841.2

EXPERIMENTAL BIOLOGY 2008
TODAY'S RESEARCH: TOMORROW'S HEALTH

Cellulase Assay as a Method of Monitoring Grape Ripening

Daniel J. Coleman[1], Robin M. Hawley[2], Gabriele M. Cook[1], and John J. Naleway[1]; [1]Marker Gene Technologies, Inc., University of Oregon Riverfront Research Park, 1850 Millrace Drive, Eugene, OR 97403-1992; [2]King Estate Winery, 80854 Territorial Road, Eugene, OR 97405

MGT Inc.

Introduction

Cellulases are an important family of enzymes found in many plant, bacterial, fungal, and yeast species. Plants encode cellulases [EC 3.2.1.4] that catalyze the cleavage of the internal 1,4-β-glucosidic linkages of cellulose comprising their own tissue. It has been suggested that plant cellulases are essential for many facets of plant development, including abscission, fruit softening, vascular differentiation and symbiosis. A new fluorescent assay system has been developed to monitor cellulase enzyme activity in a continuous assay format at physiological pH values using the new substrate resorufin β-D-cellobioside. Analysis of cellulase activity from grape samples obtained from the Pinot Noir and Pinot Gris varietals at developmental stages from before veraison through to the final ripe berry indicate the presence of a cellulase inhibitor or inhibitors whose level changes with ripening and correlates to the total mass ratio of dissolved sucrose (BRIX) and titratable acidity (TA) for the various samples. Protein and protease assays of the same samples appear to indicate that the inhibitor compound(s) may not be protein related. The identity of the inhibitor compound(s) is currently under investigation. These methods are being developed as a method to estimate optimum harvest conditions in an automated high-throughput (HTS) format.

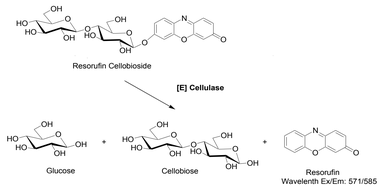
Pinot Gris

Pinot Noir

Methods

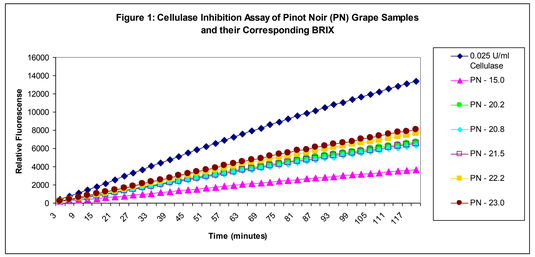
- Crushed Pinot Gris and Pinot Noir grape samples, including skin, flesh, and seeds, picked at varying developmental stages were obtained from Dr. Robin Hawley from King Estate Winery. The grape samples were filtered thru a Büchner funnel and juice was collected in 1.5 eppendorf tubes. Samples were centrifuged for 6 minutes at 7500 rpm.
- Must samples were assayed for total mass ratio of dissolved sucrose (BRIX) and titratable acidity (TA).
- Must samples with varying BRIX and TA values were assayed for cellulase activity and in a cellulase inhibition assay using the new substrate Resorufin β-D-Cellobioside (M1238). For inhibition measurement each assay contained 25 µl of must sample, 25 µl 0.1 U/ml Cellulase, 50 µl substrate Resorufin β-D-Cellobioside. Fluorescence was read at ex/em 550/595nm. Readings were taken at 3-minute intervals for 120 minutes.
- The same must samples were also assayed for protease activity using an FITC-labeled casien substrate (M1315). For protease measurement each assay contained 50 µl must sample, and 150 µl substrate solution (1mg/ml FITC-Casien in 100mM Sodium Phosphate, 150 mM Sodium Chloride, pH 7.6). Fluorescence was read at ex/em 485/535nm. Readings were taken at 1-minute intervals over 30 minutes.
- Total protein analysis was performed by SDS-PAGE to evaluate protein levels in different must samples with varying BRIX and TA levels. A 12% resolving gel and 4% stacking gel were used.
- The SDS-PAGE gels were stained using both Coomassie blue and silver staining.

MarkerGene Fluorescent Cellulase Assay Kit (M1245)



• Cellulases include β-1,4-D-glycosidases, endoglucanases, and exoglycanases. These enzymes cleave β-1,4-D-glycosidic bonds that link glucose units comprising cellulose producing both the disaccharide cellobiose and glucose. Activity of cellulase can be monitored with MarkerGene™ Fluorescent Cellulase Assay Kit (M1245) containing the fluorescent substrate resorufin cellobioside (M1238). Upon cleavage, the red fluorescent compound resorufin is produced and measurement at 585 nm are easily obtained in microtiter plate based assay format. Because of the low pKa of resorufin the assay can be performed at or near physiological pH values present in grape and must samples.

