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 (eg catechin) or flavan-3,4-diols (eg 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-napthol acetate, ρ-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.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Acknowledgments

I wish to gratefully acknowledge my supervisor Dr John Brooker (Rumen Microbial Genetics Group, Department of Animal Science) for his expert support and guidance of my project. I would also like to acknowledge additional support provided by Professor Cynthia Bottema (Livestock Genetics Group, Department of Animal Science).

I am indebted to the Australian Wool Research and Promotion Organisation for their provision of a generous Postgraduate Research Scholarship, without which this work would not have been possible.

I wish to acknowledge the assistance of Irene Wilkinson (Division of Clinical Microbiology, The Institute of Medical and Veterinary Science) for her help in the identification of *S. ruminantium* K2; Denise Turner (Department of Biochemistry, The University of Adelaide) for protein sequencing from PVDF membrane; and Dr Neil Shirley (Nucleic Acid and Protein Chemistry Unit, Department of Plant Science, The University of Adelaide) for assistance with HPLC separation of peptides and peptide and nucleic acid sequencing.

I would also like to express my gratitude to Deanne Lum and Jane McCarthy for facilitating the day-to-day operations of the lab; and Jenny Prosser and Rex Connelly for their support and assistance with the administration of my candidature in general.

I would like to thank my contempories in the Department of Animal Science, in particular fellow members of the Rumen Microbial Genetics Group, for making my time as a research student memorable and enjoyable.

Finally, I would like to express my gratitude to my wife, Trudi, for her support, understanding and unwavering patience, all of which aided me in the completion of this project.

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List of Abbreviations

3,4-DHP	3-hydroxy-4-1(H)-pyridone
API	Analytical Profile Index
AR	analytical reagent
BCIP	bromochloroindoyl phosphate
BDH	British Drug Houses
bp	base pairs
BSA	bovine serum albumin
CLSM	confocal laser scanning microscopy
CPM	counts per minute
Da	Daltons
DIFP	diisopropylfluorophosphate
DM	dry matter
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOP-PCR	degenerate oligonucleotide primed-polymerase chain reaction
DTT	dithiothreitol
eg	example
EMP	Embden-Meyerhof-Parnas
EPS	exopolysaccharide
G/C	guanine/cytosine
GAME	gallic acid methyl ester
GC	gas chromatography
HPLC	high performance liquid chromatography
ie	that is
IEF	isoelectric focusing
IPTG	isopropylthiogalactoside
mBHI	modified Brain Heart Infusion
MCS	multiple cloning site
mM10	modified M10
NBT	nitroblue tetrazolium
PAGE	polyacrylamide electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
PMSF	phenylmethylsulphonyl fluoride
PRPs	proline rich proteins

PVDF	polyvinylidene difluoride
RO	reverse osmosis
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SEM	scanning electron microscopy
spp.	species
SSC	standard saline citrate
TFA	trifluoracetic acid
TIS	transcription initiation site
T_m	melting temperature
TMAC	tetramethyl ammonium chloride
UTR	untranslated region
v/v	volume per volume
VFA	volatile fatty acid
VFI	voluntary feed intake
w/v	weight per volume

CHAPTER 1 Introduction and review of literature.

1.1 Introduction

Ruminant animals gain their energy from the digestion of fibre resulting from the action of bacteria, fungi and protozoa in their rumen, the first section of their gastrointestinal tract. This classic example of a symbiotic relationship allows ruminants to subsist on fibrous, low protein feeds which are indigestible by most non-ruminant animals. Furthermore, ruminants are able to digest many plants which are toxic to monogastric animals, including humans, because rumen microorganisms can transform many toxic ingredients to harmless compounds (Gregg, 1995). However, there are examples of plants which contain antinutritive or toxic compounds which inhibit ruminant digestion and therefore overall animal productivity. Common examples of plant secondary compounds which affect the nutritive value of forages are alkaloids such as pyrrolizidine, cyanogenic and coumarin glycosides, terpenes such as saponins, simple acids such as fluoroacetate and oxalate, amino acids mimosine and indospicine, and polyphenols such as tannins. In some cases, initially innocuous compounds are converted by rumen bacteria to toxic metabolites; such as the production of cyanide from cyanogenic glycosides, while in other cases, toxicity is believed to be due to an inability of the rumen microorganisms to metabolise the compounds responsible.

This project concentrates on one class of plant secondary compounds known to adversely affect ruminant nutrition; the tannins. In the following literature review, a general overview of ruminant digestion is provided followed by a discussion of tannins and their impact on ruminant digestion. General reviews of rumen microbiology can be found in "The Rumen Microbial Ecosystem (ed. Hobson, 1988) and "Nutritional Ecology of the Ruminant" (van Soest, 1987).

1.2 Review of literature

1.2.1 Overview of ruminant digestion and nutrition

1.2.1.1 Anatomy and function of the rumen

The stomach of the ruminant is divided into four compartments; the rumen, reticulum, omasum and abomasum. In the newborn, the first two compartments, (rumen and reticulum), are relatively undeveloped and ingested milk is diverted by a tube-like fold of tissue known as the

oesophageal or reticular groove directly to the omasum and abomasum (McDonald *et al*, 1988). As the infant begins to eat solid food, the reticulum and rumen enlarge greatly, until in the adult they comprise 85% of the total capacity of the stomach. The rumen, the largest structure, constitutes from 10-15% of the total mass of the animal (Russell and Bruckner, 1991). The oesophageal groove does not function in the adult under normal feeding conditions, and both food and water pass into the reticulo-rumen.

Ingested food is diluted with large amounts of saliva, firstly during eating and again during rumination. Typical quantities of saliva produced per day are 150 l in cattle and 10 l in sheep (McDonald *et al*, 1988). Rumen contents often occur in two phases: a lower liquid phase in which the finer food particles are suspended, and a drier upper layer of coarser solid material. On average, the solid material accounts for 7-15% of the weight of the rumen contents, water making up the balance.

The breakdown of food is accomplished partly by physical and partly by chemical means. Rumen contents are continually mixed by rhythmic contractions of its walls, and during rumination, material at the anterior end is drawn back into the oesophagus and returned to the mouth by a wave of muscular contraction. Any liquid is quickly re-swallowed, but coarser material is thoroughly chewed before being returned to the rumen. Re-chewing reduces particle size and increases the surface area exposed to microorganisms (Russell and Bruckner, 1991).

