CHARACTERISATION OF A TANNIN ACYLHYDROLASE FROM
A RUMINAL SELENOMONAD

Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
Faculty of Agricultural and Natural Resource Sciences

by

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Summary

Tannins are naturally occurring, phenolic polymers found in a variety of plants. They contain a large number of phenolic hydroxyl groups which allow them to form cross-links with proteins and other macromolecules. Based on their chemical structure, they have been divided into two groups; hydrolysable and condensed. Hydrolysable tannins consist of a central carbohydrate core, usually glucose, to which a number of gallic or ellagic acid molecules are esterified, and which can be hydrolysed into their component sugars and phenolic acids. Condensed tannins have no carbohydrate core nor ester bonds and are formed by condensation of flavan-3-ols (e.g. catechin) or flavan-3,4-diols (e.g. leucocyanidin).

The inhibition of ruminant digestion by dietary tannins is a significant factor limiting livestock production in many areas of the world. Ingested tannins can inhibit ruminant digestion by complexing with proteins and minerals forming complexes which are poorly digestible and by inhibiting microbial growth and metabolism. However, not all ruminants are adversely affected by tannins. It has been observed that feral ruminants in Australia, such as goats and camels, thrive on tannin-rich feeds. This has led to the proposal that such animals may contain novel rumen microorganisms which detoxify ingested tannins or are immune to their antimicrobial properties. One such organism, a tannin-resistant *Streptococcus*, has been previously isolated from the rumen of a feral goat browsing tannin-rich *Acacia* sp. and the presence of this organism appears to be correlated with a diet containing tannins. Given the chemical diversity of tannins it is unlikely that a single organism is responsible for the detoxification of tannins; rather, detoxification may be the result of a consortium of microorganisms.

Therefore, the aim of this PhD project was to screen feral goat rumen fluid for the presence of new organisms that may play a role in the detoxification of tannins and to investigate their mechanism(s) of action.
To this end, an enrichment experiment was conducted to screen rumen fluid from feral goats for anaerobic bacteria capable of growing in the presence of high levels of *Acacia* condensed tannin. Four morphologically-distinct bacteria were isolated as a result of this enrichment, confirming that resistance is a property shared by more than one organism. One isolate was chosen at random for further characterisation and was identified as a strain of *Selenomonas ruminantium* subspecies *ruminantium* on the basis of fermentation of carbohydrates, volatile fatty acid production profile, morphology and DNA-DNA hybridisation. This isolate was arbitrarily designated strain K2. *S. ruminantium* K2 was shown to be not only tannin-resistant but also able to grow on tannic acid, a hydrolysable tannin consisting of esters of glucose and gallic acid, as a sole energy source. This property has never previously been reported in an anaerobic bacterium and raised the possibility that there may be a link between this activity and the ability of feral goats to consume a diet high in tannins. Purified *Acacia* condensed tannin did not support growth of K2, suggesting that a mechanism for growth on hydrolysable tannin could be found in the fundamental differences in chemical structure between the two classes of tannins. Paper chromatography of tannic acid revealed that K2 was not growing on contaminants of tannic acid such as free glucose. Furthermore, K2 was unable to grow on gallic acid or other phenolic acid monomers as sole energy sources. When K2 was grown on tannic acid as the sole energy source, gallic acid was released from tannic acid into the growth medium, indicating hydrolysis of the galloyl glucose esters. Subsequent experiments demonstrated enzymatic hydrolysis of tannic acid by a crude cell lysate. This enzymatic activity has never previously been demonstrated in any bacterium and provided an explanation for the growth of K2 on hydrolysable tannin and the lack of growth on purified condensed tannin. It was proposed that this bacterium obtained energy for growth from tannic acid by fermenting the glucose released by enzymatic hydrolysis of the tannin. Such activity is characteristic of a tannin acylhydrolase (EC 3.1.1.20); an enzyme previously described in aerobic fungi of the genii *Aspergillus*, *Penicillium*, *Trichoderma* and *Candida*.

