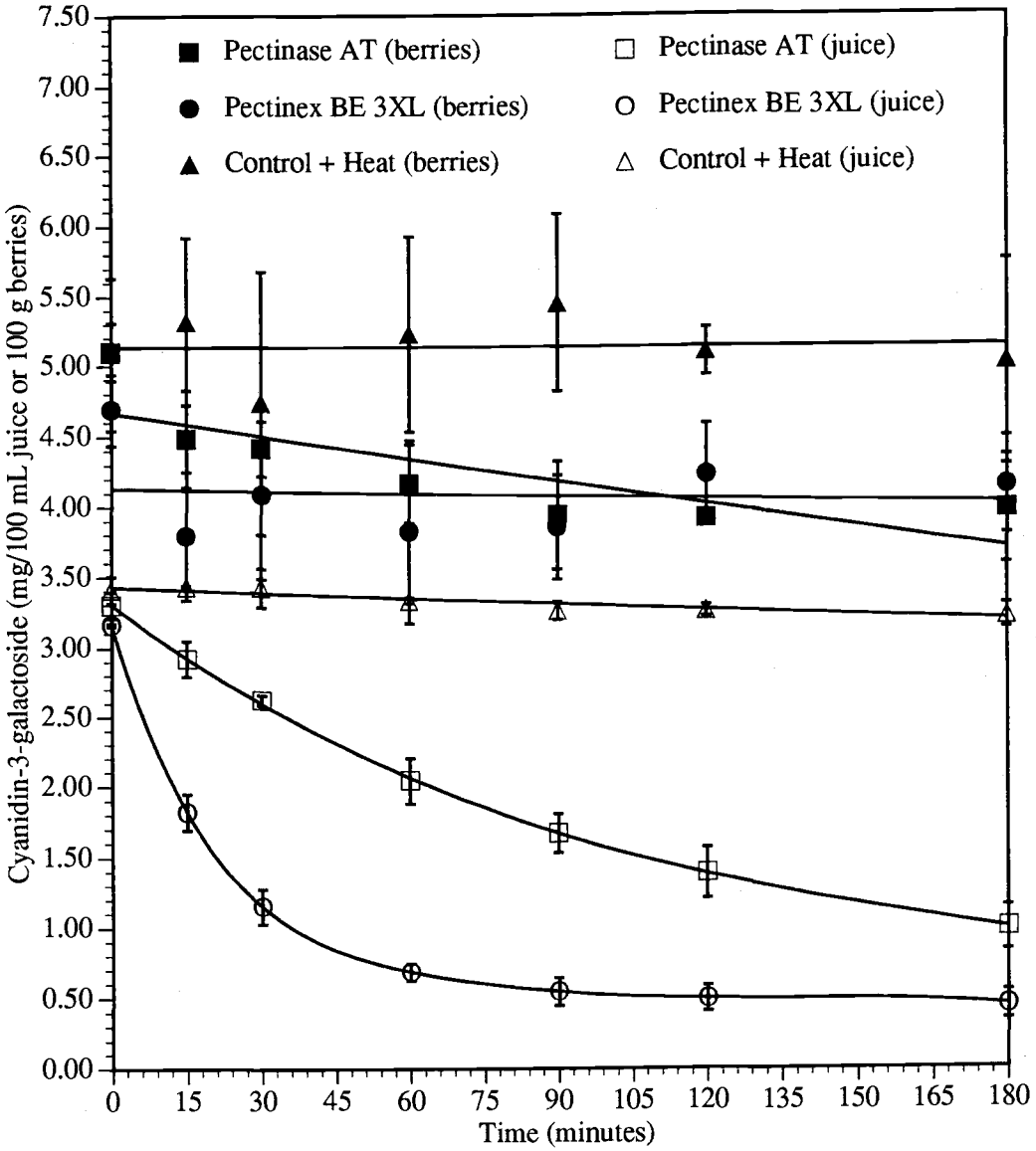

Figure 3.6 Changes in cyanidin-3-galactoside concentration with time for crushed cranberries and cranberry juice treated with Pectinase AT and Pectinex BE 3XL at 50°C.

The amount of cyanidin-3-galactoside destroyed was compared (Figure 3.7) for the different experiments. Pigment destruction was clearly higher with clarified juice than when enzymes were acting on crushed fruit. While the screening procedure demonstrated the potential for anthocyanin pigment degradation, it would not accurately predict the amount of pigment loss that may occur during processing. Enzymes which showed pigment losses in the screening assay should be further tested on a larger scale before being applied in a commercial operation. This should be applicable to any fruits which contain notable levels of anthocyanin-ß-galactosides. These include other *Vaccinium* fruits such as highbush blueberry (*Vaccinium corymbosum*) and ligonberry (*Vaccinium vitis-idaea*) which contain substantial ß-galactosides; also cyanidin-3-ß-galactoside is the major anthocyanin of apple (*Malus pumila*) peels, red pear (*Pyrus communis*) peels and chokeberry (*Aronia melanocarpa*) fruit (Macheix et al., 1990).

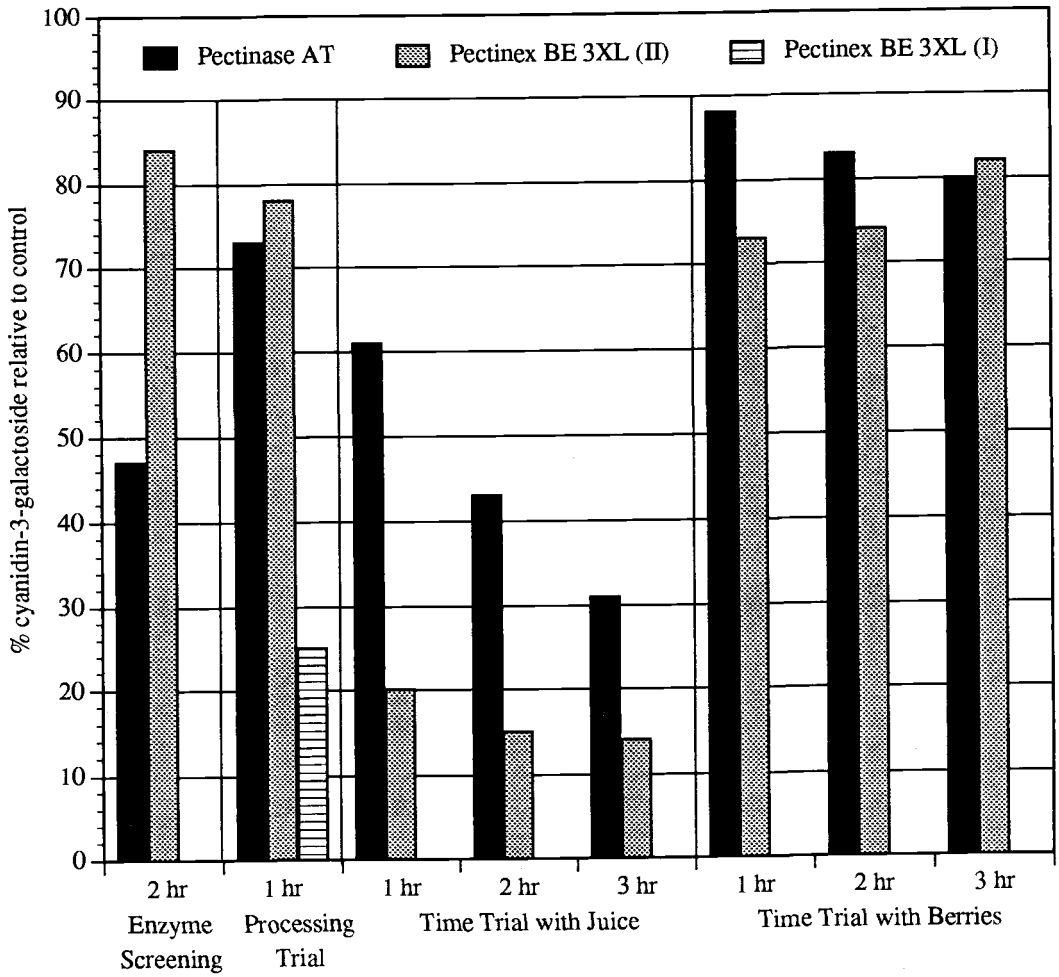

Figure 3.7 Percent cyanidin-3-galactoside relative to control for Pectinase AT and Pectinex BE 3XL treatments in four experimental treatments. Conditions for each experiment were: enzyme screening - 0.1% both enzymes, 2 hr, 50°C; juice processing trial - Pectinase AT- 0.07%, 1-1.5 hrs, 50°C; Pectinex BE 3XL- 0.025%, 50°C (I)- enzyme added to mash for 1 hr and to juice for 5.4 hr, (II)- enzyme added to mash only for 6.4 hr; berry vs. juice trials - Pectinase AT- 0.07%, 1, 2 and 3 hr, 50°C; Pectinex BE 3XL- 0.06%, 1, 2 and 3 hr, 50°C.

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CHAPTER 4

USE OF ANTHOCYANIN ANALYSES TO SCREEN FOR ß-GLUCOSIDASE ACTIVITY IN JUICE-PROCESSING ENZYMES

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ABSTRACT

A screening procedure utilizing boysenberry juice as a substrate, which combined HPLC and spectrophotometric analyses, was used to measure ß-glucosidase activity of enzyme preparations used for juice processing. Enzyme preparations (26) were evaluated at two dosage rates. At the mean recommended dosage, one preparation produced a significant decrease in total monomeric anthocyanin and cyanidin-3-glucoside, relative to a control, indicating ß-glucosidase activity. At 0.1% dosage, four enzymes produced a significant decrease in cyanidin-3-sophoroside and two enzymes caused an increase in cyanidin-3-rutinoside, indicating ß-1,2-glucosidase activity. ß-glucosidase activity in juice processing enzymes was much lower and less prevalent than that found previously for ß-galactosidase. *Botrytis cinerea* was found to degrade anthocyanins in boysenberry juice.

INTRODUCTION

Pectolytic enzymes are used in juice processing to increase juice yield and facilitate color extraction (Lanzarini and Pifferi, 1989; Voragen and van den Broek, 1991), but under certain processing conditions some enzyme preparations may degrade pigments in fruit juice by hydrolytic removal of the glycoside substituents of the anthocyanins. Anthocyanin decomposition is an undesirable side-activity in fungal enzyme preparations that has been reported in: raspberry juice (Jiang et al., 1990) and wine (Withy et al., 1993), strawberry juice (Tanchev et al., 1969; Blom, 1983), cherry nectar (Blom and Juul, 1982), blackberry wine, jellies, and jams (Yang and Steele, 1958), and chrysanthemin, isolated from blackberry (Huang 1955; 1956). Blom (1983) reported the presence of ß-glycosidase in a commercial enzyme preparation when he noted a decrease in pelargonidin-3-glucoside in strawberry juice with a subsequent increase in the pelargonidin aglycon. After isolation, purification and characterization he reported it as anthocyanin-ß-glycosidase (Blom, 1983; Blom and Thomassen, 1985). Jiang et al. (1990) also noted

changes in individual pigments and in total anthocyanin content in raspberry juice due to a commercial enzyme preparation and reported it as ß-1,2-glucosidase activity.

Commercial enzyme preparations are crude fungal preparations, containing impurities such as extraneous enzymes, proteins, mucilage and melanoidins (Martino et al., 1994). Researchers have isolated ß-glucosidase (McCleary and Harrington, 1988; Unno et al., 1993; Martino et al., 1994), ß-galactosidase (Bahl and Agrawal, 1969) and α-L-arabinosidase (Lerouge et al., 1993) from commercial enzyme preparations and all have the potential to destroy anthocyanins.

Mold-contaminated (mainly *Botrytis cinerea*) fruit has also been shown to degrade anthocyanins in strawberry wine (Pilando et al., 1985), strawberry juice (Rwabahizi and Wrolstad, 1988), red raspberry juice (Boyles and Wrolstad, 1993), and red wines (Cassignard et al., 1977), with increasing levels of mold contamination in the fruit accelerating color degradation. Pilando and colleagues (1985) suggested that the decrease in anthocyanin was due to ß-glucosidase activity in the mold.

We previously developed a method involving HPLC and spectrophotometric assays to screen for ß-galactosidase activity, using cranberry juice as a substrate (Wightman and Wrolstad, 1995). We found that enzyme preparations contained various degrees of this side activity which destroyed the galactoside pigments, most noticeably cyanidin-3-galactoside. Pigment destruction was more pronounced when the enzymes were allowed to act on clarified juice than on crushed fruit. Cranberry juice was an effective system to monitor ß-galactosidase and α-arabinosidase activities due to the substantial quantity of these pigments. However, it was not an ideal system to measure ß-glucosidase activity since only a small percentage (c.a. 5%) of glucosidically-bound anthocyanins were present.

Anthocyanins with ß-glucosidic linkages are the most prevalent pigments in anthocyanin-colored fruits (Macheix et al., 1990). Boysenberries (*Rubus* hybrid) contain three ß-glucoside pigments: cyanidin-3-glucoside, cyanidin-3-sophoroside and cyanidin-3-

glucosylrutinoside (Barritt and Torre, 1975; Torre and Barritt, 1977), thus providing an appropriate system for examining ß-glucosidase and ß-1,2-glucosidase activities of enzyme preparations. Our objective was to utilize the analytical screening method previously developed (Wightman and Wrolstad, 1995) to measure ß-glucosidase activity of enzyme preparations which may be used in fruit juice processing. We also employed a modified version of this method to test *Botrytis cinerea* (gray mold), commonly found on overripe fruits in the field, for ß-glucosidase activity which could destroy anthocyanins.

MATERIALS AND METHODS

Juice and Enzyme Samples

Boysenberry, evergreen blackberry and red sour cherry concentrates (68°Brix each) were obtained from Kerr Concentrates, Inc. (Salem, OR) and diluted to 10, 10 and 14°Brix, respectively, with distilled water prior to analysis. Single strength red raspberry juice was produced in the Dept. of Food Science and Technology, Oregon State University pilot plant for a previous study. The following enzymes were evaluated in the screening assays: AR 2000, Cytolase® M219, Cytolase® PCL5, Rapidase® AB Clear, Rapidase® BE, Rapidase® EX Color (Gist-brocades, Inc., King of Prussia, PA); Clarex® L, Clarex® ML, Pearex® 5XL, Vinemax C (Solvay Enzymes, Inc., Elkhart, IN); Bioberry, Biocellulase TRI, Biopectinase 200C, Biopectinase 300L, Biopectinase 7X (Quest International, Sarasota, FL); Novoferm 12, Pectinex® BE 3XL, Pectinex™ Ultra SP-L, Pectinex™ 1XL, Ultrazyme 100G, Vinozyme L (Novo Nordisk Ferment, Ltd., Dittingen, Switzerland); Rohapect® B1L, Rohapect® D5L, Rohapect® FL, Rohapect® MB, Rohapect® VR Super L (Rohm, Darmstadt, Germany).

Strawberry Mold

Botrytis cinerea strain 246 was obtained from Dr. Ken Johnson, Dept. of Botany and Plant Pathology, Oregon State University. This strain was isolated from strawberries in the Willamette Valley, Oregon.