The mucosa lining the rumen, reticulum and omasum does not secrete mucus, digestive enzymes or acid, and digestion results from microbial action. Fermentation of feedstuffs in the reticulo-rumen yields short-chain volatile fatty acids (mainly acetic, propionic and butyric), carbon dioxide, methane, ammonia, microbial cells, heat and occasionally lactic acid. Ruminants use the volatile fatty acids and microbial protein as sources of energy and amino acids respectively, but production of methane, heat and ammonia can be the cause of a loss of energy and nitrogen to the animal (Russell and Bruckner, 1991; Mackie and White, 1990). Carbon dioxide and methane are lost by eructation (belching) and the volatile fatty acids are

mainly absorbed through the rumen wall. The microbial cells, together with undegraded food components, pass to the abomasum and intestines; there they are digested by enzymes secreted by the host animal, and the products of digestion are absorbed. The quality and quantity of rumen fermentation products is therefore dependant on the types and activities of microorganisms in the reticulo-rumen.

The acids produced by fermentation are theoretically capable of reducing the pH of rumen liquor to 2.5-3.0, however, under normal conditions the pH is maintained at 6.8-7.2 (McDonald *et al*, 1988; Yokoyama and Johnson, 1988). This is achieved by phosphate and bicarbonate in the saliva acting as buffers in addition to the rapid absorption of the acids and ammonia. The average composition of the gas mix in the rumen is : 65% CO_2, 27% CH_4, 7% N_2, 0.6% O_2, 0.2% H_2 and 0.01%H_2S (Yokoyama and Johnson, 1988). The rumen temperature remains relatively constant and close to that of the host animal (38°- 42°C) (Yokoyama and Johnson, 1988).

The rumen is often described as a continuous culture device because feed, water and saliva enter the rumen at regular intervals, and because digesta are washed out to the lower gut. It provides an ideal environment for maintaining a stable, anaerobic microbial population. Typical estimates of population sizes are 10^9-10^{10} bacteria/ml and up to 10^6 protozoa/ml of rumen contents (Russell and Bruckner, 1991; Yokoyama and Johnson, 1988; McDonald *et al*, 1988). Anaerobic fungi have also been shown to be present and are said to play an important role in fibre digestion, being responsible for 40-70% of plant material digestion (Akin and Rigsby, 1987; Akin *et al*, 1990). Their numbers have not been precisely quantified but one estimate suggested they account for 8% of the total biomass in the rumen (Kemp *et al*, 1984). Rumen fungi are the primary invaders of plant material in the rumen and are believed to make plant fibre more accessible to bacteria (Theodorou *et al*, 1988).

Bacterial diversity within the rumen ecosystem is large- M.P. Bryant has described 22 genera and 63 species. Recent evidence indicates that another level of diversity; genetic diversity

within a given phenotype, may be considerably greater than species diversity. Studies on the genetic diversity of *Butyrivibrio fibrisolvens* (Hudman and Gregg, 1989), *Prevotella ruminicola* (Hudman and Gregg, 1989) and *Selenomonas ruminantium* (Zhang *et al*, 1991) suggest that large genetic variation within individual species may be commonplace in rumen bacteria.

1.2.1.2 Carbohydrate digestion, fermentation and metabolism

The breakdown of carbohydrates in the rumen can be divided into two stages. The first stage is the breakdown of complex carbohydrates to simple sugars which is brought about by extracellular microbial enzymes; and hence is analogous to digestion of carbohydrates in non-ruminants (McDonald *et al*, 1988). The bulk of carbohydrate in ruminant feeds is in relatively insoluble polymers; cellulose, hemicellulose, pectin and starch (Baldwin and Allison, 1983). Cellulose and hemicellulose are the main carbohydrate components of plant cell walls, comprising 40-59% and 20-30% of the dry mass of vascular plants respectively (Ward, 1981). Hence, the breakdown of cellulose and other resistant polysaccharides is the most important digestive process occurring in the rumen. *Ruminococcus flavefaciens, R. albus* and *Fibrobacter succinogenes* are the predominant cellulolytic bacteria in the rumen ecosystem and are extremely efficient in breaking down cellulose (Mackie and White, 1990). The first stage in the breakdown of carbohydrates is best summarised by Figure 1.1.

