Annual Report to the American Vineyard Foundation

Project Title: Chemical Characterization of Small Polymeric Pigments in Wines and Red Grape Skin Extracts

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Summary

Polymeric pigments are important because they are the stable form of color in wines. They are thought to be formed by reaction of monomeric anthocyanin pigments with tannins or flavan-3-ols, such as catechin or epicatechin. During the 1999 season we observed a class of low molecular weight polymeric anthocyanin pigments in wines. In new wines these pigments were a large percentage of the anthocyanin color *not* bleached by SO_2, thus classifying them as polymeric pigments. On the other hand they were not precipitated by protein, which suggested that they had a low molecular weight compared to typical tannins. This observation raised many practical questions, but also brought up several important questions regarding the chemical nature of the small polymeric pigments (SPP), which we addressed in this project during the 2000 season.

One of the objectives of the work was to devise a purification scheme that would permit separation of small polymeric pigment (SPP) from monomeric anthocyanins and large polymeric pigment (LPP). We were successful in developing a procedure to purify SPP based on column chromatography on a Toyopearl HW-40(F) column and eluting the monomeric anthocyanins and the tannin fraction separately with different solvents. Using standard SO_2 bleaching to assay polymeric pigments, we were able to show that the SPP actually resides in the monomeric fraction from the column. In the course of this work we discovered that the SPP could be partially bleached by SO_2. This was an unexpected result, but indicates that further work needs to be done with polymeric pigments to determine the extent to which they are affected by SO_2 bleaching. This is important because most assays for polymeric pigment rely on SO_2 bleaching to distinguish them from monomeric anthocyanins. If polymeric pigments are indeed bleached by SO_2, it means that such assays give an underestimate of the amount present, and that monomeric anthocyanins are overestimated.

An major accomplishment in this work during the past year has been the synthesis of an SPP dimer containing catechin as the extension unit and malvadin-3-glucoside as the terminal unit. The availability of this dimer by an unambiguous chemical synthetic route will enable us to confirm the structure of the naturally occurring dimer found in grape skin extracts and wine. The availability of the synthetic dimer will also enable us to determine if anthocyanin pigments having that configuration (catechin-malvadin-3-glucoside) are affected by SO_2 bleaching. The chemical reaction by which we created the dimer may also point the way to understanding how polymeric pigments are formed in wines during aging.

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Objectives and Experiments Conducted to Meet Stated Objectives
The objectives for the 200 season were to

- Devise a purification scheme that will permit separation of small polymeric pigment (SPP) from monomeric anthocyanins and large polymeric pigment (LPP)

- Determine the mean degree of polymerization (mDP) in an isolated SPP fraction, and whether the anthocyanin moiety is present as an extension unit or a terminal unit.

- Synthesize an SPP dimer with malvadin-3-glucoside as a terminal unit and determine if it is bleached by SO_2 under conditions that bleach monomers.

Summary of Major Research Accomplishments and Results (by Objective)

Objective 1. Devise a purification scheme that will permit separation of small polymeric pigment (SPP) from monomeric anthocyanins and large polymeric pigment (LPP)

We devised a purification procedure using a Toyopearl HW-40(F) column that can separate small polymeric pigments (SPP) found in wines from large polymeric pigments (LPP). The ethanol is first removed from the wine by rotary evaporation and the aqueous fraction is applied to the Toyopearl column. We found that the monomeric anthocyanins can be eluted from the column using ethanol/water/trifluoroacetic acid (11:9:0.001) and the tannin fraction can be eluted with acetone/water (3:2).

In order to determine whether the SPP resided in the monomeric anthocyanin fraction or the tannin fraction, we removed the solvent from each fraction using a rotary evaporator and then analyzed for SPP using a combined protein precipitation assay and standard SO_2 bleaching. In this system SPP is not bleached by SO_2 and not precipitated by protein. The results show that the majority of the SPP resides in the monomeric anthocyanin fraction with less than 10% remaining in the LPP fraction, which eluted from the column in the acetone/water fraction.

Since the SPP was found to be mainly in the "monomeric anthocyanin" fraction, we focused on this portion for further purification of SPP. The ethanol was removed from the ethanol/water/trifluoroacetic acid fraction and by repeated extraction of the residual aqueous phase with isoamyl alcohol we were able to partition most of the anthocyanin monomer into the alcohol phase with the bulk of the SPP remaining in the aqueous phase. Thus, we were able to obtain an aqueous solution that contained only a small amount of polymeric pigment that precipitated with protein (LPP) and which we thought should be nearly free of monomeric anthocyanins. We considered this material our purified SPP fraction, thus completing the first objective, which was to purify the SPP.

Our criteria for the presence of polymeric pigment during the purification scheme was bleaching with SO_2, which is known to bleach monomeric anthocyanins but thought to have little or no effect on polymeric pigments. However, when we subjected our purified SPP fraction to SO_2 bleaching we found that it bleached 23%. This result suggested that the either the SPP could be bleached by SO_2 or that the purified SPP fraction contained monomeric anthocyanin. We confirmed the absence of anthocyanin in the purified SPP fraction by reversed phase HPLC, suggesting that SPP can be bleached by SO_2. This is an unexpected but notable result. It suggests that using SO_2 bleaching to estimate polymeric pigment gives an underestimate of the amount of SPP present. Preliminary results suggest that LPP is also bleached to some extent by SO_2. In the coming year we have proposed to determine the extent to which polymeric pigments (both SPP and LPP) bleach with SO_2. This will be very important for all analyses that rely on SO_2 bleaching to estimate the level of polymeric pigments in grape skin extracts or wines.