Enzyme Screening

Enzymes were screened in boysenberry juice at their mean recommended dosage and at 0.1% (w/v). For screening with evergreen blackberry, red raspberry and red sour cherry, selected enzymes were added at 0.1% (w/v). Enzymes were diluted appropriately with distilled water and 0.1 mL was added to 10 mL boysenberry juice in 125 mL Erlenmeyer flasks. Samples were heated and prepared for HPLC and spectrophotometric analyses as in Wightman and Wrolstad (1995). Select enzymes, which had recommended conditions different from those above were screened in boysenberry juice (along with controls) under the following conditions: Biopectinase 200C, 2°C, 3 weeks; Vinemax C, 25°C, 2 days; Novoferm 12 and AR 2000, 25°C, 3 weeks. Sodium benzoate (0.1%) was added as a preservative in the controls. All treatments were performed in triplicate.

Mold Screening

Mold spores were added to 10 mL of filtered (0.45 μm) red raspberry and boysenberry juice in 125 mL erlenmeyer flasks. They were incubated in a 25°C waterbath for 7 days to form a mycelia mat. Controls were prepared without mold addition; 0.1% sodium benzoate was added as a preservative. Samples were analyzed by HPLC for individual pigments.

Total Anthocyanins

Monomeric anthocyanin pigment content was determined by the pH differential method of Wrolstad (1976) using a Shimadzu UV160U double beam spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). Anthocyanin content was calculated as mg cyanidin-3-glucoside/100 mL juice using a molar absorptivity of 29,600 and a molecular weight of 445.2 (Wrolstad, 1976). All samples were analyzed in duplicate.

HPLC of Anthocyanins

The sample cleanup procedure I of Hong and Wrolstad (1990) was used to prepare anthocyanins for analysis, using activated C-18 Sep-Pak cartridges (Waters Associates, Milford, MA). A Perkin-Elmer Series 400 Liquid Chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with a Hewlett-Packard diode array detector, Model 1040A, a Hewlett-Packard Series 9000 computer (Hewlett-Packard Co., Palo Alto, CA) and a Beckman Autosampler, Model 501 (Beckman Instruments, Inc., San Ramon, CA) was used. Column: ODS (C-18), 25 cm x 4.6 mm, 12 nm pore, 5 µm particle size (PolyLC, Inc., Columbia, MD) with a C-18 guard column (Spherisorb ODS-2 5U, 10 x 4.6 mm, Alltech Associates, Inc., Deerfield, IL) attached before the column, ambient temperature. Solvents: A = 100% CH_3CN and B = 5% CH_3CN, 10% glacial acetic acid and 1% H_3PO_4 in aqueous solution. Conditions: boysenberry, initial 2% A and 98% B, 15 min linear gradient to 12% A, 5 min linear gradient to 22% A, followed by 5 min equilibration at starting conditions between injections; evergreen blackberry and red sour cherry, isocratic elution with 8% A and 92% B; red raspberry, initial 5% A and 95% B, 15 min linear gradient to 10% A, followed by 5 min equilibration at starting conditions; 1.0 mL/min. Injection volume 50 µL, anthocyanin detection was at 520 nm, 20 nm band-width. All samples were analyzed in duplicate.

Specific Enzyme Determination

ß-glucosidase activity of enzyme preparations was measured by the method of Dopico et al. (1989) at 34°C, pH 4.5 with *ortho*-and *para*-nitrophenol-ß-D-glucopyranoside as substrates. One unit of enzyme was defined as that amount which liberated 1 µmol of *o*-or *p*-nitrophenol/min under specified conditions and with extinction coefficients of 21,300 and 18,300, respectively (Dawson et al., 1986). The specific activities of enzymes were expressed as units/mg of protein in the preparation. Protein was determined by Kjeldahl method (AOAC, 1984). Specific activities and protein determinations were performed in triplicate.

Experimental Design and Statistical Analysis

Experimental designs for enzyme screening assays, specific enzyme activity determinations and protein measurements were randomized complete block designs with enzymes as treatments. All data sets were analyzed by a general linear models procedure with SAS® (Proprietary Software Release 6.04 Copyright 1985, 86, 87 SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Boysenberry juice contains four main anthocyanins (Figure 4.1): cyanidin-3-sophoroside (cyd-3-soph) ~40%; cyanidin-3-glucoside (cyd-3-glu) ~35%; cyanidin-3-glucosylrutinoside (cyd-3-glurut) ~20%; and cyanidin-3-rutinoside (cyd-3-rut) ~5% (Torre and Barritt, 1977). Among these four anthocyanins there are three types of linkages: ß-glucoside, ß-1,2-glucoside and α-rhamnoside. Therefore, it is a good model system to test commercial enzyme preparations for not only ß- and ß-1,2-glucosidase, but also α-rhamnosidase.

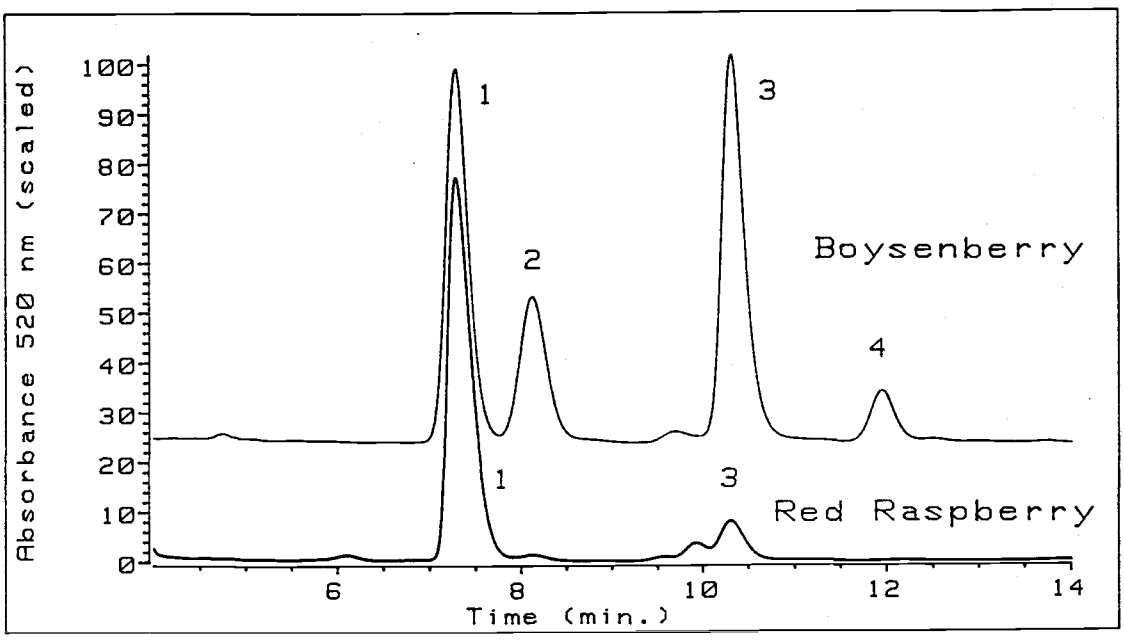

Figure 4.1 HPLC chromatogram of anthocyanins present in boysenberry and red raspberry. Peak: 1 = cyanidin-3-sophoroside, 2 = cyanidin-3-glucosylrutinoside, 3 = cyanidin-3-glucoside, 4 = cyanidin-3-rutinoside.

As Blom (1983) explained, ß-glucosidase activity would cleave off the glucose unit rendering the unstable aglycon, as in the following reaction: cyd-3-glu → cyd + glucose. In the boysenberry system, the effect of ß-1,2-glucosidase activity could be seen in the following equations (Jiang et al, 1990): cyd-3-soph → cyd-3-glu + glucose

cyd-3-glurut → cyd-3-rut + glucose

and α-rhamnosidase activity could cleave off the rhamnose unit: cyd-3-rut → cyd-3-glucose + rhamnose. Commercially available crude fungal enzyme preparations, recommended for fruit juices and wine, were screened for undesirable ß-glucosidase side-activity. As in our previous study (Wightman and Wrolstad, 1995), an enzyme/juice reaction time of 2 hr was used to reflect commercial processing practices. Two dosage rates were analyzed, the mean recommended rate to reflect actual usage rates and 0.1% (by weight) rate to standardize the assay. Both dosage amounts were well below those used

by Tanchev et al. (1969) and Jiang et al. (1990), and more closely reflected true commercial practices as well as Blom's (1983) work. Jiang et al. used up to 2.0% enzyme preparation for 4 hr at 45°C when they observed the destruction of cyd-3-soph to cyd-3-glu and cyd-3-glurut to cyd-3-rut in red raspberry juice, while Blom used 0.1% enzyme for 30 min at 50°C, when he noted a decrease in pelargonidin-3-glucoside (pgd-3-glu) and an increase in the pelargonidin aglycon in strawberry juice. Quantities of individual pigments was determined by the following equation: (% individual peak area determined by HPLC) x (total monomeric anthocyanin determined spectrophotometrically).

At the mean recommended dosage rate (Table 4.1) only one enzyme preparation, Biocellulase TRI, produced a significant decrease in both total monomeric anthocyanin as well as cyd-3-glu ($p<0.05$). Due to the large range of recommended dosages of this enzyme (0.001 to 1.0%), the mean (0.5%) was quite high in comparison to the other preparations. This quantity was also greater than that used in the 0.1% screening assay. At the 0.1% dosage level total monomeric anthocyanin content did not vary ($p>0.05$), but four enzyme preparations exhibited significant destruction of cyd-3-soph (Table 4.2). Since sophoroside consists of two glucose units, it could be destroyed by alternate mechanisms: a) ß-1,2-glucosidase activity could cleave one glucose unit from sophorose leaving cyd-3-glu, which could subsequently be destroyed by ß-glucosidase (however, no statistically significant increase in cyd-3-glu was measured), or b) enzyme activity could cleave sophorose (both glucose units) leaving the unstable aglycon. To explore these mechanisms further, enzymes that destroyed cyd-3-soph were screened using evergreen blackberry juice (Figure 4.2), which contains >80% cyd-3-glu (Rommel et al., 1992). This allowed us to determine if the enzyme preparations caused cyd-3-glu destruction, thus indicating ß-glucosidase activity. The results indicated no significant destruction ($p>0.05$) of total monomeric anthocyanin or the cyd-3-glu (Table 4.3), suggesting the activity was specific for sophorose.

Table 4.1 Total monomeric anthocyanin and individual pigments present in boysenberry juice after heating in the presence of enzyme (mean dosage) for 2 hr at 50°C

			individual pigments (mg/100 mL juice)			
Enzyme	Dosage (%w/v)	Total Monomeric Acn (mg/100 mL)	Cyd-3-soph	Cyd-3-glurut	Cyd-3-glu	Cyd-3-rut
Control	-	102.8	40.1	19.8	36.8	5.4
Ar 2000	0.003	102.1	39.7	20.0	36.3	5.3
Bioberry	0.018	103.5	40.3	20.5	37.1	5.0
Biocellulase TRI	0.500	87.2 *	38.7	19.9	22.5 *	5.3
Biopectinase 200C	0.075	102.9	40.2	20.8	36.3	5.0
Biopectinase 300L	0.001	104.0	40.6	20.9	37.0	5.0
Biopectinase 7x	0.001	102.4	39.8	20.4	36.4	5.1
Clarex L	0.003	100.7	39.2	20.0	35.8	5.1
Clarex ML	0.075	102.2	40.0	20.2	36.3	5.1
Cytolase M219	0.008	102.1	40.1	20.2	36.1	5.1
Cytolase PCL5	0.013	99.8	38.9	19.5	35.8	5.0
Novoferm 12	0.010	100.3	39.0	19.9	35.5	5.1
Pearex 5XL	0.002	105.6	41.2	20.9	37.4	5.5
Pectinex BE 3XL	0.040	100.6	39.2	19.9	35.7	5.3
Rapidase AB Clear	0.004	101.3	39.7	19.8	36.1	5.2
Rapidase BE	0.008	104.0	40.7	20.5	36.9	5.3
Rohapect B1L	0.006	101.8	39.7	19.9	36.3	5.3
Rohapect D5L	0.001	101.2	39.5	19.8	36.1	5.2
Rohapect FL	0.001	99.7	38.9	19.6	35.7	5.0
Rohapect MB	0.004	99.9	38.8	19.6	35.6	5.3
Rohapect VRSL	0.006	101.4	39.4	20.4	36.1	4.9
Ultrazyme 100G	0.001	103.4	40.5	20.4	36.7	5.4
Vinemax C	0.006	103.3	40.5	20.6	36.5	5.1
Vinozyme L	0.005	104.5	40.6	20.4	37.5	5.4
Standard errors		1.7	0.7	0.5	0.6	0.2

* significantly different from control $p<0.05$

Table 4.2 Total monomeric anthocyanin and individual pigments present in boysenberry juice after heating in the presence of enzyme (0.1%) for 2 hr at 50°C

		individual pigments (mg/100 mL juice)			
Enzyme	Total Monomeric Acn (mg/100 mL)	Cyd-3-soph	Cyd-3-glurut	Cyd-3-glu	Cyd-3-rut
Control	100.0	38.6	19.0	36.3	5.2
AR 2000	93.3	34.8 *	17.4	34.8	5.3
Bioberry	99.5	38.2	19.0	35.8	5.5
Biocellulase TRI	96.0	38.0	18.9	32.7	5.4
Biopectinase 200C	97.7	37.7	18.5	35.4	5.1
Biopectinase 300L	96.6	37.4	18.5	34.8	5.1
Biopectinase 7x	99.0	38.5	19.0	35.6	5.1
Clarex L	98.3	38.0	18.5	35.6	5.3
Clarex ML	100.2	38.5	19.3	36.1	5.3
Cytolase M219	100.3	38.9	19.3	35.9	5.3
Cytolase PCL5	98.7	38.7	18.9	35.3	4.8
Novoferm 12	96.9	37.3	18.5	35.0	5.0
Pearex 5XL	95.7	34.1 *	17.4	36.7	6.5 *
Pectinex 1XL	97.0	36.8	17.2	36.9	5.3
Pectinex BE 3XL	101.2	38.9	19.1	36.8	5.4
Pectinex Ultra SP-L	97.1	36.5 *	17.8	36.8	5.0
Rapidase AB Clear	97.9	37.4	18.5	35.8	5.3
Rapidase BE	98.8	38.0	18.6	36.2	5.1
Rapidase EX Color	101.3	39.4	19.4	36.4	5.3
Rohapect B1L	100.5	38.6	19.1	36.4	5.3
Rohapect D5L	97.6	36.8	18.0	36.6	5.4
Rohapect FL	96.9	36.5 *	18.3	35.9	5.3
Rohapect MB	99.6	38.4	19.2	35.7	5.4
Rohapect VRSL	99.6	37.7	18.8	36.0	5.2
Ultrazyme 100G	100.2	38.6	19.2	36.0	5.3
Vinemax C	98.9	37.4	18.8	35.8	5.7 *
Vinozyme L	102.5	39.4	19.6	37.2	5.4
Standard errors	1.7	0.7	0.5	1.4	0.2

* significantly different from control $p<0.05$

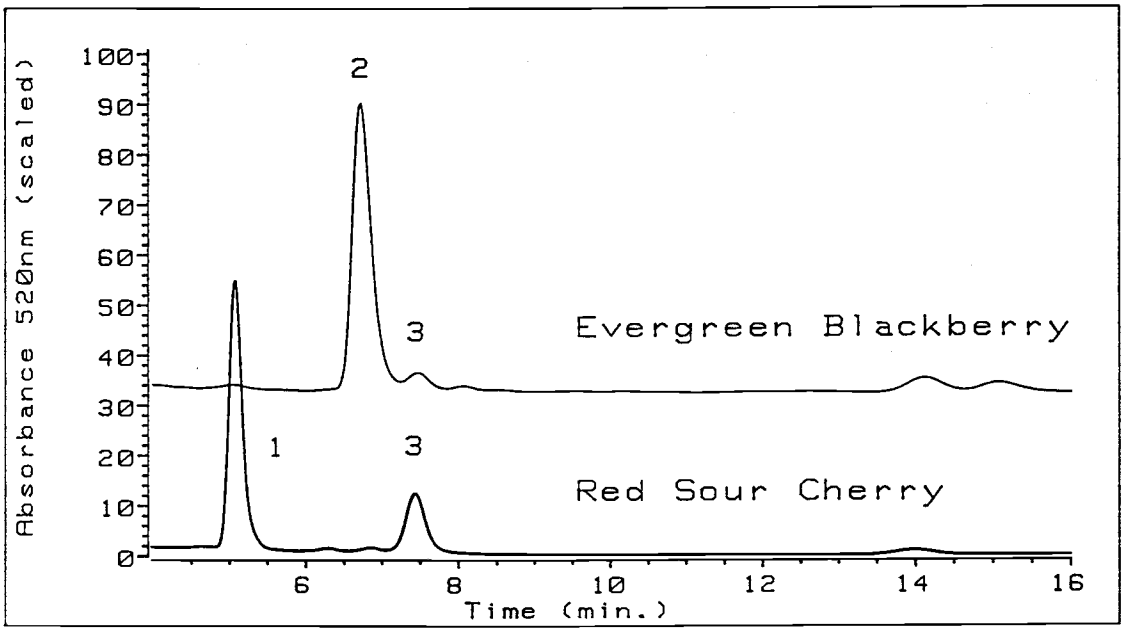

Figure 4.2 HPLC chromatogram of anthocyanins present in Evergreen blackberry and red sour cherry juices. Peak: 1 = cyanidin-3-sophoroside, 2 = cyanidin-3-glucoside, 3 = cyanidin-3-rutinoside.