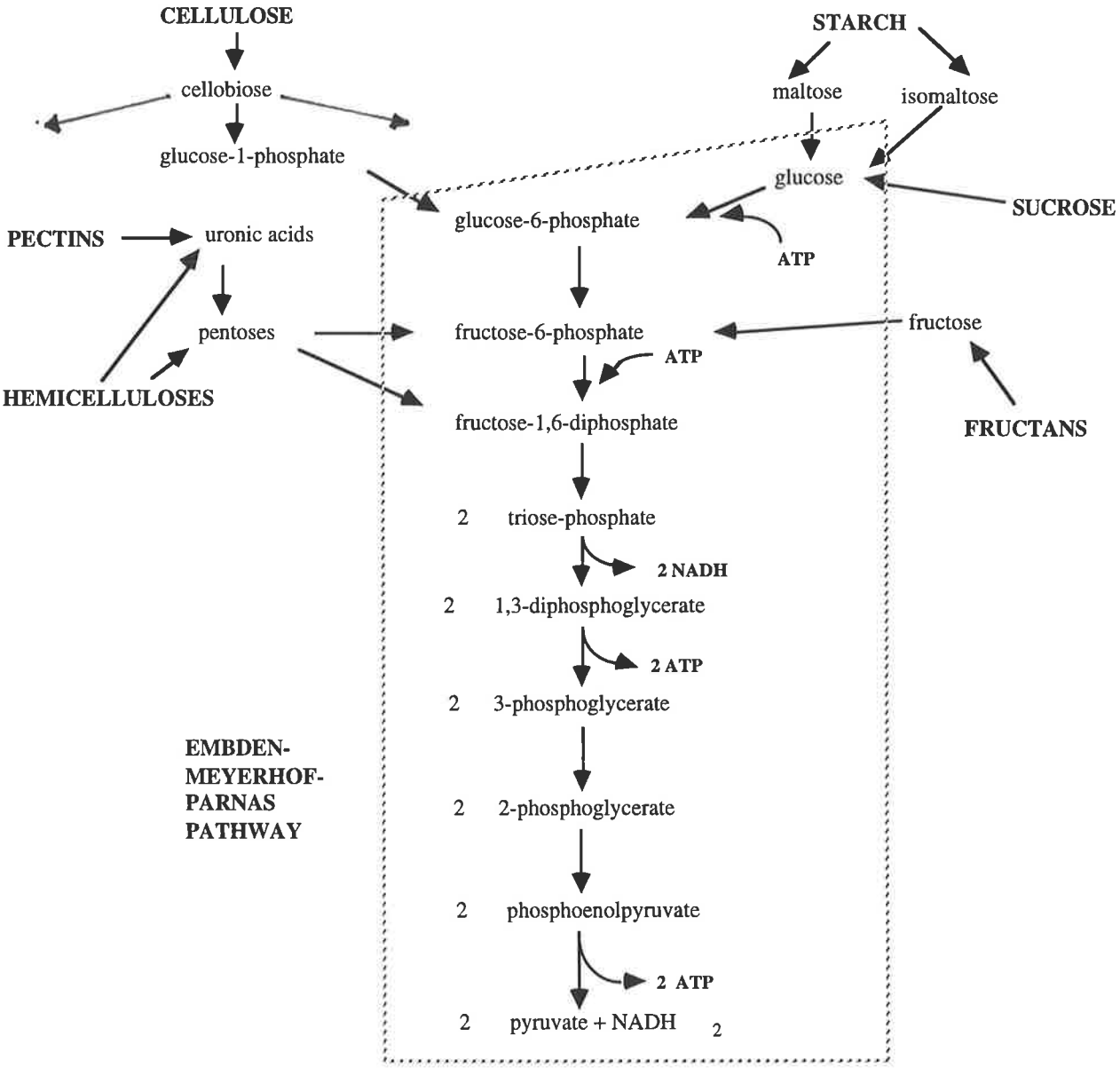


Figure 1.1 Conversion of carbohydrates in the rumen. (From McDonald *et al*, 1988; Czerkawski, 1986)

Cellulose is degraded by one or more ß-1,4-glucanases to the disaccharide cellobiose which is then converted either to glucose or glucose-1-phosphate. Starch and dextrans are first converted to maltose and isomaltose by the action of amylases, and then to glucose by maltases and maltose phosphorylases. Hemicelluloses are a group of polysaccharides often consisting of linear xylose chains with varying amounts of arabinose, uronic acids and galactose (Baldwin and Allison, 1983). The major products of hemicellulose breakdown are pentoses which are formed by enzymic action upon the ß-1,4 linkages to produce xylose. Uronic acids may be produced from pectins, which are first hydrolysed to pectic acid and methanol by pectinesterase. The pectic acid is then attacked by polygalacturonidases to produce galacturonic acids, which in turn yield xylose. Xylose may also be produced from the hydrolysis of xylans which may constitute a significant component of the dry matter of grasses (Hespell and Whitehead, 1990). The major xylanolytic bacteria in the rumen are *Butyrivibrio fribrisolvens* and *Prevotella ruminicola*, although the cellulolytic species *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens* also display xylanase activity (Hespell and Whitehead, 1990).

The second stage in the digestion of carbohydrates in the rumen involves the rapid uptake of the simple sugars produced extra-cellularly in the first stage by the microorganisms and subsequent metabolism of these sugars intra-cellularly. Fermentation of sugars is the primary source of energy for the formation of the high energy phosphate bonds (~P) of ATP that are utilised by rumen microbes for maintenance and growth (Baldwin and Allison, 1983). The primary path of hexose fermentation in rumen microbes is the Embden- Meyerhof- Parnas (EMP) pathway depicted in the centre of Figure 1.1. Two high energy phosphate bonds of ATP are used to convert hexoses to triose-phosphates and four are formed during conversion of the triose-P's to pyruvate and $NADH_2$ (Baldwin and Allison, 1983). Hence the net products are two ~P, 2 pyruvate and 2 $NADH_2$. ~~It should be noted that this energy yield from anaerobic fermentation~~

The energy yield from anaerobic fermentation is therefore substantially less than that of aerobic respiration (28-38 moles ATP/ mole glucose), however it should be noted that this is a theoretical energy yield and actual growth yields may differ (see Wallace and Russell in Hobson, 1988).

The fate of pyruvate and $NADH_2$ formed from hexose and pentose fermentation varies greatly depending on the microbes involved and incubation conditions. For the purposes of this review, I shall provide a general discussion only, (see Figure 1.2). The main end products of pyruvate metabolism are the short-chain volatile fatty acids acetate, propionate and butyrate, as well as carbon dioxide and methane. Succinate and lactate are important intermediates. The relative proportions of the volatile fatty acids in the rumen liquor vary with diet, although in general, the predominant acid is acetic. Diets high in cellulose produce the highest levels of acetate while diets high in concentrates will cause a decrease in the proportion of acetate and a rise in the proportion of propionate (McDonald *et al*, 1988). In general, molar ratios (moles acetate: propionate: butyrate) are usually near 65: 25: 10 with roughage diets and 50: 40: 10 for concentrate diets (Owens and Goetsch, in Church, 1988). The majority of the acid produced is absorbed directly from the rumen, reticulum and omasum, although some may pass through the abomasum and be absorbed in the small intestine. Some of the products of carbohydrate digestion are also used by bacteria and protozoa to form their own structural polysaccharides, especially under conditions of surplus carbohydrate (Czerkawski, 1986).

The extent of cellulose digestion in the rumen depends particularly on the degree of lignification of the plant material. The association of lignin with polysaccharide constituents of cell walls limits microbial digestion, and it has been calculated that lignin protects about 1.4 times its own mass of cell wall carbohydrates (Van Soest, 1981). Hence 80% of the cellulose may be digested in young pasture grasses containing only 50 g lignin/ kg dry matter, while in older herbage containing 100 g lignin/ kg dry matter, cellulose digestion may be less than 60% (McDonald *et al*, 1988).

