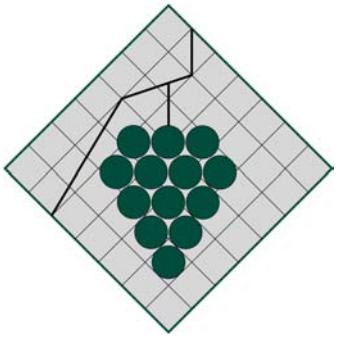




An investigation of flavonol glucosyltransferase activity in grapevine tissue

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Principal Investigator: **Christopher M Ford**

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Abstract

Flavonols are important components of the berry-derived non-pigmented phenolic compounds found in wines. In common with many plant compounds, they are conjugated to sugar molecules by enzymes called glycosyltransferases. The enzymes responsible for this in grapevine tissues have not been isolated. The research aimed to achieve a preliminary characterisation of flavonol glucosylating activity in grapevine tissues, and to suggest approaches for a future purification of the enzyme. Glucosylation of flavonols was detected in extracts from grapevine leaves, techniques for further purification were studied and intriguingly, a preliminary observation was made that specific flavonoids may be glucosylated by specific enzyme activities.

Executive Summary

This project funding was sought to provide a HECS scholarship and research support for an Honours student (Christobelle Anderson) working in the Principal Investigator's laboratory during 2003. The research undertaken for the Honours project was intended to detect, measure and isolate enzymatic activity capable of forming glucosides of flavonol compounds in soluble protein extracts prepared from grapevine tissues. Previous work by the PI and many others (**Boss, Davies, et al. (1996) Plant Physiology 111(4), 1059; Ford, Boss and Høj, 1998, J. Biol. Chem 273, 9224**; **Ford and Høj, 1998, Aus. J Grape and Wine Res. 4, 48)** has shown the existence of enzymes, glucosyltransferases, in grapevines that are responsible for glucosylating anthocyanidins, the precursors of anthocyanins, in developing grape berries. However, the flavonols, a class of compound very closely related to anthocyanidins but present at much lower levels in grapes and wine, would appear to be glucosylated by a distinct glucosyltransferase activity which as yet remains uncharacterised in grapevine tissues. Elucidation of the mechanism by which flavonol glucosylation is achieved in grapevine tissues will provide an important indication of the complexity of biosynthetic processes present within the developing grape berry, and may potentially provide a target for both viticultural management strategies to optimise levels of flavonol glucosylation, and for future experiments to modify berry composition through the use of transgenic technology.

The second aspect to the industry context lies with the increased understanding of how important grape and wine compositional parameters, in this case the flavonols, are made during berry development. Research has shown (**Haselgrove et al, 1999, Aus J Grape and Wine Research 6, 141**) that flavonol biosynthesis, unlike that of anthocyanins, occurs early in berry development; an understanding of the viticultural practices likely to impact on these

events, and of how winemaking practices may best adapt to maximise the levels of desirable flavonols during vinification, will come only from an in-depth knowledge of their biosynthetic origins in the berry.

In addition, an important part of the project was the provision of research training for an undergraduate student enrolled in the B.Ag. Sci. (Oenology) program at The University of Adelaide. By exposing students to the research environment during their undergraduate studies, and promoting this by providing payment for the HECS debt incurred during the Honours program, many benefits are incurred. First, the student may choose to continue their academic career by enrolling for a PhD degree. Second, the wine industry is supplied with winemakers and suchlike with an enhanced appreciation of the potentials, and limitations, of scientific research. Third, the independence and self-motivation that inevitably develop in the student during the course of a successful Honours year will be a major benefit both to the student and to their prospective employer (most probably within the Australian wine industry).

Background, Project Aims and Performance Targets

Grape berry composition impacts massively on the structure and organoleptic profile of finished wines. Many of the most important grape-derived compounds arise from the multitude of secondary metabolic pathways that operate during grape berry development and ripening. Foremost among these are the products of the flavonoid biosynthetic pathway, notably the anthocyanins, responsible for the colour of red wines, the flavanols, which polymerise to form condensed tannins, and the flavonols. In common with many plant secondary metabolites, many flavonoids are stabilised during their biosynthesis and beyond by the enzymatic addition of one or more sugar molecules. This is catalysed by the action of glycosyltransferase enzymes, which appear to be highly specific towards the acceptor molecules onto which they attach specific sugars. Earlier work (**Ford, Boss and Høj, 1998, J. Biol. Chem 273, 9224**) showed clearly that an enzyme responsible for forming anthocyanidin glucosides (anthocyanins) in grapevines was not the same enzyme responsible for making glucosides of the common flavonol compounds quercetin and kaempferol in the same tissues. Additional studies (**Ford and Høj, 1998, Aus. J Grape and Wine Res. 4, 48**) indicated that in soluble protein extracts prepared from grapevine leaves and berries, multiple glucosyltransferase activities were responsible for forming the many glucosides seen in these tissues.

The research undertaken in the present study was intended to demonstrate that flavonol glucosylating activity could be detected and measured in grapevine tissues, and that with the use of appropriate chromatographic techniques, an enrichment of such activity could be obtained. In addition, the flavonol glucosylating activity would be further investigated with regard to its specificity and where possible kinetic properties.