Table 4.3 Total monomeric anthocyanin and individual pigments in evergreen blackberry, red raspberry and red sour cherry after heating with enzyme (0.1%) for 2 hr at 50°C

		individual pigments (mg/100 mL juice)			
Enzyme	Total Monomeric Acn (mg/100 mL)	Cyd-3-soph	Cyd-3-glu	Cyd-3-glurut	Cyd-3-rut
BLACKBERRY					
Control	27.6	-[a]	21.7	-	1.4
Ar 2000	27.4	-	21.9	-	1.4
Biocellulase TRI	27.6	-	21.4	-	1.4
Pearex 5XL	28.7	-	22.6	-	1.4
Pectinex Ultra SP-L	28.8	-	22.8	-	1.4
Rohapect FL	28.3	-	22.3	-	1.4
RED RASPBERRY					
Control	72.4	58.2	6.3	-	-
Ar 2000	73.2	53.6 *	9.4 *	-	-
Biocellulase TRI	72.9	60.1	6.1	-	-
Pearex 5XL	73.1	53.3 *	10.3 *	-	-
Pectinex Ultra SP-L	74.2	59.2	7.6 *	-	-
Rohapect FL	73.9	60.1	7.8 *	-	-
RED SOUR CHERRY					
Control	5.9	-	-	4.2	1.4
AR 2000	5.7	-	-	4.0	1.3
Pearex 5XL	5.9	-	-	4.1	1.5
Pectinex Ultra SP-L	5.9	-	-	4.2	1.4

[a] designates anthocyanin is not present in the juice
* designates significantly different from control $p<0.05$

These enzymes were then screened using red raspberry juice (Figure 4.1) which contains cyd-3-soph (~ 80%) and cyd-3-glu (~ 11%) (Misic, 1973; Barritt and Torre, 1973, 1975; Torre and Barritt, 1977; Spanos and Wrolstad, 1987; Boyles and Wrolstad, 1993). While there was again no apparent decrease in total monomeric anthocyanin content, two preparations did significantly ($p<0.05$) decrease cyd-3-soph with a corresponding increase in cyd-3-glu, thus indicating ß-1,2-glucosidase activity. This was the same type of activity reported by Jiang et al. (1990).

At the 0.1% dosage level, two preparations produced a significant ($p<0.05$) increase in cyd-3-rut (Table 4.2). This increase would be possible if the glucose unit was cleaved off of cyd-3-glurut by a ß-1,2-glucosidase activity, leaving cyd-3-rut (although no significant decrease in cyd-3-glurut was noted). Since cyd-3-rut is found in small quantities in boysenberry juice (~ 5%), we wanted to test this hypothesis with a juice containing larger quantities of cyd-3-glurut and cyd-3-rut. Enzyme preparations that resulted in a significant increase in cyd-3-rut or a large decrease in cyd-3-glurut in boysenberry juice were subsequently tested with red sour cherry juice (Figure 4.2). Red sour cherry contains cyd-3-glurut (~ 70%) and cyd-3-rut (~ 24%) (Macheix et al., 1990). The results (Table 4.3) indicated no significant decrease ($p>0.05$) in cyd-3-glurut as well as no increase in cyd-3-rut. In correlating the boysenberry, evergreen blackberry, red raspberry, and red sour cherry results, we concluded that some of the commercial enzyme preparations do contain ß-1,2-glucosidase and ß-glucosidase activities. The extent of ß-1,2-glucosidase activity and subsequent anthocyanin destruction was not the same in each juice and may be due to a number of other factors such as other pigments present in the juice and overall quantity of total monomeric anthocyanin. Increased pigment concentration may enhance color stability through intermolecular copigmentation and self-association (Mazza and Miniati, 1993).

Due to the diverse applications of a few of the enzyme preparations (e.g. for wine or cold grape juice processing), manufacturers' recommendations regarding temperature and times varied greatly from the assay conditions in this study. For these reasons we evaluated select enzymes using boysenberry juice under conditions more closely matching manufacturers' recommendations. Juices treated with Biopectinase 200C, Vinemax C, and Novoferm 12 did not differ significantly ($p>0.05$) in total monomeric anthocyanin or individual pigments from controls held under the same conditions. However, AR 2000 samples were significantly lower ($p<0.05$) than control in total monomeric anthocyanin after 3 weeks at 25°C, 36.7 versus 59.0 mg/100 mL, respectively.

Protein content and specific activity of ß-glucosidase were compared in the enzyme preparations (Table 4.4). ß-glucosidase activity was measured using both *ortho* and *para*-nitrophenyl-ß-D-glucopyranoside (ONPG and PNPG) as substrates to determine if there was a difference in affinity. We found consistent results between the substrates; preparations that had the highest activity with ONPG also had the highest activity with PNPG. In comparing these colorimetric measurements with ß-glucosidase activity determined by HPLC (Tables 4.1 and 4.2), high activity measured by the spectrophotometric ONPG method did not always correlate to large pigment destruction. Although many of the preparations contained high ß-glucosidase activity as measured by the ONPG method, they exhibited low anthocyanin-destroying activity. We found these inconsistencies to be true in our previous ß-galactosidase study as well (Wightman and Wrolstad, 1995). These results further our belief that the HPLC assay procedure is a more reliable index of anthocyanin pigment stability in juice processing than the direct measure of ß-glucosidase activity in the preparation by conventional methods.

Boysenberry and red raspberry juices which had been inoculated with *Botrytis cinerea* were classified as heavy or light growth depending on the extent of the mycelia mat. HPLC analyses showed that heavy mold growth destroyed ß-glucosidic anthocyanins in boysenberry juice (Figure 4.3), producing a dark brown juice as compared to the control. Anthocyanin destruction was less pronounced in samples with light mold growth.

Table 4.4 Protein and ß-glucosidase activity of commercial enzyme preparations measured using two substrates

		ß-glucosidase (Units/µg protein)	
Enzyme	Protein (mg/g)[a]	ONPG[b]	PNPG[c]
Ar 2000	111.2	310	1754
Bioberry	15.3	42	248
Biocellulase TRI	86.2	84	466
Biopectinase 200C	37.3	57	316
Biopectinase 300L	43.2	12	64
Biopectinase 7x	68.6	3	15
Clarex L	5.8	87	439
Clarex ML	31.3	67	358
Cytolase M219	33.5	34	228
Cytolase PCL5	34.5	198	1013
Novoferm 12	92.5	263	1598
Pearex 5XL	65.9	187	927
Pectinex 1XL	8.2	37	188
Pectinex BE 3XL	28.7	215	1095
Pectinex Ultra SP-L	52.8	9	35
Rapidase AB Clear	8.3	14	71
Rapidase BE	19.2	29	152
Rapidase EX Color	42.6	10	58
Rohapect B1L	18.2	112	581
Rohapect D5L	25.5	76	401
Rohapect FL	24.4	40	247
Rohapect MB	15.0	19	96
Rohapect VRSL	29.0	24	142
Ultrazyme 100G	41.0	21	201
Vinemax C	51.5	95	468
Vinozyme L	13.9	15	74
Standard errors	1.6	3	20

[a] Total protein determined by Kjeldahl, using 6.25 as the multiplication factor

[b] One unit will hydrolyze 1.0 µmole of *o*-nitrophenyl-ß-D-glucopyranoside to *o*-nitrophenol and glucose per minute at pH 4.5 at 34°C. Calculated with a millimolar extinction coefficient of *o*-nitrophenol as 21.3

[c] One unit will hydrolyze 1.0 µmole of *p*-nitrophenyl-ß-D-glucopyranoside to *p*-nitrophenol and glucose per minute at pH 4.5 at 34°C. Calculated with a millimolar extinction coefficient of *p*-nitrophenol as 18.3

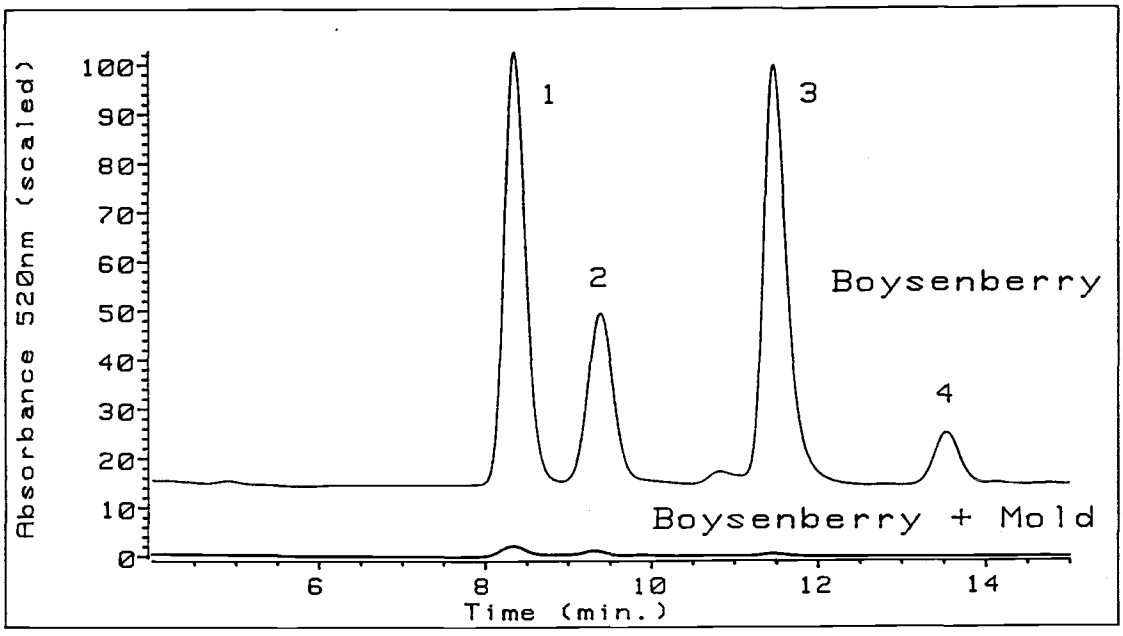

Figure 4.3 Effect of heavy mold (*Botrytis cinerea*) on boysenberry anthocyanins relative to a control. Peak: 1 = cyanidin-3-sophoroside, 2 = cyanidin-3-glucosylrutinoside, 3 = cyanidin-3-glucoside, 4 = cyanidin-3-rutinoside.

CONCLUSIONS

Anthocyanin-destroying ß-glucosidase activity in juice processing enzymes was much lower and less prevalent than that found previously for ß-galactosidase (Wightman and Wrolstad, 1995). In our previous study, ß-galactosidase activity was found in 24 out of 27 commercial enzyme preparations. These preparations produced significant ($p<0.05$) destruction of cyanidin-3-galactoside, the major pigment in cranberries. This indicated that ß-galactosidase was present in many of the commercial enzyme preparations and fruit processors should note this when working with fruits containing galactoside anthocyanins. Since ß-glucoside linkages are the most prevalent in anthocyanin containing fruits, we examined ß-glucosidase activity in commercial enzyme preparations for this study. We found when preparations were used within their recommended dosage levels there

appeared to be little pigment destruction, as determined by the screening assay. However, when excessive levels were used there was a greater possibility of anthocyanin destruction. Common gray mold (*Botrytis cinerea*) on fruit is also another possible source for anthocyanin-destroying ß-glucosidase activity, as shown by the degradation of boysenberry anthocyanins. While this work was qualitative, it can serve as a basis for future research to determine how mold contaminated fruit may effect the color quality and anthocyanins in the final juice product.

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CHAPTER 5

EFFECT OF PROCESSING ENZYMES ON ANTHOCYANINS AND PHENOLICS IN PINOT NOIR AND CABERNET SAUVIGNON WINES

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ABSTRACT

Pinot noir and Cabernet Sauvignon wines were made with four commercial enzyme preparations along with controls. Total monomeric anthocyanin and individual pigments changes were monitored throughout winemaking and storage using spectrophotometric and HPLC methods. A complete phenolic profile of the wines was determined at the completion of fermentation by HPLC. Three of the enzyme treated Pinot noir wines contained significantly less total monomeric anthocyanin than control ($p<0.05$) and destroyed the major pigment, malvidin-3-glucoside. Pigment changes were less pronounced in the Cabernet Sauvignon wines as compared to Pinot noir. Two enzyme treated Cabernet Sauvignon wines contained less ($p<0.05$) malvidin-3-glucoside, 3-glucosylacetate, and 3-glucosylcoumarate, than did the control. One of the enzymes appeared to hydrolyze quercetin glucuronide with a concomitant rise in the aglycon. Trans-resveratrol content increased in all enzyme treated wines and those that had the greatest resveratrol content also contained the greatest quantity of quercetin aglycon. Enzymes appeared to contain varying degrees of an esterase activity as shown by the cleavage of tartaric acid from caffeoyl tartrate to form caffeic acid. These activities occurred in both Cabernet Sauvignon and Pinot noir.

INTRODUCTION

Pectolytic enzymes are used in juice and wine processing to increase juice yield, facilitate color extraction and stability, and to facilitate clarification (Felix and Villettaz, 1983; Lanzarini and Pifferi, 1989; Voragen and van den Broek, 1991). Since the 1950's there have been contradictory findings as to the effect of pectic enzymes on color intensity of wines. Commercial pectolytic and macerating enzyme preparations have been reported to promote color extraction in the processing of red grapes or wine (Cruess et al., 1955; Ough and Berg, 1974; Ough et al., 1975; Shoseyov et al., 1990; Zent and Inama, 1992;

Plank and Zent, 1993). Haight and Gump (1994) reported "perceived differences" in color extraction, but no statistical difference, when comparing grape juice made with commercial enzymes versus a control with no enzyme; enzyme-treated juices had more color. Others have shown pectolytic enzymes to cause a decrease in total anthocyanin or color intensity in grape wine (Berg and Marsh, 1956; Blouin and Barthe, 1963; Montedoro and Bertuccioli, 1976) and fruit wine (Yang and Steele, 1958; Withy et al., 1993). Berg and Marsh (1956) also used pectic enzymes in port production and found that one lot increased in color intensity while one lot lost color. They suggested that the fungal enzymes that were used may have contained ß-glucosidase activity which destroyed the anthocyanins, in reference to Huang's (1955) work with blackberry pigments and wine.