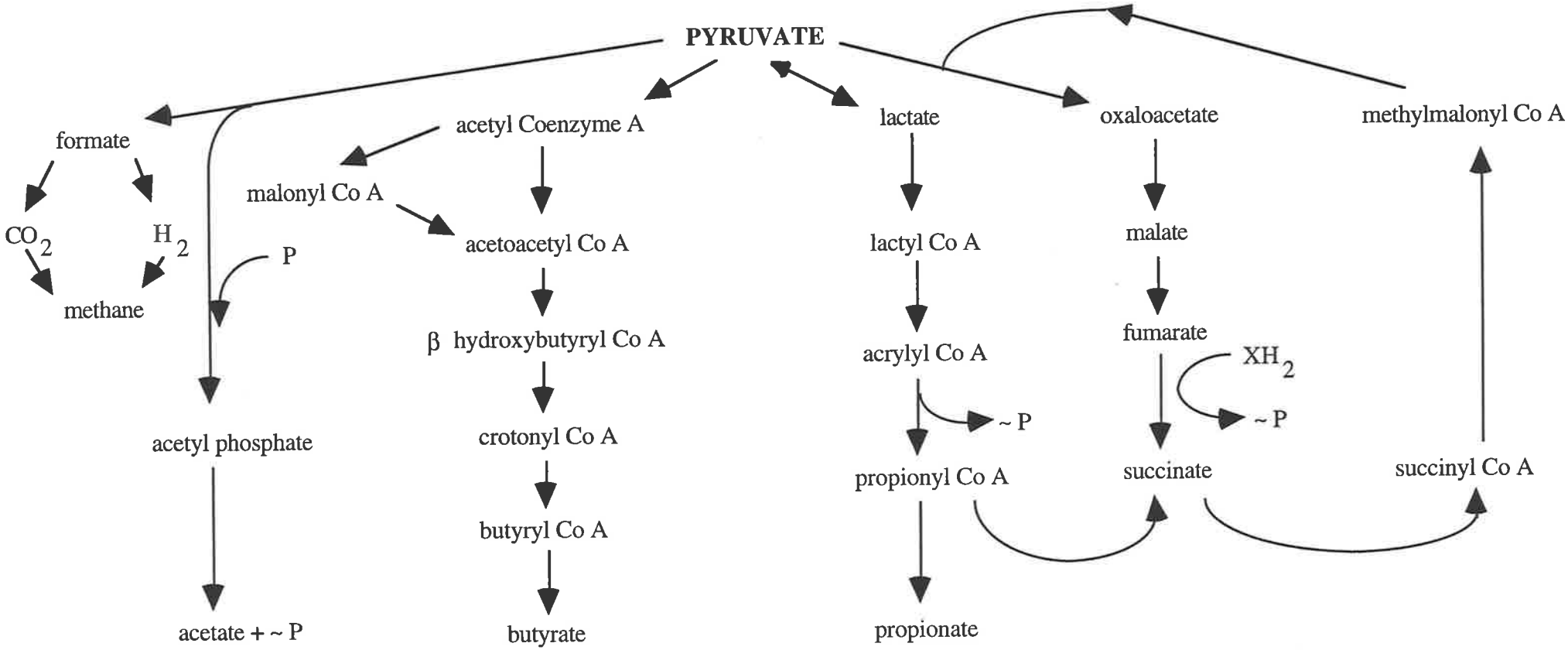


Figure 1.2 Conversion of pyruvate to volatile fatty acids in the rumen.

1.2.1.3 Protein digestion

In ruminants, the amino acid requirements are provided by microbes synthesized in the rumen and from dietary protein that is not degraded in the rumen but is intestinally digestible ("bypass" protein) (Mackie and White, 1990). A large but variable proportion (60-90%) of the dietary protein is degraded by rumen microorganisms. The rate at which the different proteins can be hydrolysed influences the proportion of undegraded dietary protein and microbial protein that is available for digestion in the small intestine.

The change in quantity and pattern of amino acids which results from the conversion of dietary protein to microbial protein in the rumen can be advantageous or disadvantageous to the ruminant, depending on the composition of the food protein. If the food protein is of good quality, value is reduced because the microbial protein is of lower digestibility and is accompanied by nucleic acids. Alternatively, proteolysis during ruminal fermentation may benefit the host animal if the microbial protein synthesized from the products is of higher biological value than the feed proteins (Mackie and White, 1990).

Proteolytic activity in the rumen is not confined to a single bacterium but is a variable property possessed by many different bacteria that may be active in the degradation of other feed constituents, mainly carbohydrates. The major protease-producing bacteria in the rumen include species of *Prevotella, Selenomonas* and *Butyrivibrio*. *Streptococcus bovis* has been shown to be important in ruminal proteolysis on high concentrate diets (Russell *et al*, 1981).

Proteolysis results in oligopeptide production, which then undergo degradation to smaller peptides and amino acids (see Figure 1.3). Currently, our understanding of the metabolic pathways involved in peptide/amino acid utilisation is limited and a detailed review of this field is not required here. In general, the transport of peptides into the cell is considered more important than the uptake of free amino acids owing to the low concentration of free amino acids in rumen fluid (Wright and Hungate, 1967). Free amino acids tend to undergo rapid deamination providing ammonia for bacterial growth (McSweeney *et al*, 1994). The

deamination and degradation of specific amino acids are of special relevance to bacterial growth in the rumen. The most important of these is the conversion of leucine, isoleucine and valine to isovalerate, 2- methylbutyrate, and isobutyrate respectively, because these branched-chain fatty acids have been shown to be either required by or highly stimulatory to the growth of some fibrolytic ruminal bacteria (El-Shazly, 1952; Bryant and Robinson, 1962, 1963).

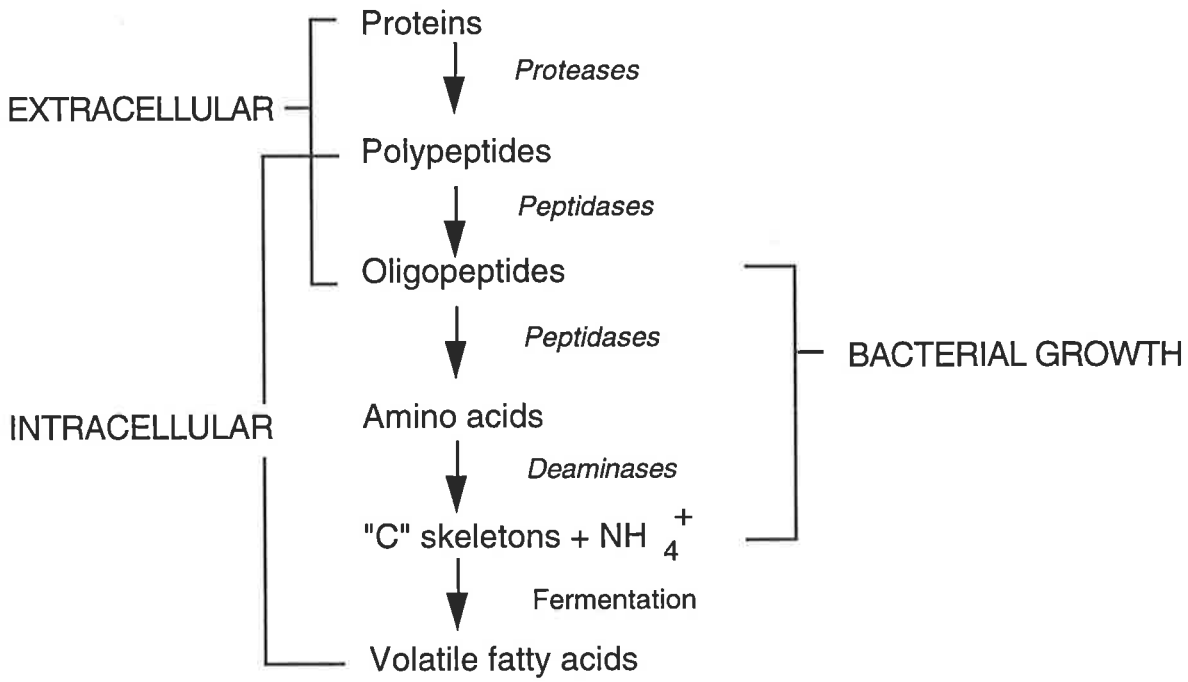


Figure 1.3 Fate of proteins in the rumen. (From Mackie and White, 1990)

Ammonia, in the form of ammonium, is the preferred source of N for most rumen bacteria. Bryant and Robinson (1962, 1963) found that 92% of ruminal bacterial isolates could utilise ammonia as the main source of N and that it was essential for the growth of 25% of isolates tested. Depending upon the diet, 60-90% of the daily N intake by the ruminant is converted to ammonia and from 60-80% of bacterial N can be derived from ammonia (Pilgram *et al*, 1970; Mathison and Milligan, 1971; Nolan and Leng, 1972).