An assay was developed to measure the enzymatic release of gallic acid from the defined substrate gallic acid methyl ester (GAME) by K2 cell-free extracts. The enzyme assay was
optimised and the pH, temperature optima and $K_m$, $V_{max}$ were determined using GAME. The enzyme has a pH optimum of 7 and a temperature optimum of 30-40°C. In cell-free extracts, the maximal rate of GAME hydrolysis was 6.3 μmol minute$^{-1}$ mg$^{-1}$ of protein and the $K_m$ for GAME was 1.6 mM. Apart from GAME, the enzyme was shown to hydrolyse gallic acid lauryl ester and tannic acid. Commonly used non-specific esterase substrates such as α-naphthol acetate, p-nitrophenol acetate and methylumbelliferyl acetate were not hydrolysed. The enzyme was not inhibited by PMSF, indicating that it does not contain a serine residue at its catalytic site. These results suggest that the enzyme may display specificity for gallic acid esters and may be a new tannin acylhydrolase rather than a non-specific esterase.

The specific activity of K2 tannin acylhydrolase was measured in both cell-free extracts and whole-cell suspensions prepared from K2 cells grown in the presence and absence of various phenolic compounds. The enzyme was produced constitutively however its activity was higher in cells grown in the presence of tannic acid, gallic acid and GAME. This result may represent the first example of bacterial gene expression regulated by tannins.

A zymogram was developed using GAME as the substrate in order to determine the enzyme's size and isoelectric point. SDS-PAGE gels and zymograms indicated that the enzyme is a single polypeptide of approximately 60kDa molecular weight. Isoelectric focusing and zymograms indicated that the tannin acylhydrolase has an approximate isoelectric point of 7.0. The enzyme was not secreted as no activity was detected in concentrated spent-media. Furthermore, the enzyme was Triton X100-extractable, however, it was not clear if it was located in the periplasm or the cytoplasm.

A preliminary screening of 19 strains of *Selenomonas ruminantium* revealed that tannin acylhydrolase activity was comparatively rare; weak activity being detected in only 3 strains apart from K2.
A variety of different approaches were taken with the aim of cloning the gene coding for the K2 tannin acylhydrolase. Plasmid and λ libraries of K2 genomic DNA were prepared and screened for expression of tannin acylhydrolase activity using a substrate-overlay technique. No tannin acylhydrolase-positive clones were identified, possibly because the *Selenomonas* promoter did not function in *E. coli*. The prokaryotic promoter selection vector pKK232-8 was employed in a bid to clone a tannin-inducible promoter which could then be used as a probe to clone the complete tannin acylhydrolase gene. Although fragments of K2 genome were cloned that functioned as promoters in *E. coli*, none of these were tannin-responsive. An attempt was made to raise rabbit polyclonal antisera to the tannin acylhydrolase which could then be used to screen a λgt11 library for the expressed β-galactosidase-tannin acylhydrolase fusion protein. No antibodies were detected, possibly because not enough protein was used or because the protein was not highly immunogenic.

Preparative isoelectric focusing was combined with SDS-PAGE and electroblotting to PVDF membrane to provide a sample of the enzyme for determining the N-terminal amino acid sequence. Thirteen amino acids (which included some gaps) were identified from the N-terminus and from this an 18 base, 24-fold degenerate oligonucleotide was designed and synthesized. This degenerate oligonucleotide was radiolabelled and used to probe the λgt11 library for the tannin acylhydrolase gene. Although positive plaques were detected, time restrictions limited further analysis. To aid in the identification of tannin acylhydrolase-positive plaques from within the λgt11 library, a sample of tannin acylhydrolase was subjected to digestion with endoproteinase-Lys C and the peptides were separated using HPLC. One peptide was sequenced and revealed 10 amino acids from which a 23 base, 48-fold degenerate oligonucleotide containing the reverse complement sequence was synthesized. The two sets of degenerate primers were used in a degenerate oligonucleotide primed PCR (DOP-PCR) strategy to amplify the intervening sequence in order to produce a specific probe which could be used to identify the tannin acylhydrolase gene within the λgt11 library. Several products were obtained, one of which (170 bp) was cloned and sequenced. This clone proved to be incorrect as the DOP-PCR product apparently arose from mispriming events and did not represent a
portion of the K2 tannin acylhydrolase gene. Other products from the DOP-PCR experiment may prove to be correct and should be investigated in future.

The exact role of tannin acylhydrolase in *S. ruminantium* K2 is not clear, however, it is hypothesised that this enzyme has evolved as a means by which this bacterium can gain energy from phenolic acid-carbohydrate esters, a widely available but microbially under-utilised resource. Future research should concentrate on determining the exact role of the tannin acylhydrolase in K2 and determining whether or not this bacterium contributes to the ability of feral goats to efficiently utilise plant material rich in tannins.