Methods

- Extracts of total soluble proteins in grapevine tissues were made using buffer solutions developed by the PI that minimise the interaction between the protein components of the tissue and its high content of polyphenolic compounds.
- Assays for the formation of flavonol glucosides were performed using a suitable donor substrate (UDP-glucose) and a range of flavonol acceptor substrates available in the PI's laboratory. Product formation was detected by HPLC separation and absorbance monitoring, and where this proved to be of inadequate sensitivity, by the use of a radiolabelled substrate, UDP-[U^{14}C]-glucose and suitable detection protocols.
- Qualitative and quantitative glucosylation assays were developed, and the formation of glucosides of a range of flavonoid compounds was studied.
- Enrichment of protein fractions for glucosyltransferase activity was examined by continuous gradient ion exchange chromatography.
- Pseudo-affinity dye chromatography, used in the purification of a number of glucosyltransferases from other plants, was tested for its usefulness in isolating the flavonol glucosylating activity.
- All separation stages were monitored by SDS-polyacrylamide gel electrophoresis to allow visualisation of the consequences of chromatographic steps on the composition of total proteins in the extracts studied.

Results and Discussion

1. Detection and measurement of flavonol glucosylating activity in grapevine tissue extracts.

Crude extracts of soluble proteins were prepared by the PI and frozen at –80C until required. Glucosylation of the flavonol quercetin was demonstrated in thawed aliquots of crude extract

using a simple assay buffer system and detection by HPLC. The level of activity was low however, and in consequence assays were developed in which the radiolabelled donor substrate UDP-[U^{14}C]-glucose was used. This enabled the sensitive detection of low levels of activity. The crude extract was desalted to remove small molecular weight compounds, among which may have been potential competitor substrates for the enzymes whose activity we were trying to measure, and concentrated prior to further enzyme assays. Quantitative determinations of the extent of flavonol glucosylation indicated that a large proportion of the activity was being lost during this step, which suggested that the successive freezing and thawing was detrimental to enzyme stability. Consequently a purification protocol using crude extract loaded directly onto an anion-exchange resin was tested. There was a clear peak of quercetin glucosylating activity eluting mid-way through the salt gradient, but again almost 90% of the activity loaded was lost during the purification. Although examination of the peak fractions revealed a high degree of difference from the loading material, the loss of activity resulted in approximately 5-fold purification for the ion-exchange step. Nevertheless, with a more stable starting material, namely a freshly prepared extract that is processed continuously from harvest to further purification steps, ion-exchange chromatography should provide a powerful means of initial separation.

2. Further purification of grapevine flavonol glucosylating enzyme activity.

Reactive dye pseudoaffinity chromatography has been successful in the isolation of several plant glucosyltransferases. The method works on the fortuitous structural similarity between various dye compounds and the nucleotide moiety of the nucleotide sugar donor substrate of the glucosylation reaction. Several dye resins were tested in a batch format for their ability to bind flavonol glucosyltransferase activity from the concentrated peak fractions obtained by ion-exchange chromatography. Increasing concentrations of salt were applied to each resin in a simple attempt to elute bound enzymatic activity. All fractions were assayed for their ability to glucosylate quercetin, and the protein composition of each examined by SDS-PAGE. Of the four resins tested, three (Reactive Red, Reactive Green and Reactive Blue) bound the added flavonol glucosyltransferase activity under low-salt conditions and did not release it substantially at salt concentrations up to 400 mM. The fourth resin, Reactive Yellow, did not bind any flavonol glucosylating activity under and salt concentration. SDS-PAGE results indicated that for dyes that bound flavonol glucosyltransferase activity, the bulk of added

proteins in the loading fraction were also bound. Conversely, Reactive Yellow resin did not appear to bind many proteins at all.

3. Substrate specificity of flavonol glucosylating activity

The flavonol glucosylating activity in crude extract and in the desalted/concentrated material, and from the peak fractions eluted from the ion-exchange column, was tested against a range of flavonols and flavonoid compounds. As expected, the crude extracts showed the ability to form glucosides of several flavonols (quercetin, kaempferol and myricetin, the latter to a slight degree only due to the age of the standard used in assays), as well as representative flavones, flavanones, isoflavones and anthocyanidins. Quantitative assays indicated that while the crude extracts glucosylated quercetin to a greater degree than kaempferol (flavonols) , and cyanidin to a greater degree than delphinidin (anthocyanidins), this pattern of substrate utilisation appeared to be reversed in the peak fractions from the ion-exchange chromatography. This intriguing result, although demanding repetition with a freshly prepared high-activity extract first and foremost, raises the possibility that individual flavonoids have specific glucosyltransferases responsible for their conjugation to sugars.

Outcomes, Conclusion and Recommendations

The work proposed for this Honours project has been successfully completed. An enzyme activity directing the glucosylation of flavonols in grapevine tissue extracts was identified and characterised, and important preliminary steps completed towards the eventual purification of the enzyme.

The Honours student successfully completed all aspects of the program on time and to a very high standard. In addition she was necessarily enrolled in a further two full-time (6 hours per week contact time and at least that again in private study) courses. The final thesis for the Honours program was submitted on Monday November 3 2003; a seminar will be presented in early December 2003 following which the examiners will meet and determine the final honours grade to be awarded.

It is hoped that a new honours student will continue this work in 2004. If a suitable student is identified, it is proposed that further extracts be prepared and immediately subjected to chromatographic separation with no intervening freezing stages. Subsequently, the flavonol glucosyltransferase-enriched fractions will be carefully examined to determine if substrate

specificities and glucosylation rates are indeed different following separation by ion-exchange chromatography.

Appendix 1: Communication

The results of work carried out in this project will be incorporated into the appropriate sections of research papers to be published in due course.

Appendix 2 Intellectual Property

Not applicable to this report.

Appendix 3 References

Citation details were presented in the text.

Appendix 4: Staff

Principal Investigator – Dr Christopher M Ford
Honours Student – Christobelle Anderson (January to November 2003)
Second supervisor – Dr Ewald Swinny (Discipline of Wine and Horticulture, School of Agriculture and Wine, The University of Adelaide)

Appendices 5 and 6

Not applicable to this report.