The ammonia in rumen liquor is the key intermediate in the microbial degradation and synthesis of protein. A diet deficient in protein or containing protein that resists degradation will lead to a low concentration of ammonia and hence a slow growth rate of rumen microorganisms, which in turn will mean that the breakdown of carbohydrates will be retarded. Alternatively, if protein

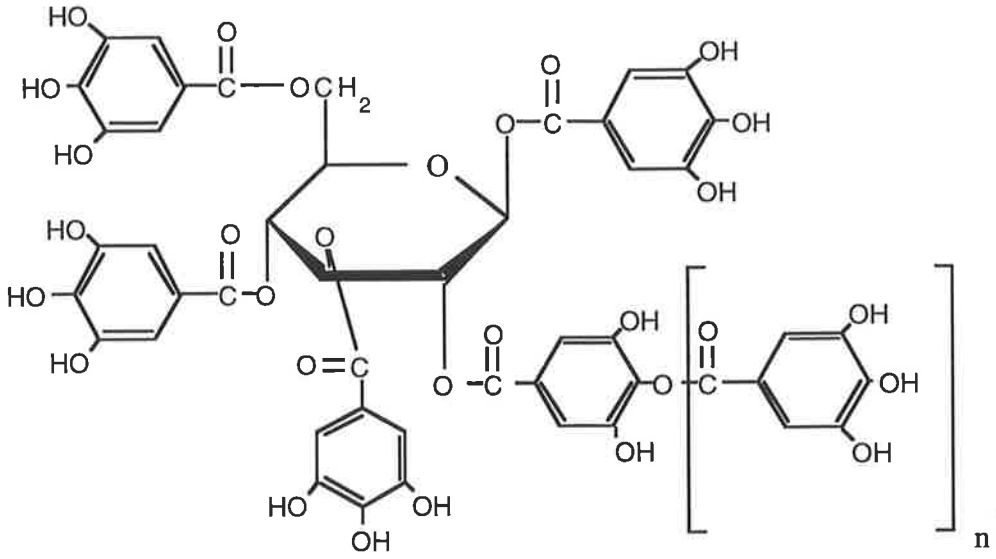
degradation proceeds more rapidly than synthesis, ammonia will accumulate in the rumen liquor and the optimum concentration may be exceeded. When this occurs, ammonia is absorbed from the rumen into the bloodstream and transported to the liver where it is converted to urea (McDonald *et al*, 1988). This urea may be excreted in the urine as waste or under conditions of low ammonia concentration, it may be transported back to the rumen and converted to microbial protein.

1.2.2 Overview of tannins

1.2.2.1 Structure

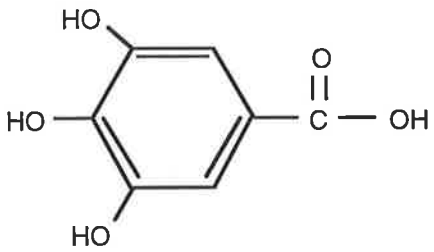
The term tannin was introduced in 1796 and referred to substances with the ability to tan leather (Ribereau-Gayon, 1972). It is now generally used to include any naturally occurring compound of high enough molecular weight (> 500) and containing a large number of phenolic hydroxyl groups to enable it to form effective cross-links with protein and other molecules (Kumar and Singh, 1984). There are two main groups of tannins, hydrolysable and condensed (proanthocyanidins), which may be differentiated by their structure and reactivity towards hydrolytic agents (Kumar and Singh, 1984).

Hydrolysable tannins (average molecular weight 500-3000) contain a central core of glucose to which a number of molecules of gallic acid (3,4,5-trihydroxybenzoic acid) or hexahydroxydiphenic acid are bound by ester linkages (Bernays *et al*, 1989; Kumar and Vaithiyanathan, 1990). Hydrolysable tannins can be readily hydrolysed by hot mineral acids and bases and by certain enzymes (Kumar and Vaithiyanathan, 1990). They may be subdivided into gallotannins or ellagitannins, depending on the acids obtained from this hydrolysis. Tannic acid, an example of a gallotannin, is illustrated in Figure 1.4. On hydrolysis, gallotannins yield gallic acid (Figure 1.5) and ellagitannins produce hexahydroxydiphenic acid which is isolated as its stable dilactone, ellagic acid (Figure 1.5) (McLeod, 1974).