Anthocyanin decomposition is an undesirable side-activity in fungal enzyme preparations that has been reported in other fruits as well: blackberry wine, jellies, and jams (Yang and Steele, 1958), chrysanthemin isolated from blackberry (Huang 1955; 1956) cherry nectar (Blom and Juul, 1982), cranberry (Wightman and Wrolstad, 1995), raspberry (Jiang et al., 1990), and strawberry juices (Tanchev et al., 1969; Blom, 1983). Enzyme concentration has also been shown to have a profound effect on wine color. Bulgarian wines prepared from pectic enzyme-treated Zaitchine grapes contained almost 18% more color than a control wine when 0.3% enzyme was used, but 60% less color than control when 0.7% of the same enzyme was used (Neubeck, 1975).

Commercial enzymes are typically crude fungal preparations, containing impurities such as extraneous enzymes, proteins, mucilage and melanoidins (Martino et al., 1994). Researchers have isolated ß-glucosidase (McCleary and Harrington, 1988; Unno et al., 1993; Martino et al., 1994) from commercial enzyme preparations. This enzyme can cleave the sugar from the anthocyanin, leaving the unstable aglycon which spontaneously transforms into a colorless form (Huang, 1956). Evidence of anthocyanin-destroying ß-glycosidase activity (Blom, 1983; Blom and Thomassen, 1985) and ß-1,2-glucosidase activity (Jiang et al., 1990) in commercial preparations has been reported.

In our laboratory, we have developed a method involving HPLC and spectrophotometric assays to screen for possible ß-galactosidase (Wightman and Wrolstad, 1995) and ß-glucosidase (Wightman and Wrolstad, unpublished) activity utilizing cranberry and boysenberry juice, respectively. We found that commercial enzyme preparations contained various degrees of these side activities which destroyed both galactoside and glucoside pigments. The objective of this research was to utilize this analytical method to determine if grape and wine pigments could also be destroyed by ß-glucosidase activity from commercial enzyme preparations. Pinot noir contains five ß-3-glucosides, delphinidin (dpn), cyanidin (cyd), petunidin (ptd), peonidin (pnd), and malvidin (mvd), while Cabernet Sauvignon contains these five anthocyanins plus acylated forms (Macheix et al., 1990). We also wanted to determine if acylation had an inhibitory effect on the destructive properties of the enzymes. In conjunction with possible anthocyanin degradation, we monitored other phenolic compounds to determine what effect the enzyme preparations had on them.

MATERIALS AND METHODS

Enzymes

The following enzymes were evaluated at their highest recommended dosage for wine (w/v): AR 2000 (0.005%), Cytolase® PCL5 (0.005%), Rapidase® EX Color (0.005%), Gist-brocades, Inc., King of Prussia, PA; Rohapect® VR Super L (0.010%), Rohm, Darmstadt, Germany. Another treatment, Cytolase PCL5 (0.02%), was evaluated only in Pinot noir. Control treatments with no enzyme addition were also made. All treatments were prepared in triplicate lots.

Grapes and Winemaking

Pinot noir grapes were harvested from Woodhall III Vineyard, Willamette Valley, Oregon in Sept 94, with initial measurements of: pH 3.15; 0.560 g tartaric acid/100 mL; 23.5°Brix. Cabernet Sauvignon grapes were harvested from the same vineyard in Oct 94, with initial measurements of: pH 3.25 ; 0.726 g tartaric acid/100 mL; 22.4°Brix. Grape clusters were randomly divided into 3.5 kg lots. Each lot was crushed, destemmed manually, and 50 mg/L SO_2 was added. After 1 hr, enzymes were added and the musts were mixed thoroughly and inoculated with yeast, 0.2 g/L, Levure Bourgorouge RC212 (Lalvin, Lallemand, Inc., Montréal, Canada). Fermentation on the skins took place in 3.8 L wide-mouth jars in a 25 to 30°C waterbath. Musts were punched down twice daily. After fermentation the wines were inoculated with *Leuconostoc oenos* (OSU Lalvin, Lallemand, Inc., Montréal, Canada). Upon completion of malolactic fermentation, the wines were cold-stabilized at 5°C and remained there until bottling.

Samples

Samples from each lot were collected for analyses: from the starting must before SO_2 addition (0 days), during maceration (1, 2, 3, 5, and 7 days), after pressing (9 days), at the end of alcoholic fermentation (28 days), at the end of malolactic fermentation (92 days), after cold stabilization (114 days), and 1.5 months later (160 days). Sample vials were filled completely to minimize oxygen contact and immediately frozen at -10°C. Titratable acidity and pH were measured prior to freezing on days 0, 28, 92, and at bottling (Zoecklein et al., 1990). Day 0 samples were also analyzed for °Brix. At bottling (Pinot noir, day 190; Cabernet Sauvignon, day 174), wine samples were analyzed for percent alcohol with an ebulliometer (Zoecklein et al., 1990) and for total phenols by the Folin-Ciocalteu method (Singleton, 1988).

Total Anthocyanins

Monomeric anthocyanin pigment content, percent polymeric color and color density were determined on filtered (0.45 μm, Millipore Corporation, Bedford, MA) samples by the pH differential method of Wrolstad (1976) using a Shimadzu UV160U double beam spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). Anthocyanin content was calculated as mg malvidin-3-glucoside/L wine using a molar absorptivity of 28,000 and a molecular weight of 493.5 (Niketic-Aleksic and Hrazdina, 1972).

HPLC of Anthocyanins

Samples were filtered (0.45μm) prior to analysis. A Perkin-Elmer Series 400 Liquid Chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with a Hewlett-Packard diode array detector, Model 1040A, a Hewlett-Packard Series 9000 computer (Hewlett-Packard Co., Palo Alto, CA) and a Beckman Autosampler, Model 501 (Beckman Instruments, Inc., San Ramon, CA) were used. Column: PLRP-S, 25 cm x 4.6 mm, 5 μm particle size (Polymer Laboratories, Inc., Amherst, MA) with a guard column (PLRP-S, 30 x 5 mm, Polymer Laboratories, Inc., Amherst, MA) attached before the column, ambient temperature. Solvents: A = 100% CH_3CN and B = 4% H_3PO_4; 1.0 mL/min. Pinot noir conditions: 10 min at 10% A and 90% B, 15 min linear gradient to 15% A, 2 min linear gradient to 35% A, 5 min isocratic at 5% A, followed by 10 min linear gradient to 50% A. The column was re-equilibrated to the starting solvent for 20 min between runs. Cabernet Sauvignon conditions: 10 min at 10% A and 90% B, 25 min linear gradient to 20% A, 20 min linear gradient to 30% A, 7 min linear gradient to 100% A, followed by 5 min isocratic at 100% A. The column was re-equilibrated to the starting solvent for 25 min between runs. Injection volume 50 μL; anthocyanin detection was at 520 nm, 20 nm bandwidth; phenolics at 360 nm, 10 nm bandwidth. Peaks were identified by spectral comparison to known standards and by retention times.

Individual Pigment Calculations

Individual pigment quantities were estimated by the following equation: (percent peak area of total monomeric anthocyanins from HPLC chromatogram) x (total monomeric anthocyanin determined spectrophotometrically).

HPLC Analysis of Anthocyanidins

The same HPLC, detector/integrator system, column and conditions as used for anthocyanin analyses were used. The hydrolysis and concentration procedures described by Hong and Wrolstad (1986) were used with an initial sample of 2 mL Pinot noir wine directly adsorbed onto a Sep-Pak without NaCl addition. With Cabernet Sauvignon, the anthocyanins were first saponified by the method of Hong and Wrolstad (1990) and then hydrolyzed as above. UV-Vis spectra were taken directly from chromatographic runs.

Color Measurements

At bottling all lots were analyzed for CIE L*, a*, b* values with a HunterLab ColorQUEST® Sphere with Universal Software version 2.2.2 (Reston, VA). Analysis was done in the reflectance mode, specular included, with a 10° observer angle and D65 light source. Three readings were averaged for each lot and then the values for the 3 lots per treatment were averaged. Chroma $(a^{*2} + b^{*2})^{1/2}$ and hue angle $(\tan^{-1} b^*/a^*)$ were calculated from CIE a* and b*.

HPLC Analysis of Phenolics

A complete chromatographic analysis of each lot at day 28 (end of alcoholic fermentation) was determined using the procedure of Price et al. (1995). Samples were centrifuged in an Eppendorf Microfuge (5415 C) for 5 min at 13,000 RCF prior to

injection. The same HPLC equipment and elution gradient as cited were used for both Pinot noir and Cabernet Sauvignon samples. Spectra were recorded at 280, 320, 360, and 520 nm.

Experimental Design and Statistical Analysis

Experimental design for the Pinot noir and Cabernet Sauvignon trials was a split in time with fermentors in a completely randomized design having enzymes as treatments (Steel and Torrie, 1980). All data sets were analyzed by a general linear models procedure with SAS® (Proprietary Software Release 6.04 Copyright 1985, 86, 87 SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Enzymes were chosen for this study based on manufacturers' recommendation for use in red wines, except AR 2000 which is recommended for use in white wines. This enzyme was added to the trial as a positive control since it is marketed as containing high enzymatic activity to "transform the non-aromatic terpenic precursors into free aromatic terpenols" in white wines, thus containing ß-glucosidase which has been shown to destroy anthocyanins.

Total Anthocyanin

In Pinot noir total monomeric anthocyanin over time differed between the treatments ($p<0.05$). Overall, Pinot noir wines treated with Rapidase EX Color and Rohapect VRSL did not significantly differ ($p>0.05$) from the control in total monomeric anthocyanin content. However, with AR 2000 and both concentrations of Cytolase PCL5, total monomeric anthocyanin was significantly reduced ($p<0.05$).

The main difference among the treatments was observed within the first 2 days of fermentation (Figure 5.1). Twenty-four hr after inoculation with yeast there was almost a two-fold difference among treatments in the amount of anthocyanin extracted from the skins; Cytolase PCL5 (0.005%)-treated musts contained the most anthocyanin and control the least. At this point the pectolytic enzymes were breaking down cell walls and releasing pigments from vacuoles within the skin (Voragen and van den Broek, 1991). After 48 hr, the difference between treatments was less than 1.2-fold. Also by day 2 the destructive effect of AR 2000 on anthocyanins was apparent, with a significant reduction in anthocyanin content compared to other treatments for the rest of the process. The amount of monomeric anthocyanin in the must peaked at day 9 (pressing) for most of the treatments except for both Cytolase PCL5's , for which the peak was day 7. The inset of Figure 5.1 shows that total anthocyanin contents due to enzyme-treatment are parallel after day 28 and follow the same basic polymerization and subsequent loss of monomeric anthocyanin trend, with the only difference being the level of anthocyanin present. The effect of a four-fold difference in enzyme concentration is quite profound as seen between Cytolase PCL5 0.005% and 0.02%.

Rates of pigment extraction among treatments of Cabernet Sauvignon did not vary as greatly as in Pinot noir (Figure 5.2). Differences in total monomeric anthocyanin are not apparent until day 7 when the amount of monomeric anthocyanin in AR 2000 begins to decrease. The amount of pigment extraction peaked in the other treatments on day 7. As with Pinot noir, the rate of disappearance of monomeric anthocyanin among treatments was the same, with the starting point set by day 9. Over the entire time, AR 2000 and Cytolase PCL5 are significantly different from control, $p<0.05$ and 0.055, respectively.

The amount of polymeric anthocyanin, measured at 520 nm by HPLC, followed the inverse trend of the monomeric; treatments with the highest monomeric anthocyanin had the lowest percent of polymeric anthocyanin (Figure 5.3) for Pinot noir and Cabernet

Sauvignon. Percent polymeric anthocyanins, determined spectrophotometrically, also followed this pattern.

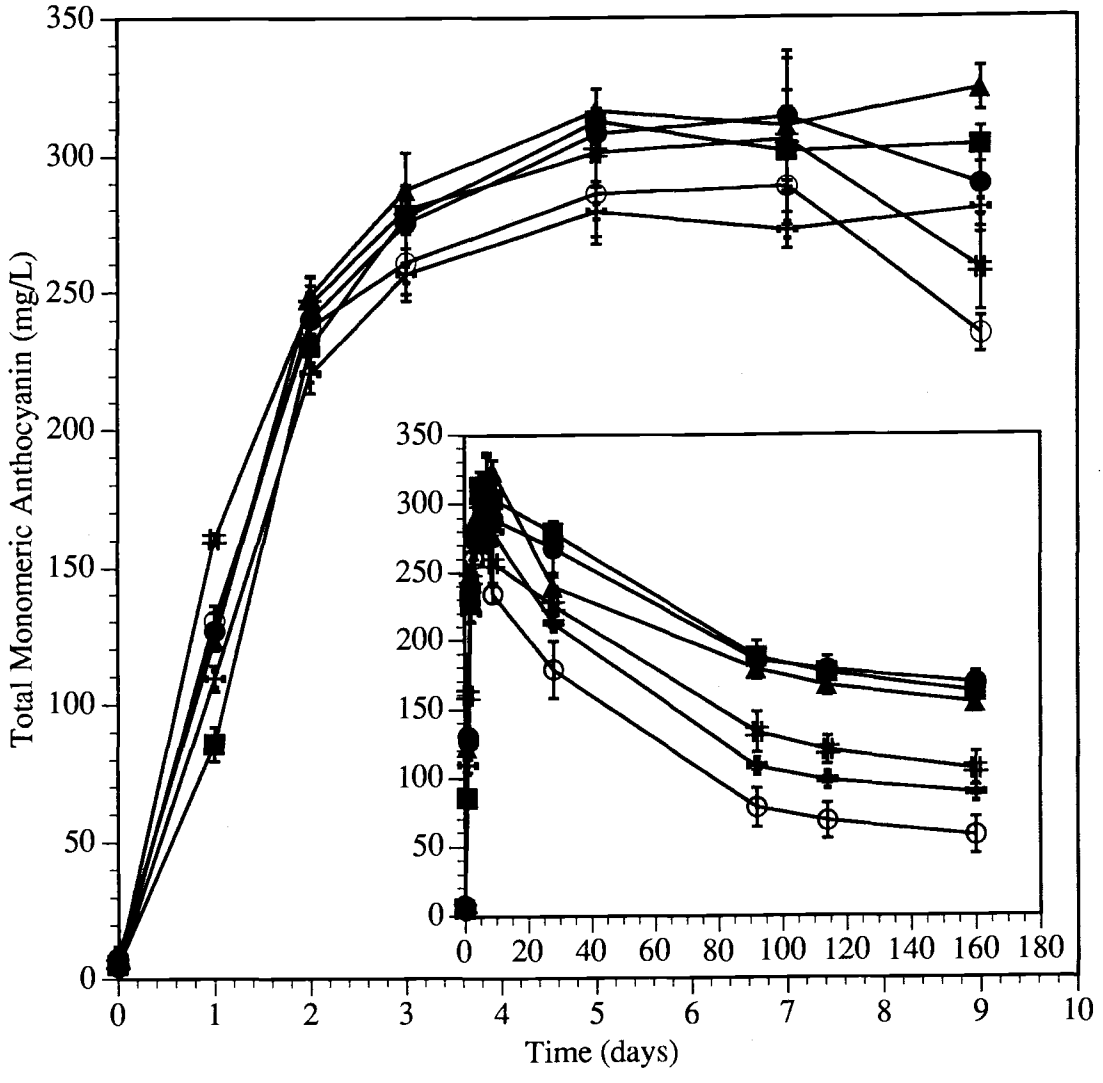

Figure 5.1 Total monomeric anthocyanin in Pinot noir wine; ■ Control, ● Rapidase EX Color, ▲ Rohapect VRSL, ✣ AR 2000, # Cytolase PCL5 (0.005%), and o Cytolase PCL5 (0.02%).