Cellulase Inhibition



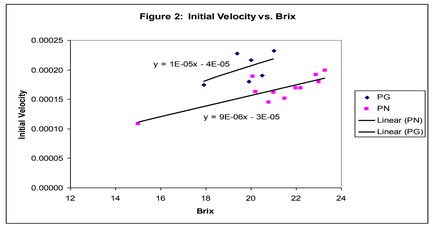
Potential Cellulase Inhibitors Present in Grapes

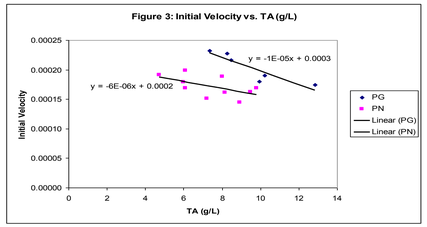
Inhibitor	Ki Values [mM]
Cellobiose	1.6, 11, 34
Glucosamine	0.4
Glucose	0.5, 1.4, 7.4
Sodium hypochlorite	0.051

•Above: Ki values of known inhibitors of cellulase most likely in grape samples. Sodium hypochlorite is used as a pesticide to treat grape phylloxera, a common pest in commercial grapevines.

•Figure 1: Cellulase Inhibition Assay. Pinot Noir grape samples with varying BRIX levels were assayed for cellulase inhibition. All grape samples assayed exhibited cellulase inhibition and an inverse correlation between BRIX and cellulase inhibition was found.

Initial Velocities





•Figures 2 and 3: Initial Velocities of Cellulase Assay vs. BRIX and TA (total acids) values for various must samples. For each sample, 25 uL of must, 25 µl 0.1 U/ml cellulase, and 50 µl substrate Resorufin β-D-Cellobioside were added to a 96-well microtiter plate (clear, flat bottom). Fluorescence was recorded using a Perkin-Elmer HTS 7000 BioAssay Reader, with 550nm excitation filter and 595nm emission filter. Fluorescence readings were taken at 3-minute intervals for 120 minutes. Fluorescence values of blank (50 µL substrate reagent added to 50 µL reaction buffer) were subtracted at each time point. Initial velocities were calculated by determining the tangent slope for the first 9 minutes. Data analysis was performed using Microsoft Excel linear regression software.

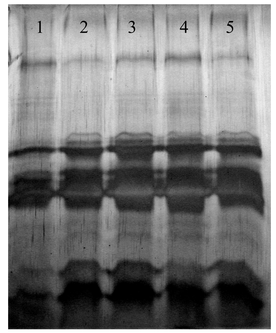


Figure 4. SDS-PAGE gel analysis of proteins from Pinot Gris grapes with increasing BRIX levels. BRIX levels: lane 1 = 17.9; lane 2 = 19.4; lane 3 = 20.0; lane 4 = 20.5; lane 5 = 21.0.

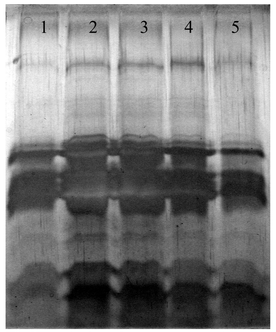


Figure 5. SDS-PAGE gel analysis of proteins from Pinot Noir grapes with increasing BRIX levels. BRIX levels: lane 1 = 20.1; lane 2 = 21.0; lane 3 =22.0; lane 4 = 22.9; lane 5 = 23.3.

Discussion and Conclusions

We measured cellulase activity in grape and must samples from Pinot Gris and Pinot Noir grape varieties and found no activity in any grape samples tested.

However, the presence of a cellulase inhibitor or inhibitors was found in both Pinot Gris and Pinot Noir grape varieties correlating to ripening and veraison. Previous publications have shown a positive correlation between cellulase activity and berry abscission.[1] It is thought that this cellulase activity is localized primarily in the cell wall, middle lamella, and paramural bodies of abscission zone cells[2] where the grape is attached to the stem. This may explain why mature berries have exhibited less cellulase inhibition. As the berry matures cellulase activity may be localized to the abscission zone and inhibited in other areas of the berry.

We believe that the cellulase inhibition assay may be a convenient, high-throughput method to monitor grape ripening. An inverse relationship between ripening (measured using the typical BRIX and TA analyses) and cellulase inhibition was found.

Our data indicates that the cellulase inhibitor in the grape samples is most likely a small molecule as the samples assayed showed no protease activity (data not shown). And SDS-PAGE gels (Figures 4 and 5) did not show a correlation between protein levels and BRIX values.

Cellulase inhibition has also been reported in grape leaves. Porter and Schwartz identified these cellulase inhibitors to be tannins.[3] Condensed tannins are known to be present in grape skin and seeds. The grape samples tested where in contact with their skin and seeds possibly providing a source of the inhibitory tannin. Our next objective will be to measure tannin levels in these same samples.

References

1. Yun, D., Ying W., Yunfei, L. Effects of high CO_2 and low O_2 atmospheres on the berry drop of 'Kyohn' grapes. Food Chem. 100: 768–773. 2007.
2. Baird, L.M., and Reid, P.D. Cellulase activity and localization during induced abscission of *Coleus blumei*. J. Plant Growth Reg. 11:129-134. 1992.
3. Porter, W.L., Schwartz, J.H. Isolation and description of the pectinase-inhibiting tannins of grape leaves. J. of Food Sci. 27:416-418. 1962.