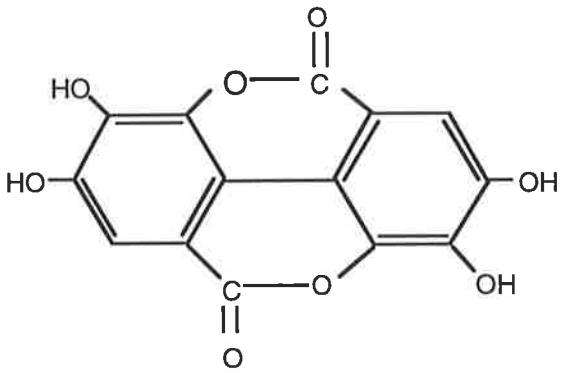


n = 1, 2, 3

Figure 1.4 Tannic acid



Gallic acid

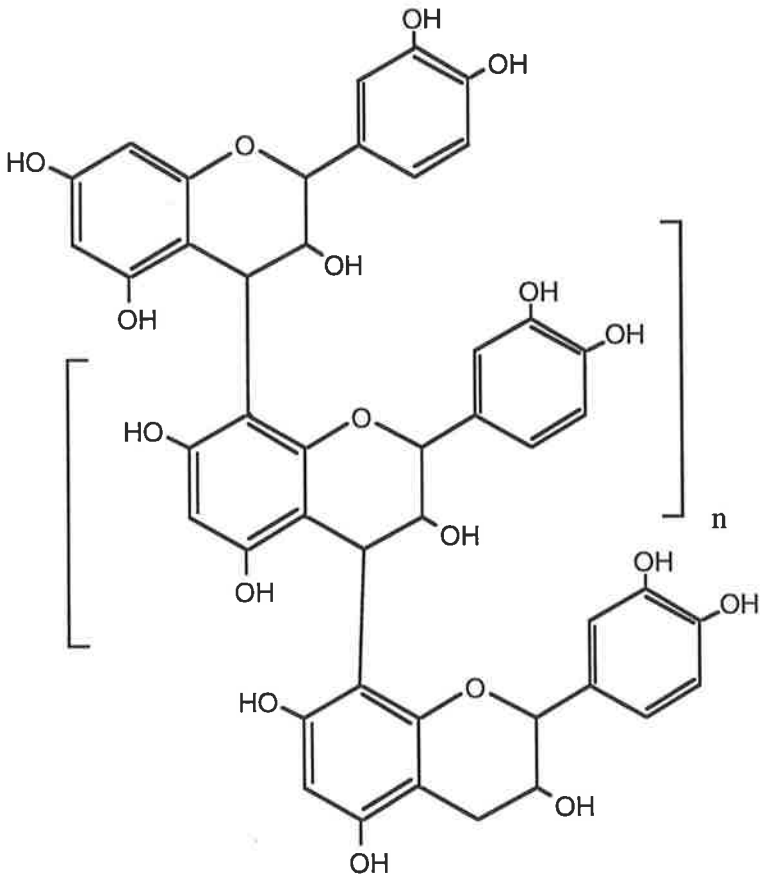


Ellagic acid

Figure 1.5

Kumar and Singh (1984) list the following as potential livestock feeds that are rich in hydrolysable tannins: green pods of *Ceratonia siliqua*, leaves of *Quercus robus* , acorns of *Quercus incana* , acorns of European Oak (*Quercus pedunculata*) and deoiled sal (*Shorea robusta*) seed meal. Yellow wood (*Terminalia oblongata*), an important part of the diet of cattle in northern Australia during the dry season, is known to contain hydrolysable tannins (McSweeney *et al*, 1988).

The condensed tannins (average molecular weight 1000-20 000) are the most widely distributed in vascular plants (Bernays *et al*, 1989) and they represent the principle tannins of forages. They are derived by carbon-carbon polymerisation of leucoanthocyanidins (flavan-3,4-diols) and catechins (flavan-3-ols) (Waghorn, 1990) and they have no carbohydrate core (McLeod, 1974). Condensed tannins are also called proanthocyanidins because upon heating in acid solution, they yield small amounts (5-15%) of the corresponding anthocyanidins, cyanidin and delphinidin (Bernays *et al*, 1989). Figure 1.6 illustrates a condensed tannin.



n = 4 - 5

Figure 1.6 Condensed tannin (from Sorghum grain)

Condensed tannins have been demonstrated in legume pasture species such as *Lotus corniculatus* (Birdsfoot trefoil) and *Lotus pedunculatus* (Lotus major), in sainfoin (*Onobrychis viciifolia*), in dock (*Rumex obtusifolius*), in several species of acacia such as *Acacia aneura* (Mulga) and in sorghum grain (Waghorn, 1990; Kumar and Singh, 1984). Condensed tannins exhibit a wide range of molecular weights, ranging from about 6000 in lotus to 28 000 in sainfoin (Waghorn, 1990). Molecular weights in the region of 1700-2000 have been observed for the condensed tannin of sorghum grains (Kumar and Singh, 1984).

1.2.2.2 Reactivity

Tannins react with protein forming tannin-protein complexes. In the case of vegetable tannage, the collagen chains are cross-linked by appropriate polyphenols to give leather (Kumar and Singh, 1984). Tannins have also been shown to complex with polysaccharides such as starch, cellulose and hemicellulose (Loomis, 1974; Mole and Waterman, 1987a; Price *et al*, 1980), and with fats, nucleic acids (Takechi and Tanaka, 1987), amino acids (Mole and Waterman, 1987b) and minerals (Kennedy and Powell, 1985).

A variety of chemical interactions between tannins and other macromolecules have been postulated: covalent interactions, ionic interactions, hydrogen bonding and hydrophobic interactions (Bernays *et al*, 1989). It is generally considered that the most common form of interaction between tannin and protein involves hydrogen bond formation between the protein amide carbonyl and the phenolic hydroxyl groups (Hagerman and Butler, 1980). The process of complex formation may also involve hydrophobic interaction between the aromatic ring structure of the phenolic compounds and hydrophobic regions of the protein (Loomis, 1974; Hagerman and Butler, 1980). The tannin-protein complex formation is claimed to be pH dependent and reversible (Bernays *et al*, 1989), and in principle, both protein and polyphenols can be recovered unchanged from the complex (Kumar and Singh, 1984). However, under certain conditions (eg pH >8, O_2), the polyphenols may become oxidised to a quinone, and the oxidised form may then form covalent bonds with nucleophilic groups (-SH, -NH_2) on the protein, making the tannin-protein complex irreversible (Kumar and Singh, 1984).

The structure of the tannin and the structure of the protein both play important roles in the relative affinities of the protein for the tannin and vice versa. For example, Proline Rich Proteins (PRPs), a class of salivary proteins found in many species such as humans, rats, deer, monkeys and rabbits, are bound several orders of magnitude more strongly than small, compact proteins (Bernays *et al*, 1989). Asquith *et al* (1987) state that proteins that have open, loose conformations and relatively high molecular weights efficiently bind tannin because the two polymers can freely interact and hence formation of hydrogen bonds is facilitated. In general, PRPs fit these criteria due to their size, conformational mobility, high content of proline and random-coil structures (Asquith *et al*, 1987). PRPs found in saliva of some species (rats, mice and deer) contain carbohydrate in amounts up to 40% by weight (Asquith *et al*, 1987). Glycosylation of PRPs may enhance affinity for tannins by maintaining the proteins in relatively open configurations (Asquith *et al*, 1987). In their study, molecular modelling of one particular mouse salivary proline-rich glycoprotein indicated that steric interference between the oligosaccharides and the three sequential proline residues is minimised when the polypeptide chain is in an extended rather than compact conformation (Asquith *et al*, 1987).

Recent evidence shows that a high proline content and large molecular size are not essential for a protein to interact strongly with a tannin. Yan and Bennick (1995) have reported the discovery of another class of human salivary proteins with an even greater affinity for precipitating tannin than PRPs. These proteins are members of the group called histatins; they have a very low molecular weight (3-4,000 Da) and contain no proline. According to Yan and Bennick, the most striking feature of histatins is their high level of basic amino acids, especially histidine. In one particular histatin, histidine, arginine and lysine made up 60% of the total amino acids. The contribution of these basic amino acids to tannin binding has not yet been elucidated however it is hypothesised that [illegible] interactions between the amino side-chain groups of arginine, lysine and histidine and the aromatic rings in tannins may be responsible. In addition, it is suggested that a lack of secondary structure may make the protein more accessible to tannin binding.