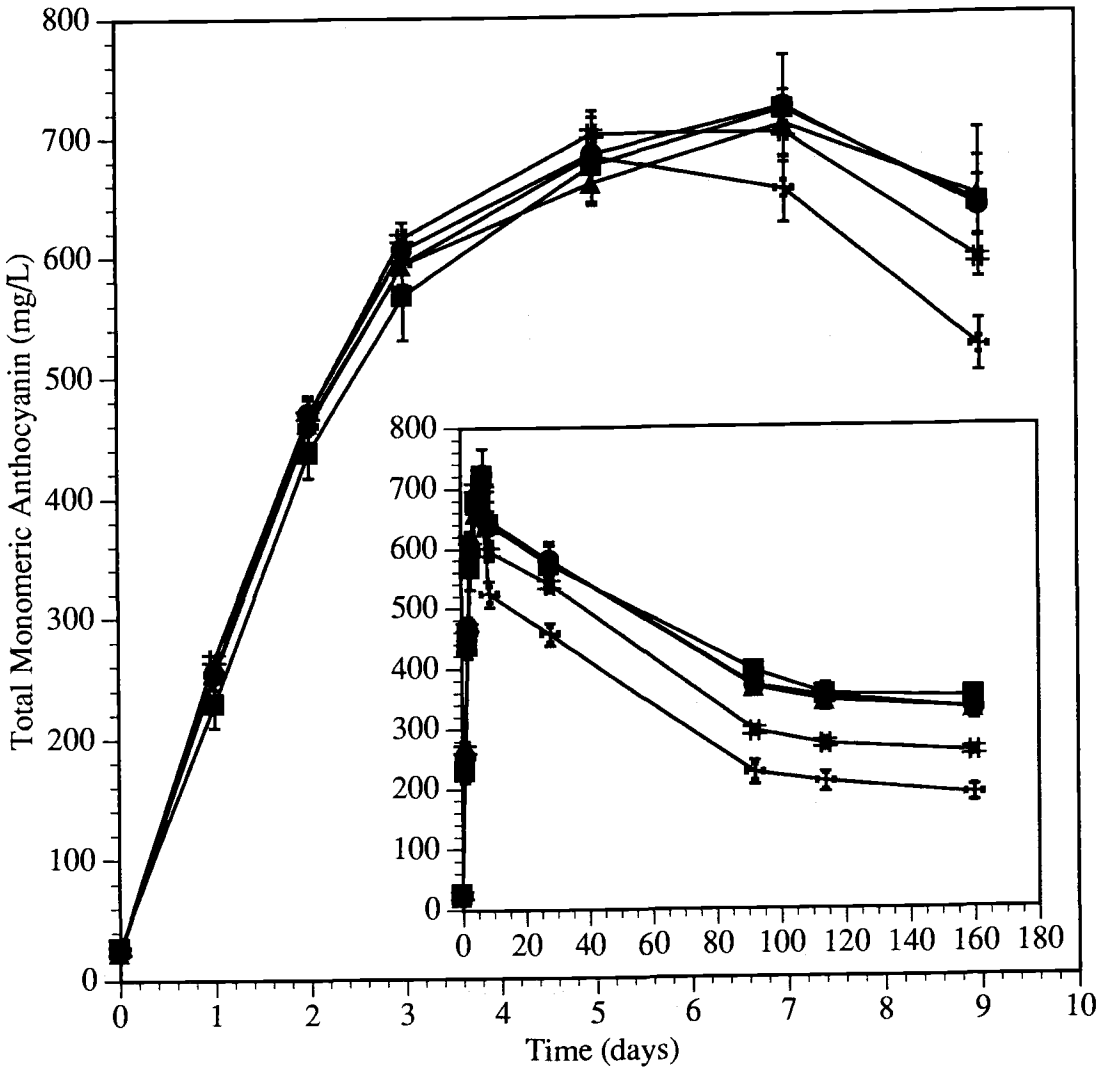

Figure 5.2 Total monomeric anthocyanin in Cabernet Sauvignon wine; ■ Control, ● Rapidase EX Color, ▲ Rohapect VRSL, ✣ AR 2000, # Cytolase PCL5 (0.005%).

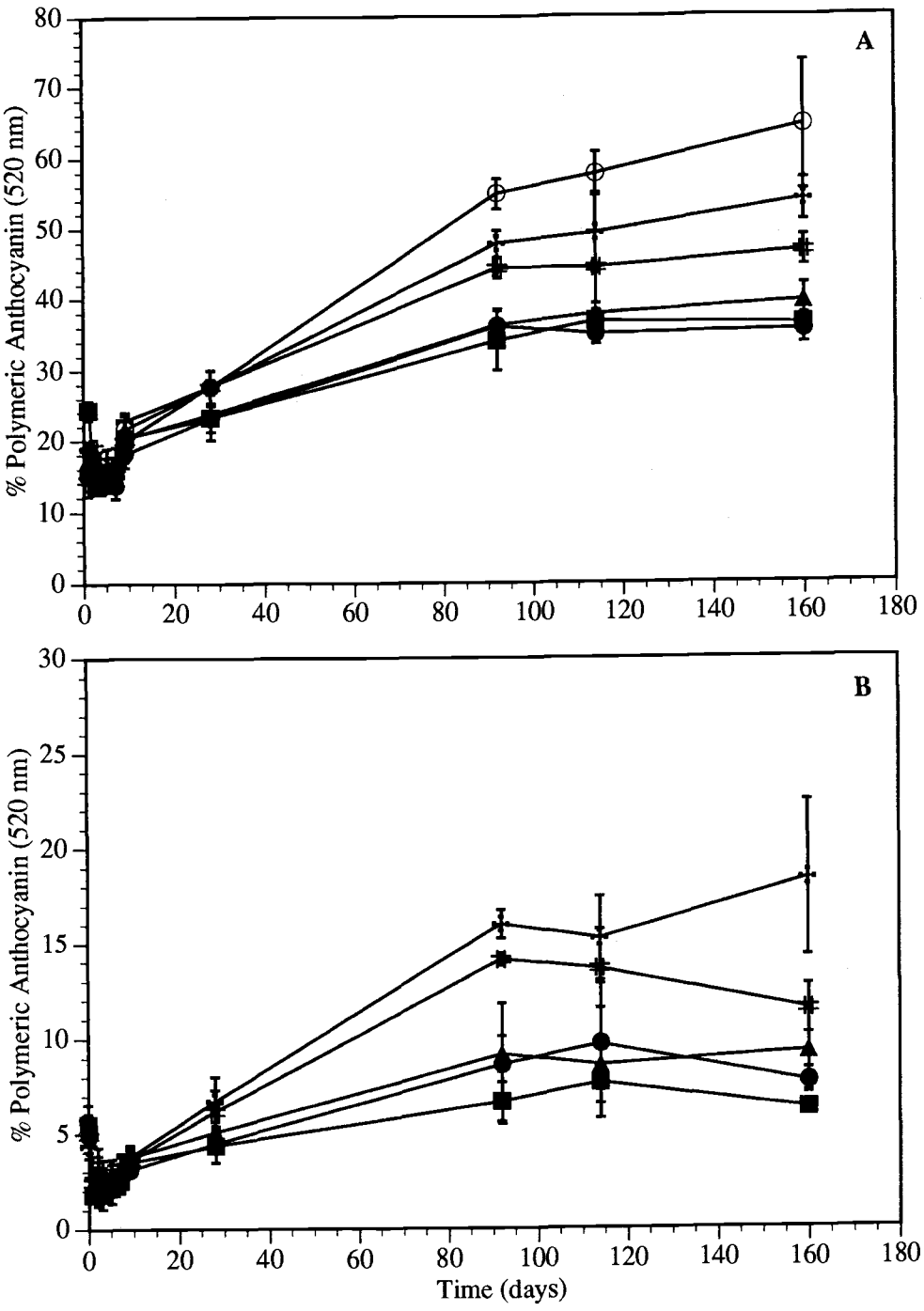

Figure 5.3 Percent polymeric anthocyanin in Pinot noir (A) and Cabernet Sauvignon (B) wines determined by HPLC, 520 nm; ■ Control, ● Rapidase EX Color, ▲ Rohapect VRSL, ✣ AR 2000, # Cytolase PCL5 (0.005%), and o Cytolase PCL5 (0.02%).

Individual Pigments

Individual pigments were monitored by HPLC (Figures 5.4 and 5.5). In general the extraction and disappearance of individual anthocyanins followed the same patterns as total monomeric anthocyanin. AR 2000 and Cytolase PCL5 (0.005% and 0.020%)-treated wines were significantly lower ($p<0.05$) than control in pnd-3-glucoside and mvd-3-glucoside (Figure 5.6). With ptd-3-glucoside, only wines treated with AR 2000 and Cytolase PCL5 0.020% were significantly different ($p<0.05$) from control, both were lower. There were no significant differences ($p>0.05$) in cyd-3-glucoside content due to enzyme treatment and only Rapidase EX Color caused a significant increase ($p<0.05$) in dpn-3-glucoside, relative to control.

A distinct peak appeared in chromatograms of AR 2000-treated Pinot noir wines which matched the retention time of malvidin aglycon. In comparing the spectra of the aglycon and the new peak, the latter contained a higher 280:520 nm ratio of the peak's spectra than that of the aglycon, indicating polymerization (Kantz and Singleton, 1990).

Cabernet Sauvignon contains the same anthocyanins as well as acetic and coumaric acylated forms. Due to the large number and therefore relatively small quantities of some pigments, the following anthocyanins were monitored by HPLC: dpn, cyd, ptd, pnd, mvd-3-glucoside, mvd-3-glucosylacetate, and mvd-3-glucosylcoumarate. Acetic acid acylated pigments other than mvd (other acetates) were monitored as a group as well as coumarate acylated pigments other than mvd (other coumarates). Individual pigments followed the same general trend, peaking after 7 to 9 days and then slowly decreasing. Mvd-3-glucoside and its acylated forms are shown in Figure 5.7. Overall, AR 2000 was the only treatment significantly different ($p<0.05$) from control in dpn-3-glucoside, ptd-3-glucoside, other acetates and mvd-3-glucosylacetate. AR 2000 and Cytolase PCL5 were both significantly different ($p<0.05$) from control in mvd-3-glucoside and mvd-3-glucosylcoumarate. Rapidase EX Color was significantly different ($p<0.05$) from control,

higher, in pnd-3-glucoside and mvd-3-glucosylcoumarate. There were no significant differences between treatments in cyd-3-glucoside or other coumarates. Acylation did not appear to inhibit anthocyanin destruction.

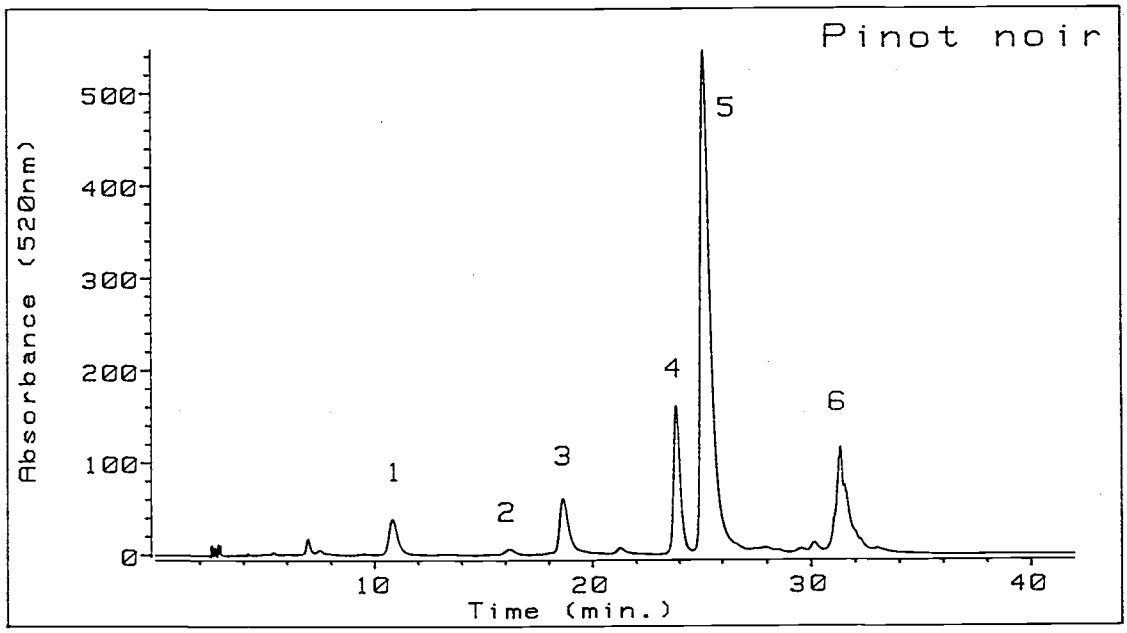

Figure 5.4 HPLC chromatogram, at 520 nm, of Pinot noir anthocyanins. Peak 1 = delphinidin-3-glucoside, 2 = cyanidin-3-glucoside, 3 = petunidin-3-glucoside, 4 = peonidin-3-glucoside, 5 = malvidin-3-glucoside, 6 = polymeric anthocyanins.

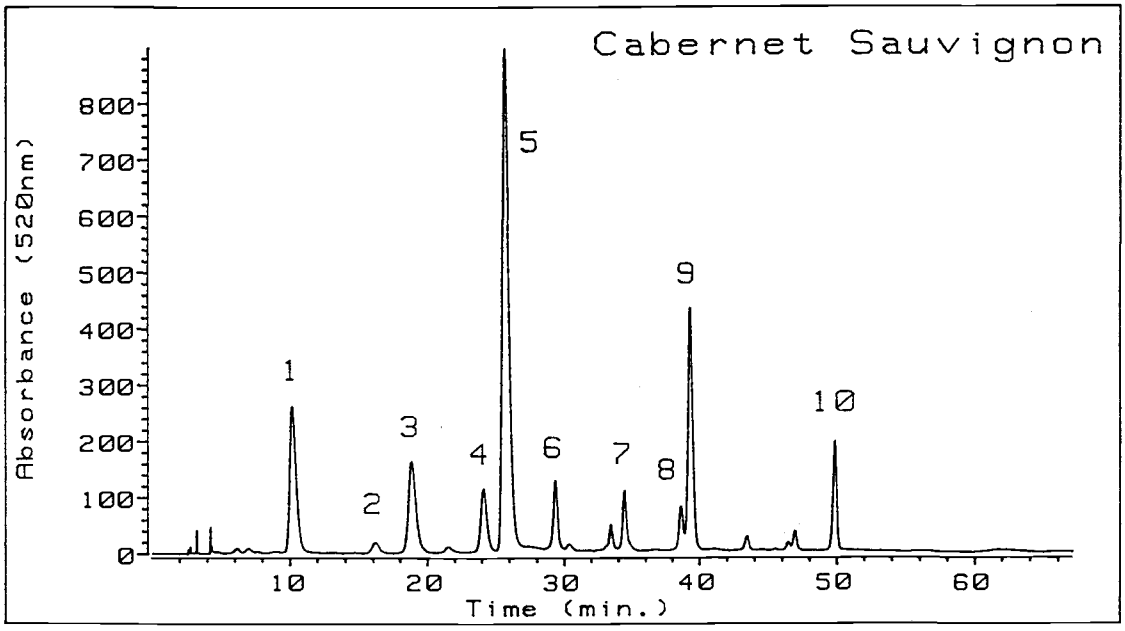

Figure 5.5 HPLC chromatogram, at 520 nm, of Cabernet Sauvignon anthocyanins. Peak 1= delphinidin-3-glucoside, 2 = cyanidin-3-glucoside, 3 = petunidin-3-glucoside, 4 = peonidin-3-glucoside, 5 = malvidin-3-glucoside, 6 = delphinidin-3-glucosylacetate, 7 = petunidin-3-glucosylacetate, 8 = peonidin-3-glucosylacetate, 9 = malvidin-3-glucosylacetate, 10 = malvidin-3-glucosylcoumarate. Polymeric anthocyanins elute in older wines near 62 min.

