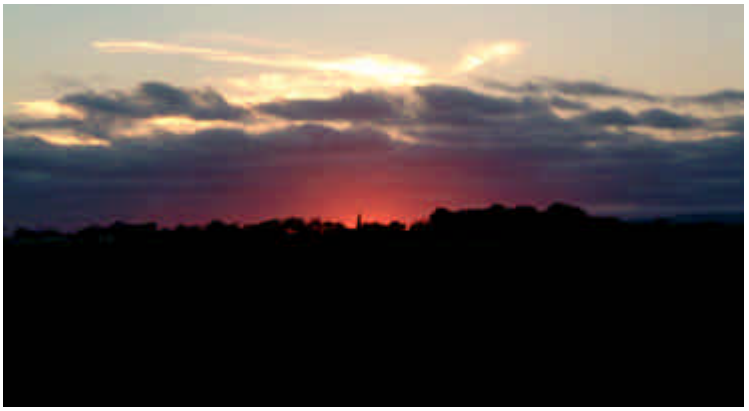
Grape and Wine Research and Development Corporation
Final Report

Improving Methods to Analyse Grape Seed and Grape Skin Tannins by Enzyme Hydrolysis



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 00/3**

Principal Investigator: **Assoc. Prof. J.D. Brooker**

Research Organisation: **University of Adelaide**

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Executive Summary
Improving methods to analyse grape seed and grape skin tannins by enzyme hydrolysis

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Condensed tannins from grape seeds and skins are important contributors to wine colour and organoleptic properties, and the description of their structures is important in identifying key factors for improvement of viticultural techniques. Unfortunately, complex tannins containing gallic acid residues interfere with procedures to chemically identify and study these tannins. The aim of this project is to use enzyme extracts from bacterial and fungal sources to remove gallic acid groups from grape seed and grape skin tannins. This will provide a less complex tannin preparation and will enable established analytical methods to be more effectively carried out. The outcome will be to provide valuable information on structures of grape seed and skin tannins, which can then be used to develop viticultural techniques that may ultimately improve red wine colour stability and organoleptic properties.

Enzyme extracts.
Fungal tannase preparations (crude, partially purified, and highly purified) were prepared from enzyme supplied by the Kikkoman Corporation, Japan. We also have obtained the gene encoding the fungal tannase. Tannin-induced bacterial tannase extracts were prepared from growing bacterial cultures.

Enzyme activity
Tannin extracts and highly purified tannins were obtained by extraction from grape seeds and skins. The activity of the fungal and bacterial tannase against a standard compound (gallic acid methyl ester), as well as pure tannins and crude tannins from grape seed and grape skins was tested. The results showed that enzyme action yielded gallic acid from all substrates, but with varying activities. This suggests that where condensed tannins contain gallate esters, the tannase enzyme can cause degradation, with the complex tannins yielding more gallic acid. However, with crude grape tannin extracts, activity was quite low, suggesting that either the concentration of gallate esters may not be high enough or other tannin components inhibit the reaction.

Products of fungal and microbial tannase hydrolysis were analysed. Results show that enzyme action reduced the level of phloroglucinol monomers, indicating that even with large purified tannins, the enzyme is not inactivated. This suggests that the enzyme may be a valuable additional tool for analysis of complex condensed tannins from grapes, and could yield a modified profile that may be simpler to interpret than that of the parent compound. Tannase activity was not able to destroy the carbon framework of the tannins, which may be beneficial in the interpretation of structural information.

Screening for microorganisms from winery waste.
We have also isolated microorganisms from winery waste sites for the presence of tannin-transforming enzyme activities. A number of bacteria enriched from grapemarc dump sites have been isolated, and, in future work, these will be analysed for tannin degrading activities. Some of these may add to the activity of tannase to provide structural information on complex tannins and will complement research to chemically define tannin structures from wine grapes.

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Project Title	Improving methods to analyse grape seed and grape skin tannins by enzyme hydrolysis
Project No	UA 00/3
Organisation	Adelaide University
Location	Wine Science and Microbiology Laboratories, Waite and Roseworthy campuses
Supervisor	Associate Professor J.D. Brooker
Staff	Dr. G. Jones, Mr. H. Jones
Funding	$29,000
Time span	2000/2001

Objectives

- To use fungal and bacterial enzyme extracts to remove carbohydrate from grape seed and skin tannins
- To analyse products of enzyme hydrolysis
- To examine other potential microbial sources of enzymes

Final outcome

Enzyme extracts.

Fungal tannase preparations (crude, partially purified, and highly purified) were prepared from enzyme supplied by the Kikkoman Corporation, Japan. We also have obtained the gene encoding the fungal tannase.