The reactivity of a tannin towards protein is strongly influenced by the molecular size of the tannin and the conformational flexibility and mobility of the polyphenols (Bernays *et al*, 1989). The affinity of tannin for protein increases linearly from those with a molecular weight of 576 to those with a molecular weight beyond 1134 (Kumar and Singh, 1984). According to Kumar and Singh (1984), the minimum molecular weight is about 350 for effective protein precipitation. When the molecular weight is large, ie greater than 5000, the condensed tannins are weakly soluble in physiological solutions, even in the absence of protein, and consequently protein precipitating ability is very low.

1.2.2.3 Measurement of tannins

Methods for the qualitative and quantitative determination of plant tannins have to date been difficult to obtain. Knowledge of the variation in tannin structures and activities has improved in recent years and more specific methods of quantitative determination have been devised (Inoue and Hagerman, 1988; Bernays *et al*, 1989). Currently there are a variety of techniques which depend either on chemical analysis or on relative ability to bind with various proteins. The choice of technique will depend on the major point of interest - tannin chemistry (ie structure, amounts and properties) or potential biological activity.

At present, there are reliable methods for quantifying condensed tannins and total phenolic contents of plants however the methods for quantifying hydrolysable tannins have until recently been inadequate. A method for determining gallotannin content of plants has been devised by Inoue and Hagerman (1988). In this assay, total tannins ie condensed, gallotannins and ellagitannins are first extracted from plant leaves with 70% aqueous acetone. The extract is then hydrolysed with acid and gallic acid in the hydrolysate is then reacted with rhodanine (2-thio-4-ketothiazolidine) and assayed spectrophotometrically. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with a maximum absorbance at 518nm (Inoue and Hagerman, 1988). The rhodanine assay determines free gallic acid. In order to determine gallotannin, two separate rhodanine assays must be performed on each sample - one

aliquot assayed before hydrolysis to quantitate free gallic acid and another assayed after hydrolysis to quantitate total gallic acid. The difference between the two values represents the gallic acid released from esters. It should be noted that this value is not exclusively gallotannin because a) gallic acid esters found in higher plants include mono- and di- galloyl esters as well as gallotannin; and b) ellagitannin may contain some galloyl moieties (Inoue and Hagerman, 1988).

The rhodanine assay is very specific for gallic acid - no interferences from other plant phenolics, including ellagic acid or condensed tannins have been observed. To date, no reliable method for quantifying ellagitannins has been developed. This situation needs to be rectified in order to carry out complete studies of tannin biosynthesis, accumulation and function in plants.

Much effort has gone into the development of methods for measuring condensed tannins. The presence of proanthocyanidins in plant tissue has been traditionally determined by their conversion to anthocyanidins (cyanidin and delphinidin) in hot mineral acid solution - either from ground tissue or suitable extracts (Porter *et al*, 1986). Methods based on this principle have been continually refined and improved to the extent that heating the substrate in n-butanol-conc. hydrochloric acid (95:5 v/v) to estimate proanthocyanidins is virtually the universal practice. The hydrolysis of proanthocyanidins to anthocyanidins (cyanidin and delphinidin) has been shown by Porter *et al* (1986) to occur by an auto-oxidation following acid catalysed cleavage of the interflavonoid bonds. The yield of anthocyanidin is critically affected by trace impurities of transition metal ions, and Porter *et al* (1986) have improved the method by showing that the addition of iron salts to the reaction mixture provides consistent yields of anthocyanidins. Another method that has been used widely is the vanillin-HCl method of Broadhurst and Jones (1978). This method is specific for flavanols. However, flavanols that are commonly present in plants but not necessarily in the form of condensed tannins, such as catechin and epicatechin, will react; perhaps leading to an incorrect conclusion unless an independent assay is used to verify the results. A third method for the measurement of condensed tannins is the H_2S0_4 procedure first published by Bate-Smith and Rasper (1969).

Like the butanol-HCl method, this method also relies on the production of a bluish purple chromophore resulting from oxidative cleavage of condensed tannin and release of anthocyanidins. Bae *et al* (1993) evaluated four condensed tannin assays and concluded that the H_2SO_4 assay was the most suitable for measuring condensed tannins in microbial cultures because it was the least susceptible to interference from media components.

The choice of a reference standard to be used in determining the condensed tannin content of a particular sample must be carefully considered. Two types of tannin standards can be used: absolute standards and relative standards (Hagerman and Butler, 1989) Determining the *absolute* amount of tannin in a plant sample is not an easy task and for many experiments is not necessary. To do so would require a tannin standard that was purified from the particular plant in question and this in itself is a laborious task fraught with complications and compromises. For example, the yield and to some extent structure of the purified tannin is dependent on the extraction solvent and conditions (temperature, light and presence of oxygen). In fact, so many factors influence tannin purification, it is generally accepted that obtaining a representative pure tannin for the absolute determination of tannin may well be impossible.

Most studies determine the tannin content relative to a commercially available standard. Non-tannin phenolics have often been used, such as catechin in the case of the vanillin-HCl assay and cyanidin in the case of the butanol-HCl assay.

Potential biological activity of tannins has traditionally been related to the degree of protein precipitation that can be obtained (Bernays *et al*, 1989). This result has been used to define tannins in operational or ecologically functional terms (Bernays *et al*, 1989). The choice of protein must be carefully considered however, because as stated previously, proteins vary in their affinity for tannins. Protein precipitation methods have generally used either haemoglobin (Bate-Smith, 1973; Schultz *et al*, 1981) or bovine serum albumin (BSA) (Hagerman and Butler, 1978), although the leaf protein ribulose-1,5-bisphosphate carboxylase (Martin and Martin, 1983)and ß-glucosidase (Becker and Martin, 1982) have also been used.

As described earlier, there are two fields of interest with regard to measuring tannins in plants - chemical analysis or protein binding ability. Results from the two fields of study cannot be positively correlated. For example, by measurement of total phenolics and condensed tannins of one particular plant it is impossible to make a prediction of protein precipitation ability of the crude extracts of the leaves from that plant.

Controversy also exists with different methods designed to measure the same properties of tannins. For example, there are at least three different methods used to quantify protein precipitation capacity, all using BSA. In one method, the precipitation of I^{125}-labelled BSA is measured radiochemically (Hagerman and Butler, 1980); in another method, the amount of unprecipitated BSA is measured colorimetrically (Martin and Martin, 1982). In a third method, the precipitation of blue dye-labelled BSA is determined colorimetrically (Asquith and Butler, 1985). Although BSA is used as the test protein in each case, the results from these methods are not comparable (Hagerman and Robbins, 1987). Hence the differences between the assays make it impossible to directly compare experiments from different laboratories which routinely use different tests.