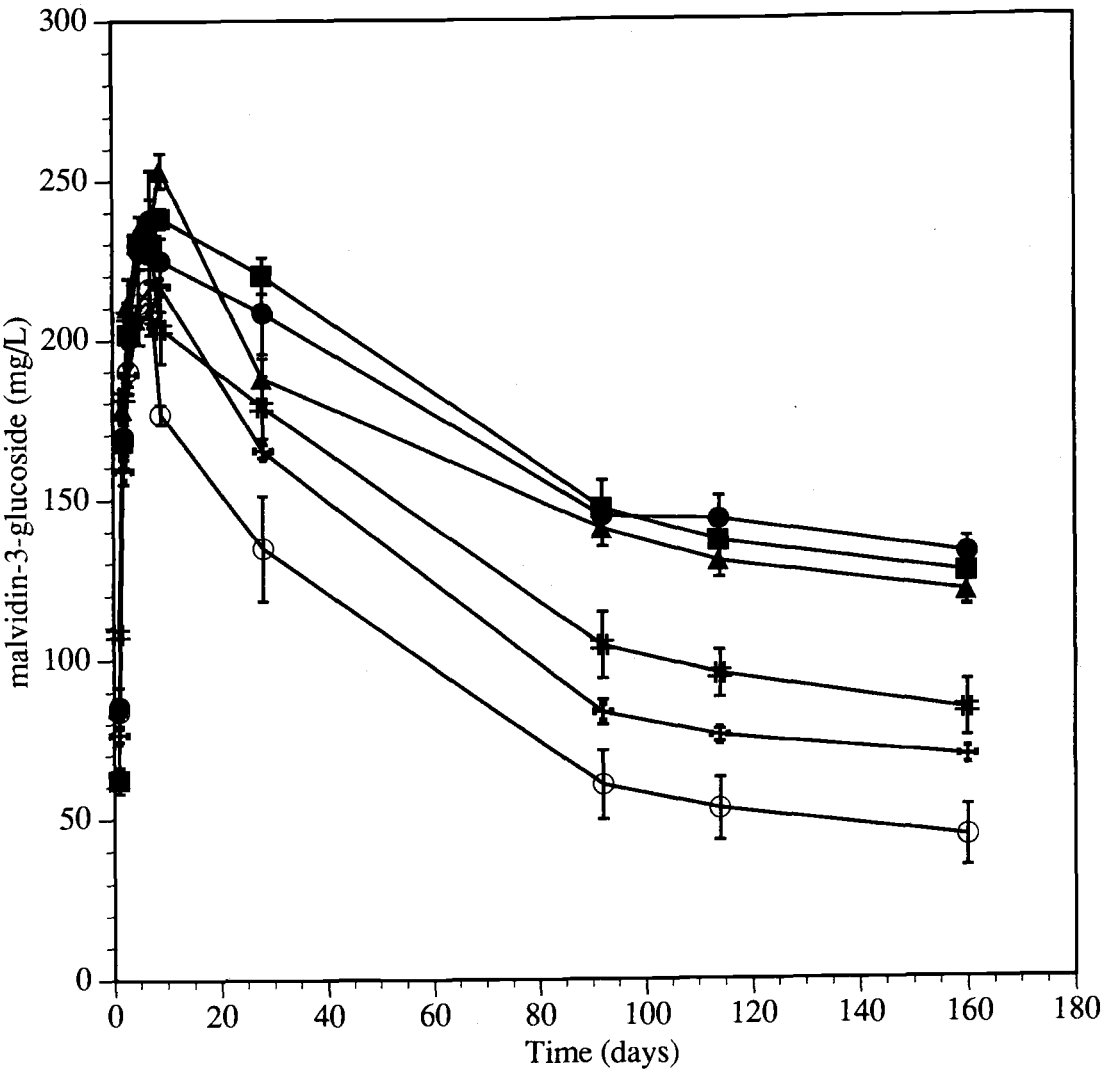

Figure 5.6 Quantity of malvidin-3-glucoside present in Pinot noir wine.

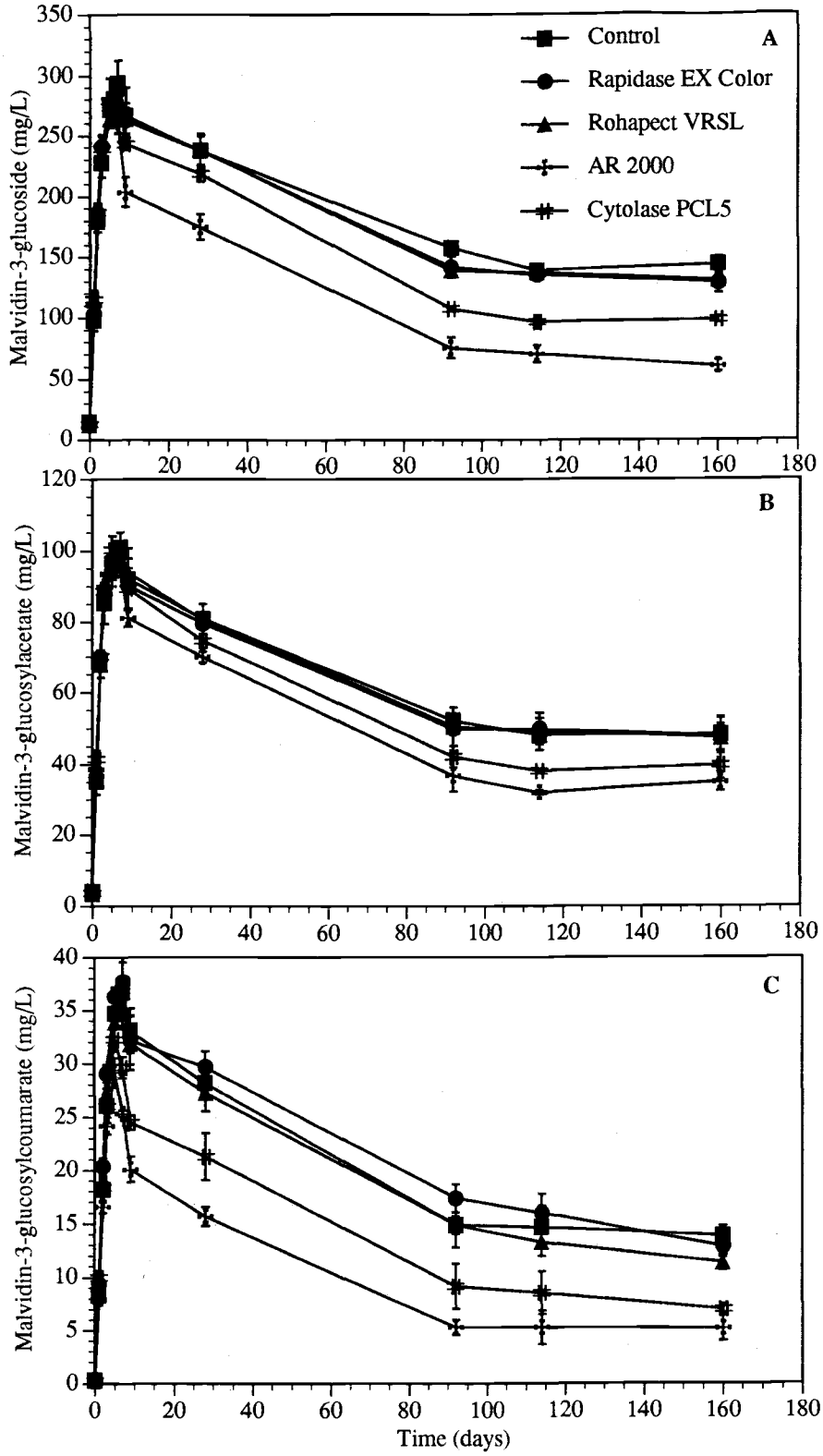

Figure 5.7 Quantity of malvidin-3-glucoside and its acylated forms present in Cabernet Sauvignon wine. A: malvidin-3-glucoside, B: malvidin-3-glucosylacetate, C: malvidin-3-glucosylcoumarate.

Color Measurements

Color measurements for Pinot noir and Cabernet Sauvignon are given in Table 5.1. L* represents lightness to darkness (100 and 0, respectively), a* indicates green to red-purple, b* represents blue to yellow. Chroma provides a measure of the intensity of color, while hue angle (0° = red-purple, 90° = yellow, 180° = bluish-green, and 270° = blue) indicates the sample color itself (McGuire, 1992). AR 2000 and both Cytolase PCL5-treated wines were significantly different ($p<0.05$) from control in chroma and hue angle for Pinot noir and chroma for Cabernet Sauvignon.

Table 5.1 Color measurements of Pinot noir and Cabernet Sauvignon

	L*	a*	b*	chroma[a]	hue[b]
PINOT NOIR					
Control	25.40	6.03	1.49	6.21	13.79
AR 2000	25.79	8.38*	2.22*	8.67*	14.86*
Rohapect VRSL	25.44	6.52	1.70	6.74	14.64
Rapidase EX Color	25.42	6.33	1.60	6.53	14.16
Cytolase PCL5 (0.005%)	25.80	8.98*	2.48*	9.32*	15.39*
Cytolase PCL5 (0.020%)	26.12*	10.02*	2.80*	10.41*	15.45*
CABERNET SAUVIGNON					
Control	24.51	0.70	0.18	0.73	14.54
AR 2000	24.57	1.64*	0.35	1.68*	12.73
Rohapect VRSL	24.38	0.74	0.14	0.75	11.42
Rapidase EX Color	24.49	0.69	0.17	0.71	13.78
Cytolase PCL5 (0.005%)	24.65*	1.58*	0.37	1.62*	13.16
Std error Pinot noir	0.13	0.68	0.23	0.71	0.32
Std error Cabernet Sauv.	0.04	0.13	0.03	0.13	1.55

[a] chroma = $(a^{*2} + b^{*2})^{1/2}$
[b] hue = $\tan^{-1}(b^*/a^*)$
* significantly different from control ($p<0.05$)

Phenolics

A complete phenolic profile was determined by HPLC on the wine samples at the completion of fermentation (Figure 5.8). Many factors may effect the concentration of phenolics in wine, such as processing variables which increase skin extraction resulting in higher levels (Ramey et. al., 1986; Merida et al., 1991). The anthocyanin data showed that enzyme treatments resulted in differences in anthocyanin extraction and polymerization. In examining the phenolic profiles, many differences were also noted among treatments, especially between AR 2000 and Cytolase PCL5 (0.02%) and the other treatments.

Quercetin

Relative levels of quercetin glucoside, glucuronide and aglycon were determined (Figure 5.9). In general, the order for the three forms, from least to greatest amount, in Pinot noir was: glucoside, aglycon, and glucuronide. The most noticeable exception is AR 2000 and to a lesser extent Cytolase PCL5 (0.02%)-treated wines. Both preparations appear to contain a glucuronidase-type activity which cleaved the glucuronide leaving quercetin aglycon, as seen by the lowering of quercetin glucuronide with a concomitant rise in quercetin aglycon. Although there was not as much difference between the three forms of quercetin in Cabernet Sauvignon, the same glucuronidase-type activity was noted with AR 2000.

Treatments which showed the highest levels of quercetin aglycon also showed the highest degree of polymerization (refer to Figure 5.3). This supports the work of Price et al. (1995) who also found a higher level of polymerization in wines made from grapes containing higher levels of quercetin aglycon.

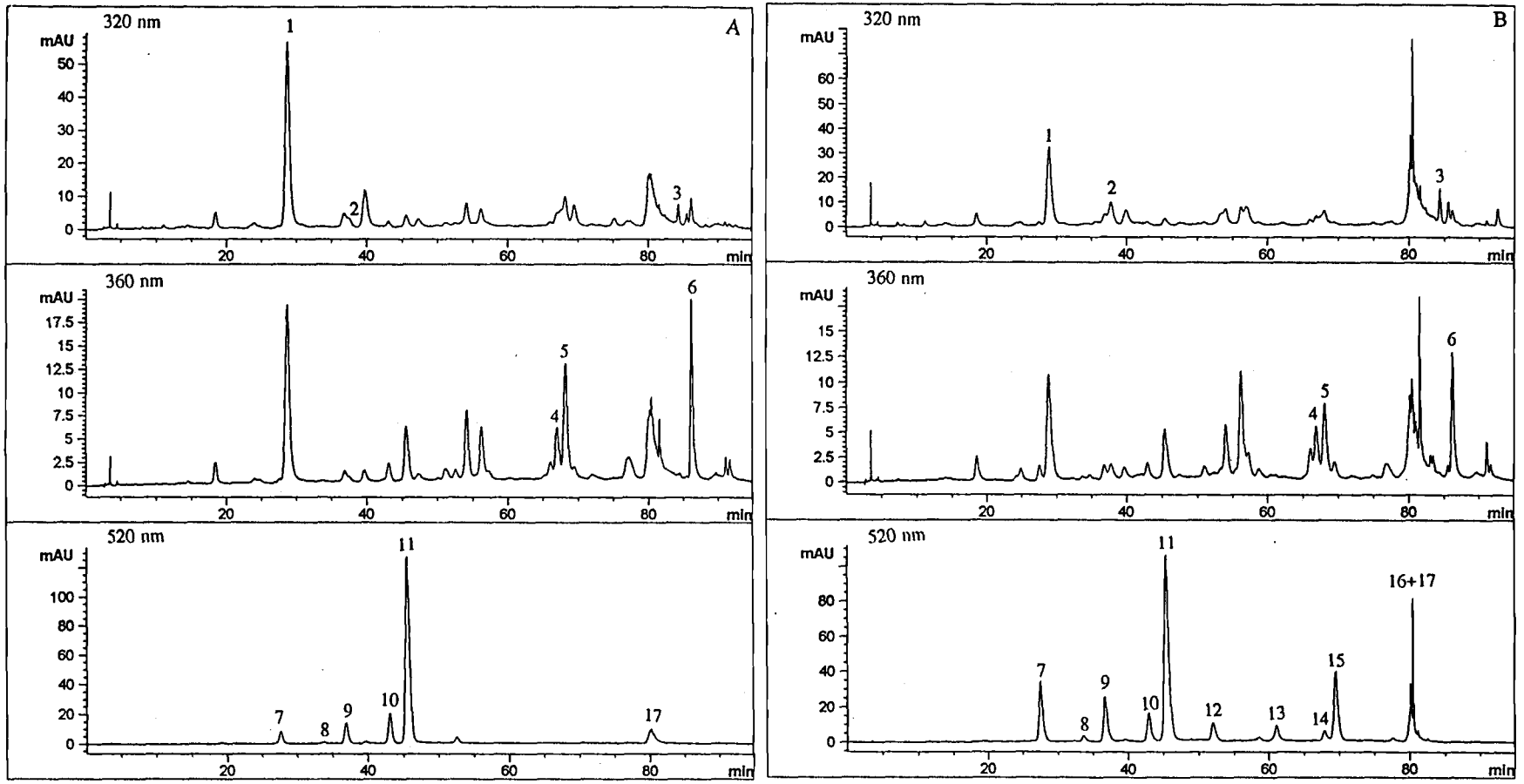

Figure 5.8 HPLC chromatogram, at 320, 360, and 520 nm, of Pinot noir (A) and Cabernet Sauvignon (B) phenolics. Peak 1 = caftaric acid, 2 = caffeic acid, 3 = *trans*-resveratrol, 4 = quercetin glucoside, 5 = quercetin glucuronide, 6 = quercetin aglycon, 7= delphinidin-3-glucoside, 8 = cyanidin-3-glucoside, 9 = petunidin-3-glucoside, 10 = peonidin-3-glucoside, 11 = malvidin-3-glucoside, 12 = delphinidin-3-glucosylacetate, 13 = petunidin-3-glucosylacetate, 14 = peonidin-3-glucosylacetate, 15 = malvidin-3-glucosylacetate, 16 = malvidin-3-glucosylcoumarate, 17 = polymeric anthocyanin.