Objective 2. Determine the mean degree of polymerization (mDP) in an isolated SPP fraction, and whether the anthocyanin moiety of the SPP is present as an extension unit or a terminal unit.

This objective required a purified preparation of SPP, which we have now obtained. Work on this objective is still underway, and we are using the thiolytic procedure described in the literature on an aliquot of purified SPP. In order to be certain as to whether the anthocyanin moiety of SPP is present as an extension unit or a terminal unit it is critical that the purified SPP fraction be free of monomeric anthocyanin. The isoamyl alcohol partitioning is effective but a small amount of monomeric anthocyanin apparently remains. Since we also found that the SPP is bleached with SO_2, it means that HPLC is the only way we can confirm that all of the monomeric anthocyanin has been removed from the purified SPP fraction. We are currently purifying additional SPP for the thiolysis experiments. When we have enough material, an aliquot of the SPP fraction will be heated for 10 minutes at 60 °C with an equal volume of 5% toluene-α-thiol in methanol containing 0.2M HCl. The products will be separated by HPLC and monitored at 280nm and 520nm. Malvadin-3-glucoside will be subjected to the same thiolytic conditions to determine whether or not it is stable under the conditions of thiolysis, and samples of SPP will be spiked with malvadin-3-glucoside and subjected to thiolysis for comparison with SPP samples after thiolysis. This experiment will provide evidence for anthocyanins as extension units or terminal units in polymeric pigments.

Objective 3. Synthesize an SPP dimer with malvadin-3-glucoside as a terminal unit and determine if it is bleached by SO_2 under conditions that bleach monomers.

The synthesis of the SPP dimer represented an important part of the work undertaken in this project for the 2000 season. Our proposal was to use the 4-thiobenzylether of catechin in a reaction with malvadin-3-glucoside at neutral pH to form the dimer. The only commercially available starting material was taxifolin, which had to be reduced to the 3,4-diol before the 4-thiobenzylether of catechin could be formed by reaction of the diol with phenylmethanethiol. We first reduced taxifolin with $NaBH_4$ and monitored this reaction by thin layer chromatography (TLC) to confirm that the reduction proceeded to near completion. We found that with sufficient $NaBH_4$ added in small aliquots reduction of taxifolin to the 3,4-diol was nearly quantitative.

After reduction of the taxifolin to yield the diol, we next added phenylmethanethiol in acetic acid to form the 4-benzylsulfanylcatechin. This reaction was also monitored by TLC and after several experiments to optimize the yield of 4-benzylsulfanylcatechin, we obtained nearly 50% yield of a product that had the right characteristics by TLC. The putative product was purified by column chromatography on Sephadex and the elution was monitored by TLC. Fractions containing the suspected 4-benzylsulfanylcatechin were pooled, the solvent was removed and the identity of the product was verified by proton NMR. We had thus prepared the 4-benzylsulfanylcatechin required for the proposed synthesis.

The reaction we proposed to use for synthesis of the SPP dimer was entirely novel for obtaining anthocyanin adducts, but had been used successfully by other workers to produce procyanidin dimers. They obtained the procyanidin B-3 dimer when catechin was allowed to react with 4-benzylsulfanylcatechin. If we were to properly perform the reaction we proposed it was important to confirm that we could successfully repeat the reaction conditions used by other workers. We combined catechin with 4-benzylsulfanylcatechin under the same conditions they used and obtained a reaction product consistent with the B-3 dimer. Identity of B-3 dimer was confirmed by HPLC-MS in which we observed a peak with exactly the right molecular weight. Although we did not purify the B-3 dimer, we were confident that our conditions were correct for conducting the reaction we proposed.

We combined 4-benzylsulfanylcatechin with malvadin-3-glucoside that we obtained commercially. The reaction gave a new compound that eluted just ahead of malvadin-3-glucoside on HPLC. The product shows absorbance at 520nm, which is consistent with it being an anthocyanin adduct, and it has precisely the right mass based on HPLC-MS. Thus, we believe we have successfully prepared the SPP dimer as proposed in our third objective. We have examined different solvent conditions to optimize the amount of product formed, and we have prepared enough of it for a proton and carbon NMR analysis to confirm its chemical structure. In order to obtain the product free of starting material we are currently negotiating to use a preparative HPLC in the Chemistry Department on campus. When we have purified enough of the final product, the NMR analysis will be done to confirm the structure. Having the catechin-malvadin-3-glucoside will enable us to determine whether or not this compound bleaches with SO_2, which was part of the objective.

Outside Presentations of Research
Thus far we have made no outside presentations of this research.

Research Success Statements
In the past four years this project has provided growers and winemakers with an easy and inexpensive way to measure tannins in grape berry extracts and wines. Further developments in the analysis during the past two years to include traditional SO_2 bleaching has extended the capability of the assay to include determination of polymeric pigments in wines and grape extracts. This assay is currently being used in several wineries as a tannin and polymeric pigment management tool. It has proven to be reliable, easy to implement, and inexpensive to run.

Application of the assay developed in this research project has led to the recognition that the polymeric pigments found in wines can be separated into two functional classes; large polymeric pigments (LPP) that precipitate with protein, and small polymeric pigments (SPP) that do not.

Recent results from this project suggest that polymeric pigments may be affected by SO_2 bleaching. By determining the extent of bleaching, we will be able to provide assays that give more accurate values for the amount of monomeric and polymeric pigments in wines. Polymeric pigments are important because they are the stable form of color in red wines, and this research has provided new ways to investigate their formation and evolution during wine aging.

Funds Status

The funds remaining will be expended by March 31,2001 for support of the Research Assistant and supplies for additional sample analysis related to this project.