Tannin-induced bacterial tannase extracts were prepared from growing cultures of *Selenomonas ruminantium* K2 in the presence of tannic acid, or gallic acid methyl ester (GAME). Cell extracts were obtained by ultrasonic cell disruption, and were assayed using defined and grape tannin substrates. Products of digestion were analysed by HPLC.

Enzyme activity

Tannin extracts and highly purified tannins were obtained by extraction from grape seeds and skins. Activity of the fungal tannase against a standard compound (gallic acid methyl ester), as well as pure epigallocatachin gallate, total chardonnay tannin and phloroglucinol adducts of epigallocatechin gallate were carried out. The results showed that enzyme hydrolysis yielded gallic acid from all substrates, but with varying activities. Against methyl gallate, one unit of crude enzyme released 215.9 nmoles of gallic acid/minute (specific activity of 1,668 nmoles/mg/min), whereas activity against epigallocatachin gallate was as high as 731 nmoles gallic acid /minute (up to 21,007 nmoles/mg/min). Tannase activity against epicatechin gallate phloroglucinol adduct was even higher at 3,776.5 nmoles gallic acid /min (108,404 nmoles/mg/min) although activity against a crude tannin extract from chardonnay was very low at 6.4 nmoles gallic acid/min (143 nmoles/mg/min). This suggests that where condensed tannins contain gallate esters, the fungal tannase enzyme can mediate hydrolysis, with the complex tannins yielding more gallic acid. However, with crude grape tannin extracts, either the concentration of gallate esters may not be high enough or other tannin components inhibit the reaction. Similar reactions using bacterial extracts were carried out. Results show that in some cases, activity was even higher than with fungalpreparations. The bacterial enzyme was active against GAME, with a specific activity of 3,374 nmoles/mg/min, 2 fold higher than the fungal tannase, tannic acid (11,224 nmoles/mg/min), and epigallocatechin gallate (1046 nmoles/mg/min). Tannase activity using either enzyme preparation against grape seed tannin

was insignificant, but this was reflected in abnormally high backgrounds, and may indicate interference from endogenous compounds giving false rhodanine absorbance readings. Fungal tannase was also active against Riesling seed extract (1206 nmoles/mg/min) and Shiraz skin tannin (144 nmoles/mg/min). These activities are low compared with purified substrates, possibly reflecting the presence of inhibitory compounds in crude extracts.

Products of fungal and microbial tannase hydrolysis were analysed by HPLC, GC-MS and acid hydrolysis in the presence of phloroglucinol. Results show that enzyme hydrolysis reduces the level of phloroglucinol monomers, indicating that even with large tannins, the enzyme is not inactivated. This suggests that the enzyme may be a valuable additional tool for analysis of complex condensed tannins from grapes, and could yield a modified profile that may be simpler to interpret than that of the parent compound. Tannase activity was not able to destroy the carbon framework of the tannins, which may be beneficial in the interpretation of structural information.

Screening for microorganisms from winery waste.
An honours student has been screening microorganisms from winery waste sites for the presence of tannin-transforming enzyme activities. A number of bacteria enriched from grapemarc dump sites have been isolated, and, in future work, these will be analysed for tannin degrading activities. Some of these may complement the activity of tannase to provide structural information on complex tannins.

Summary: This project has demonstrated that both bacterial and fungal tannase are active in cleaving gallate residues from commercial condensed tannins, especially phloroglucinol adducts, and showed activity against grape skin tannins. The enzyme therefore may have potential, in conjunction with phloroglucinol technology, to describe more clearly, complex tannins extracted from grapes. This work will therefore complement research being carried out by Dr. G. Jones to chemically define tannin structures from wine grapes. The purified tannase enzyme preparation will be provided to Dr. Jones for this work.

Summary of Tannase activity against various tannin substrates

Substrate	**Tannase activity (nmol/mg protein/min** 244,237,234 = fungal tannase preparations K2 = bacterial tannase			
	Tannase 244	**Tannase 237**	**Tannase 234**	**Tannase K2**
Epigallocatechin gallate	8,980	18,129	2,401	1,046
Epicatechin gallate-phloroglucinol adduct	37,892	108,404	N/A	N/A
Gallic acid methyl ester	1,668	N/A	N/A	2,320
Tannic acid	N/A	N/A	N/A	11,224
Chardonnay tannin	141	86	N/A	N/A
Grape seed tannin	N/A	N/A	N/A	5
Riesling seed extract	1,206	N/A	N/A	25
Shiraz tannin	56	57	N/A	N/A
Shiraz skin tannin	144			24