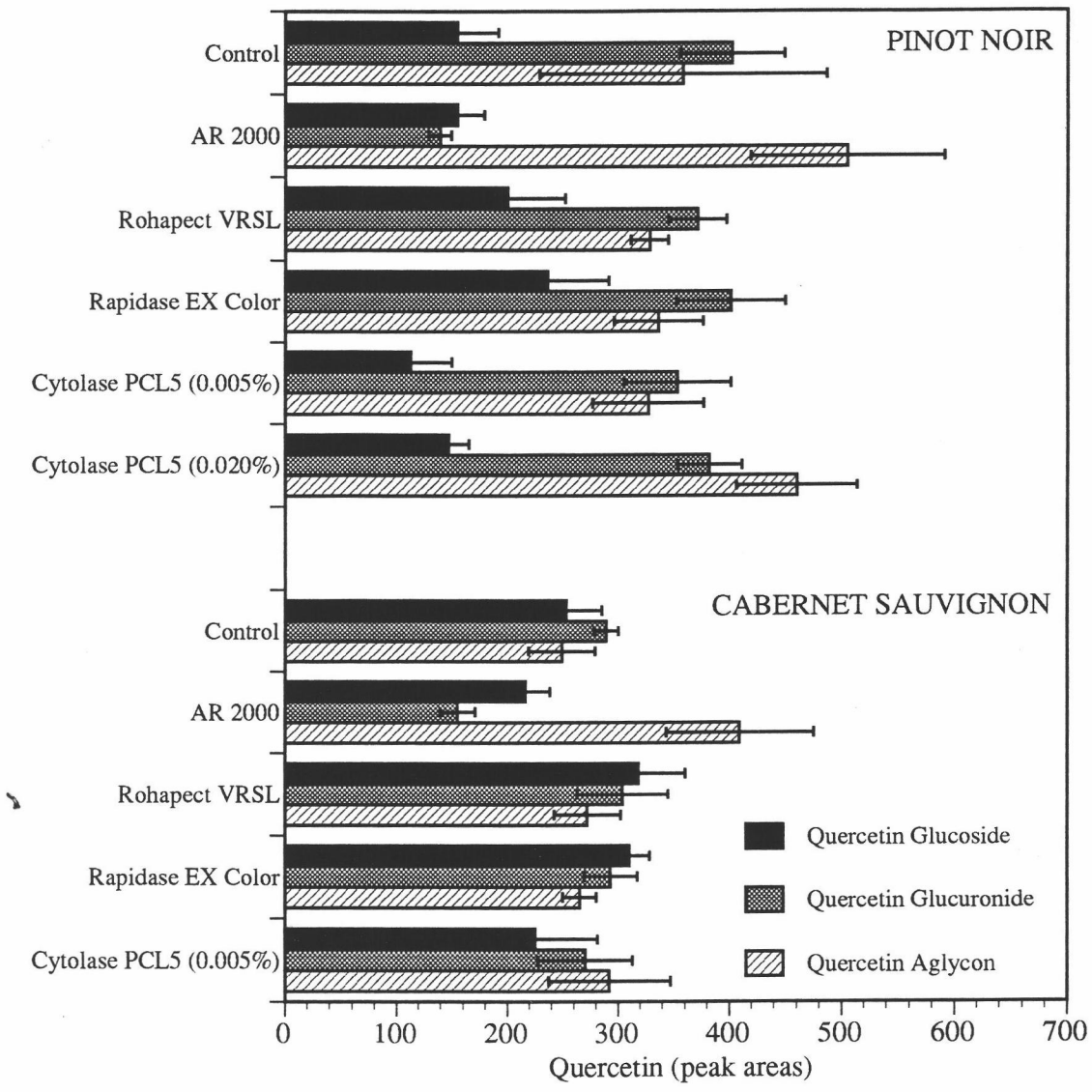

Figure 5.9 Quercetin levels in Pinot noir and Cabernet Sauvignon wine after fermentation.

Caffeic and Caftaric Acids

A relationship between caffeoyl tartrate (caftaric acid) and caffeic acid was found. Typically there is little to no caffeic acid present in grapes (Price et al., 1995) and caftaric acid is present. Some of the enzyme preparations appeared to contain an esterase activity which hydrolyzed the tartaric ester of caftaric acid rendering caffeic acid. This activity occurred in both varieties (Figure 5.10). Enzyme preparations grouped into three categories as to the extent of hydrolysis: little (Control and Rapidase EX Color), medium (Rohapect VRSL and Cytolase PCL5 0.005%), and great (AR 2000 and Cytolase PCL5 0.02%). Esterase activity has also been reported in commercial enzyme preparations for apple juice processing (Spanos et al., 1990).

trans-Resveratrol

Relative peak areas of *trans*-resveratrol also differed among treatments (Figure 5.11). In both Pinot noir and Cabernet Sauvignon, the controls had the lowest levels but greater variability was noted among the Pinot noir treatments. Two relationships involving *trans*-resveratrol were detected. First, there appears to be an inverse relationship between total monomeric anthocyanin and *trans*-resveratrol in both varieties; treatments that had lowest levels of total monomeric anthocyanin had highest levels of *trans*-resveratrol. The 3-ß-glycoside of *trans*-resveratrol (piceid) has been reported in grape skins (Waterhouse and Lamuela-Raventós, 1994; Lamuela-Raventós et al., 1995). Therefore, this relationship may be due to the 3-ß-glycoside bond being similar to the sugar linkage in the anthocyanin; ß-glucosidase could release bound *trans*-resveratrol while cleaving sugar from the anthocyanins and destroying them. Second, the amount of free *trans*-resveratrol exhibited a linear relationship to the log ratio of caftaric acid to caffeic acid. Enzyme preparations that released the largest amount of *trans*-resveratrol also contained high esterase activity.

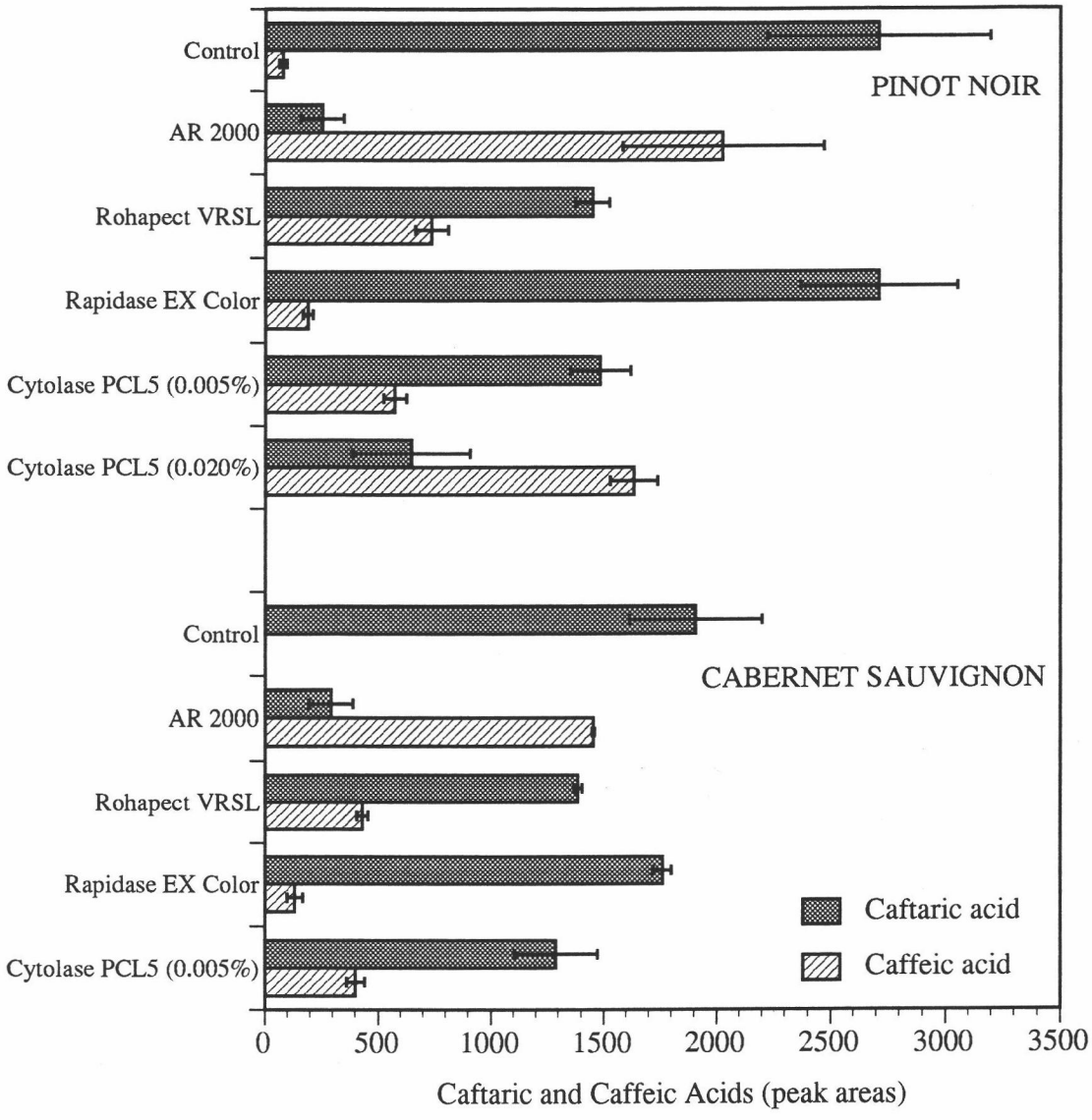

Figure 5.10 Caftaric and caffeic acid levels in Pinot noir and Cabernet Sauvignon wine after fermentation.

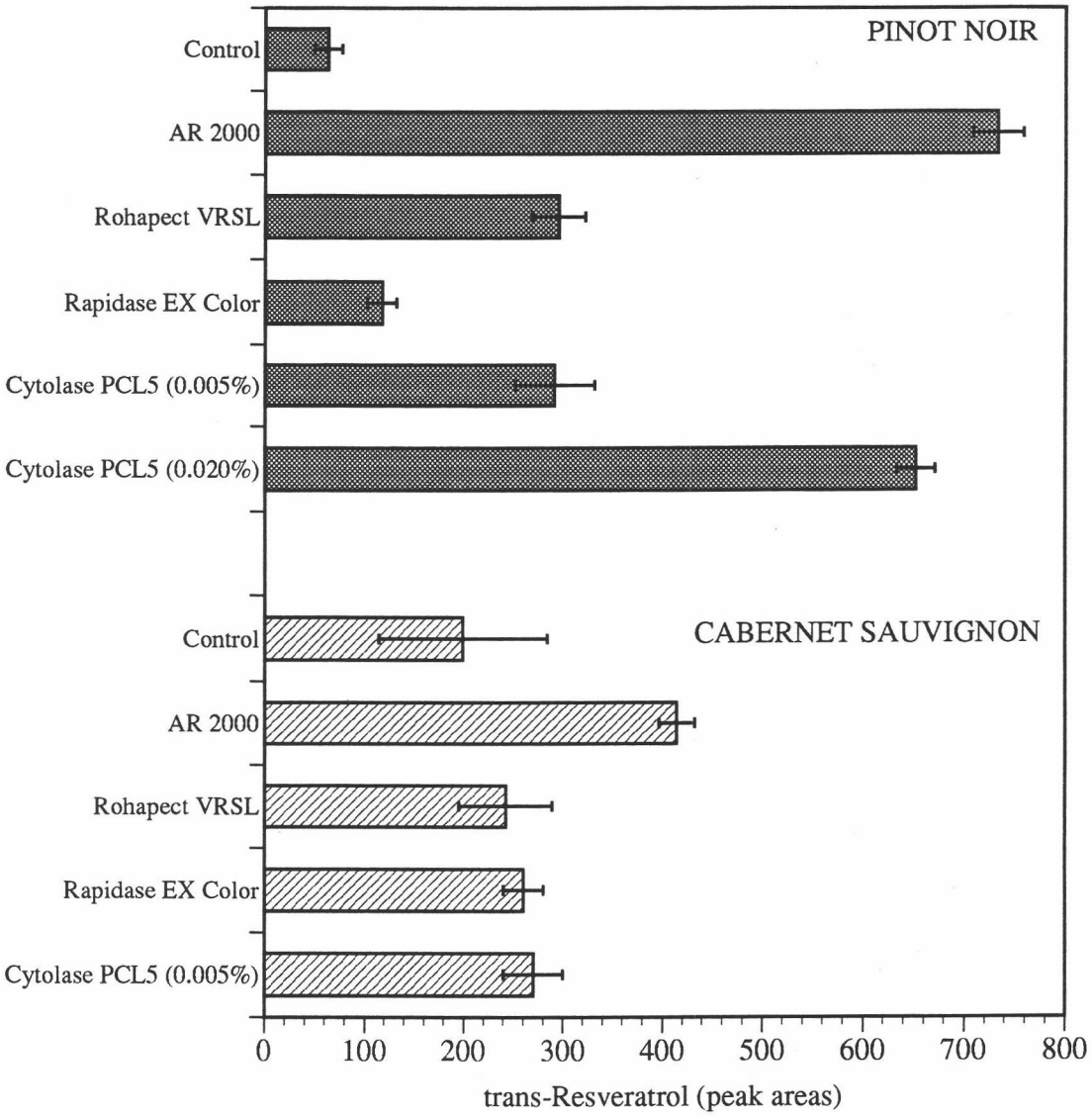

Figure 5.11 *Trans*-resveratrol levels in Pinot noir and Cabernet Sauvignon wines after fermentation.

Resveratrol is a phytoalexin synthesized by grape skin cells in response to pathogen infection, e.g. *Botrytis cinerea* (Creasey and Coffee, 1988; Jeandet et al., 1991). Researchers have found that wines produced from grapes affected 40 to 100% by *Botrytis* have lower resveratrol levels (Lamuela-Raventós and Waterhouse, 1993; Jeandet et al., 1995a) and wines made from 10% affected grapes have higher resveratrol levels than either highly or non-affected grapes (Jeandet et al., 1995a,b). Jeandet et al. (1995b) concluded that *Botrytis* infection is required for high resveratrol wine concentration but that extensive *Botrytis* development before harvest may lower resveratrol. In early *Botrytis* stages enzymes capable of degrading host phytoalexins are not present (Jeandet et al., 1993), but other enzymes are. Some of the same specific enzymes produced at early stages of *Botrytis* development might also be present in the commercial enzyme preparations, thus cleaving the glycoside from resveratrol and leaving *trans*-resveratrol.

CONCLUSIONS

Commercial enzyme treatments appeared to have similar effects on both Pinot noir and Cabernet Sauvignon wines. Two enzyme preparations, AR 2000 and Cytolase PCL5, had profound effects on wine color. Both produced significant destruction of total monomeric anthocyanin as well as individual pigments. The presence of acylating groups on malvidin-3-glucoside did not appear to inhibit this enzymatic effect. Preparations that caused the most anthocyanin degradation also produced wines that had higher amounts of polymeric anthocyanin. Increasing the enzyme concentration magnified these effects.

Enzyme preparations had a large impact on the phenolic profile as well. Preparations that resulted in wines with the lowest amount of total monomeric anthocyanins also produced wines with: lower quercetin glucuronide and higher quercetin aglycon, a larger ratio of caffeic to caftaric acid, and higher levels of *trans*-resveratrol. A better

understanding of phenols in wine is necessary to determine what impact enzymes may have on organoleptic properties.

This research showed that use of crude fungal preparations needs to be examined more closely since they may be deleterious to the final product. More work needs to be done not only on a commercial scale to examine these effects, but also on the enzymes themselves to select against the presence of undesirable side-activities or to develop cost-effective purification methods.

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CHAPTER 6. SUMMARY

The objective of this research was to develop a method involving HPLC and spectrophotometric assays to screen for ß-galactosidase, α-arabinosidase, ß-glucosidase and α-rhamnosidase activities in commercial enzyme preparations used for juice and wine processing. Cranberry juice was found to be an effective system to monitor ß-galactosidase and α-arabinosidase activities due to the substantial quantities of these pigments. However, it was not an ideal system to measure ß-glucosidase activity since only a small percentage of glucosidically-bound anthocyanins were present. The major pigments in anthocyanin-containing fruits are bound by ß-glucoside linkages. Since the major anthocyanins in boysenberry juice contain three types of linkages: ß-glucosidase, ß-1,2-glucosidase, and α-rhamnosidase, it was a good model system to test for the presence of these side-activities.

In the cranberry study we found that 24 out of 27 enzyme preparations contained various degrees of ß-galactosidase side activity which destroyed the galactoside pigments, most noticeably cyanidin-3-galactoside, the major pigment. However, no destruction of α-arabinosidase pigments was observed. Pigment destruction was determined to be more pronounced when enzymes were allowed to act on clarified juice than on crushed fruit. We found that while the screening procedure demonstrated the potential for anthocyanin pigment degradation, it would not accurately predict the amount of pigment loss that may occur during processing. Therefore, enzymes which produced pigment losses in the screening assay should be further tested on a larger scale before being applied in a commercial operation. This study showed that ß-galactosidase is present in many commercial enzyme preparations and processors should note this when working with fruits containing galactoside anthocyanins.

ß-glucosidase activity in juice processing enzymes was much lower and less prevalent than that found for ß-galactosidase. Utilizing the HPLC screening assay, we

found that when enzyme preparations were used within their recommended dosage levels there was little pigment destruction. However, when excessive levels were used there was a greater possibility of anthocyanin destruction.

In examining Pinot noir and Cabernet Sauvignon wines, which contain non-acylated and acylated pigments, respectively, we found that acylation did not inhibit enzymatic degradation of anthocyanins. While some enzyme preparations were found to degrade anthocyanins in both varieties of wine, enzymatic effect was more prevalent in Pinot noir. In conjunction with anthocyanin destruction, some of the preparations also had a profound effect on the phenolic profile of the wine. Wines which had the lowest amounts of total monomeric anthocyanins also contained: lower levels of quercetin glucuronide and higher quercetin aglycon, a larger ratio of caffeic to caftaric acid, and higher levels of *trans*-resveratrol.

This research has shown that anthocyanin degradation due to side activities in commercial enzyme preparations is a valid concern which needs to be investigated further. Processors and enzyme suppliers need to be aware of the possibility of this type of problem occurring and take steps accordingly to ensure proper enzyme use as well as the need to screen enzymes before use in a commercial scale operation. These concerns will be legitimate until production of commercial preparations includes safe-guards against undesirable activities.

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APPENDIX

COMPARISON OF TOTAL PROTEIN AND ß-GLUCOSIDASE ACTIVITY IN COMMERCIAL ENZYME PREPARATIONS FROM TWO YEARS

MATERIAL AND METHODS

Commercial Enzyme Preparations

The following enzymes were evaluated: AR 2000, Cytolase® 219, Cytolase® CL, Cytolase® M102, Cytolase® M103M, Cytolase® M219, Cytolase® PCL5, Rapidase® AB Clear, Rapidase® BE, Rapidase® EX Color (Gist-brocades, Inc., King of Prussia, PA); Novoferm 12, Pectinex™ 1XL, Pectinex® BE 3XL, Pectinex™ Ultra SP-L, Ultrazyme 100G, Vinozyme L (Novo Nordisk Ferment, Ltd., Dittingen, Switzerland); Bioberry, Biocellulase TRI, Biopectinase 150L, Biopectinase 200C, Biopectinase 300L, Biopectinase 7X (Quest International, Sarasota, FL); Rohapect® 7104, Rohapect® B1L, Rohapect® D5L, Rohapect® FL, Rohapect® MB, Rohapect® TL, Rohapect® VR concentrate, Rohapect® VR Super L, research grade enzymes: EL 65-92, EL 66-92, EL 67-92, EL 68-92 (Rohm, Darmstadt, Germany); Cellulase AC, Cellulase Tr Concentrate, Cellulase TRL, Clarex® L, Clarex® ML, Hemicellulase, Pearex® L, Pearex® 5XL, Pectinase AT, Spark-L HPG®, Vinemax C (Solvay Enzymes, Inc., Elkhart, IN). Enzyme preparations were obtained in 1992 and 1994. Not all preparations were analyzed both years.

Specific Enzyme and Protein Determinations

ß-glucosidase was measured by the method of Dopico et al. (1989) at 34°C, pH 4.5 with *o*-nitrophenol-ß-D-glucopyranoside as the substrate. One unit of enzyme was defined as that amount which liberated 1 µmol of *o*-nitrophenol/min under specified conditions and

with extinction coefficient of 21,300 (Dawson et al., 1986). The specific activities of enzymes were expressed as units/mg of protein in the preparation. Protein was determined by Kjeldahl method (AOAC, 1984). Specific activities and protein determinations were performed in duplicate.

RESULTS

Total protein content (Figure A.1) and ß-glucosidase activity (Figure A.2) in commercial enzyme preparations were compared. While the amount of protein/gram of preparation was fairly consistent between years, the specific activity of ß-glucosidase was not. In general, protein values differed from 0 to 1.3-fold between years, except Pectinex BE 3XL which was 1.8-fold. ß-glucosidase activity differed 1.3 to 1.8-fold for most preparations, except Pectinex BE 3XL which varied by 21-fold.

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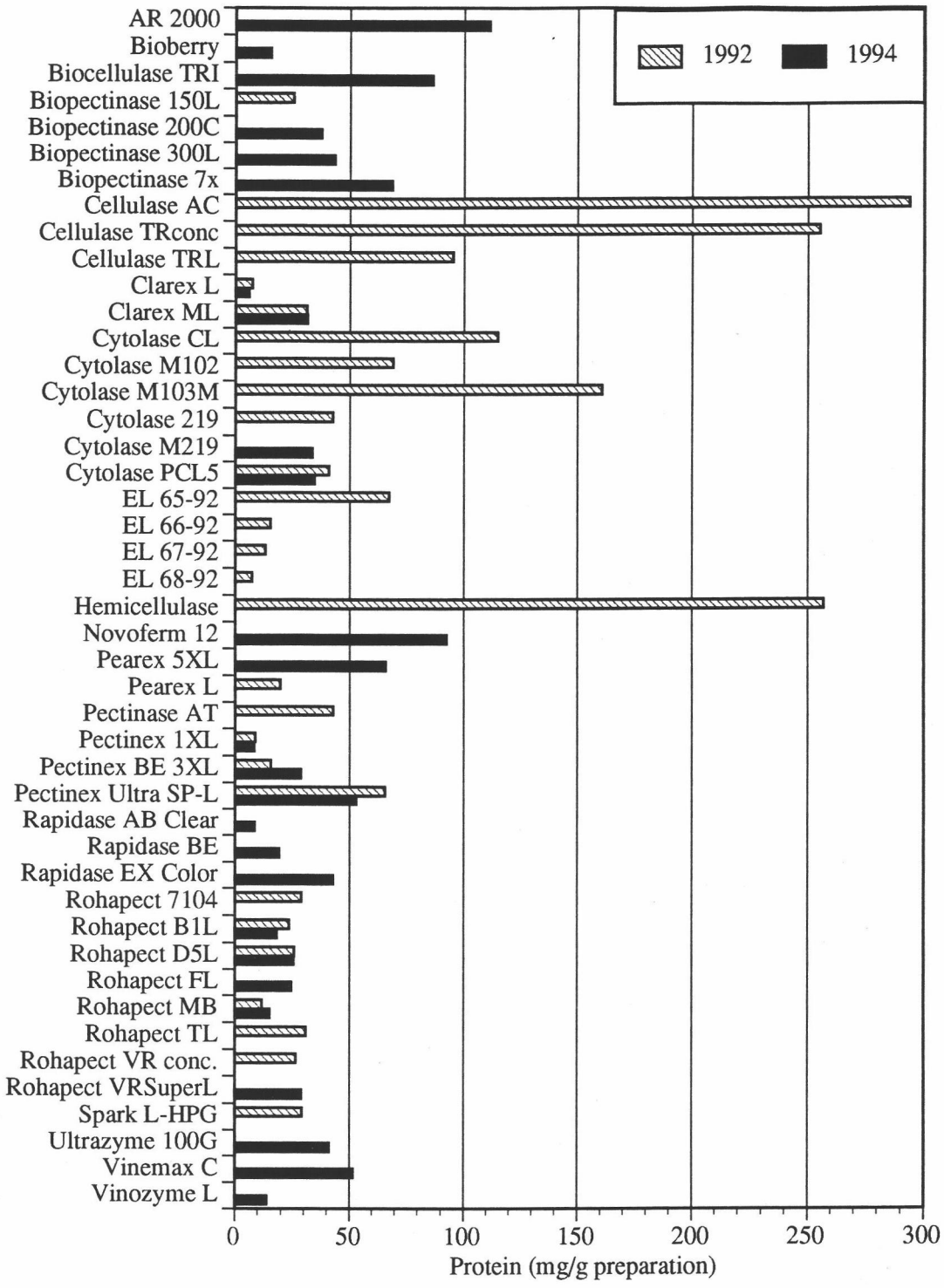

Figure A.1 Protein comparison between 1992 and 1994 lots of commercial enzyme preparations.

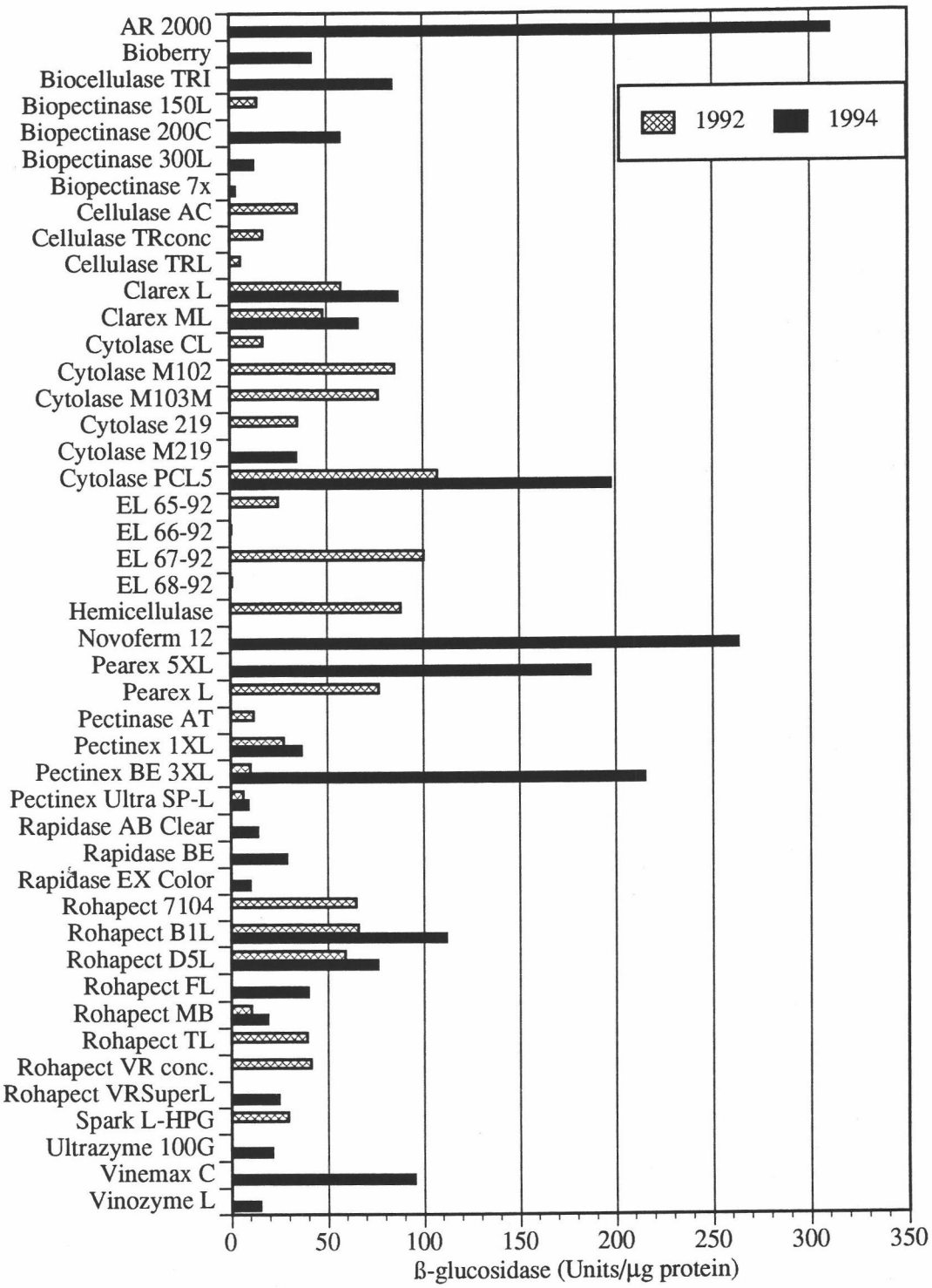

Figure A.2 Comparison of ß-glucosidase activity between 1992 and 1994 lots of commercial enzyme preparations.