1.2.3 Effects of tannins upon ruminant nutrition

1.2.3.1 Positive effects on nutrition

Several studies have shown that a low level of condensed tannins in the diet improves digestion (Barry *et al*, 1986; Waghorn *et al*, 1987; Barry, 1989; Terrill *et al*, 1992). G.C Waghorn has conducted much research into the beneficial effects of tannins and in one such study showed that a level of condensed tannin in the diet <4% DM was not toxic to ruminants and the net effect was to increase absorption of essential amino acids by 20-50% compared to tannin-free plants (Waghorn, 1990). This author has also shown that such a level of dietary condensed tannin will prevent bloat in cattle (Waghorn and Jones, 1989). Bloat occurs when an animal fails to ruminate due to a build-up of a persistent foam in the rumen. The soluble proteins present in lush pasture species such as white clover (*Trifolium repens*), red clover (*Trifolium*

pratense) or lucerne (*Medicago sativa*) have been implicated as the surfactants responsible for this foam development (Jones and Mangan, 1977). Cattle grazing pastures containing condensed tannins do not exhibit symptoms of bloat and it is thought that this is due to the removal of these proteins from solution as insoluble tannin-protein complexes.

It is believed that condensed tannins released upon mastication bind with plant proteins and form insoluble complexes in the rumen which are resistant to microbial degradation. These plant protein-tannin complexes then pass out of the rumen to the abomasum where they can dissociate under the conditions of lower pH (1.3-3.0), and enzymatic digestion of the plant proteins can commence in the duodenum. Hence, the plant protein is "protected" and its nutritive value is enhanced, both in terms of protein quality and quantity. Plant proteins can be of a higher biological value than microbial proteins: they contain higher proportions of limiting amino acids and do not contain nucleic acids. Protein quantity is enhanced because protection of plant proteins usually increases the quantity of protein passing out of the rumen for digestion in the intestines (Waghorn *et al*, 1990). For ruminant production in New Zealand, Waghorn has proposed that white clover be genetically engineered to express condensed tannin to a level of 8% DM in the leaves which would provide a dietary level of 1.5-2.5% condensed tannin assuming that white clover accounted for 20-30% of pasture DM consumed (Waghorn *et al*, 1990). In this way it is believed that ruminant productivity could be enhanced 10-15% and bloat would be eliminated (Waghorn *et al*, 1990).

1.2.3.2 Negative effects on nutrition

Physiological effects

Tannins have traditionally been seen as a plant defence mechanism against herbivory. The chemical basis for their defensive role has been attributed to their ability to precipitate plant proteins and gastrointestinal enzymes, thereby reducing both protein and fibre digestion (Robbins *et al*, 1987a).

The separation of behavioural deterrence from postingestinal effects is impossible in many studies, and furthermore, the majority of evidence for feeding inhibition is correlative (Bernays *et al*, 1989). This generalised view that herbivores avoid tannin-rich plants due to their digestibility-reducing properties is now being questioned. There have been many *in vivo* studies in ruminants that have demonstrated anti-nutritional effects of tannins. Such effects include reduced voluntary feed intake (Robbins *et al*, 1987a&b; Waghorn *et al*, 1990; Barry and Manley, 1986; Pritchard *et al*, 1988); reduced protein digestibility (Robbins *et al*, 1987a; Waghorn *et al*, 1990); reduced cell wall digestion (Robbins *et al*, 1987b); reduced sulphur retention (Pritchard *et al*, 1988; Waghorn, 1990) and decreased liveweight gain (Lowther *et al*, 1987). Overall evidence indicates that high levels of tannins (>8-10%DM) can produce negative effects on ruminant nutrition. However there is growing evidence which suggests that these negative effects may not necessarily be attributed to the inhibition of digestion alone.

Many studies that have focused on the precipitation of dietary proteins and gastrointestinal enzymes have been *in vitro* studies. Although tannins inhibit most enzymes *in vitro*, *in vivo* enzyme inhibition has not been demonstrated. A study by Blytt *et al* (1988) suggested that membrane bound enzymes are less susceptible to inhibition by tannins than soluble enzymes. Furthermore, these authors suggest that the protection of digestive enzymes from tannins by membrane components may be common. In addition, dietary tannins have opportunities to complex with a wide variety of dietary proteins before they are exposed to the major digestive enzymes (Mehansho *et al*, 1983). Hence under physiological conditions, dietary tannin may not be accessible to digestive enzymes.

A common feature of all *in vivo* studies concerned with the postingestive effects of tannins is a reduction in voluntary feed intake (VFI). Often, this reduction in VFI persists and sometimes increases throughout the course of the feeding trial (Waghorn *et al*, 1990). The reduction in VFI is believed to be due to the astringency of tannins which in turn is related to their protein-binding properties. For example, F.D Provenza has shown that goats in the USA avoid new-season growth on blackbrush (*Coleogyne ramosissima*) due to its high tannin concentration

(Provenza *et al*, 1990). While astringency is well recognised as a feeding deterrent, some authors have suggested that in specific cases, the depressed VFI is due to the ingested tannin exerting some toxic effect on the host and is not solely due to a reduction in digestibility. For example, Clausen *et al* (1990) showed that snowshoe hares (*Lepus americanus*) preferred bitterbrush (*Purshia tridentata*) tannins to blackbrush (*Coleogyne ramosissima*) tannins despite both tannins displaying similar protein-binding capacities. Structural analyses of these tannins showed that both were procyanidins (condensed tannins) of similar length however they exhibited differences in stereochemistry at C-3 and C-4. It was hypothesised that the preference of snowshoe hares for bitterbrush tannins over blackbrush tannins was a result of blackbrush tannins depolymerising in the digestive tract at a faster rate than bitterbrush tannins. In order for any tannin, either condensed or hydrolysable, to be toxic it must be able to be absorbed from the gastrointestinal tract into the bloodstream of the host. An example of this is provided by yellow-wood poisoning of sheep and cattle which is characterised by liver and kidney injury, jaundice, photosensitisation, abdominal pain and dehydration and is known to be caused by hydrolysable tannins present in Yellow-wood (*Terminalia oblongata*). Murdiati *et al* (1992) and McSweeney *et al* (1988) conducted studies on the effect of Yellow-wood tannins on digestion in sheep. These authors found that the digestion of organic matter, fibre and nitrogen from the basal diet of a tropical legume (*Stylosanthes hamata*) was unaffected by the presence of added Yellow-wood tannins and yet Yellow-wood poisoning still occurred. It was stated that hydrolysable tannins and their hydrolysis products are not absorbed by proteins as readily as condensed tannins however they are readily absorbed by the animal and exert toxic effects on the liver and kidneys. Hagerman and Robbins (1987) described the formation of soluble tannin-protein complexes (both condensed and hydrolysable) *in vitro* . It was found that at protein: tannin ratios larger than optimum, soluble tannin-protein complexes formed instead of insoluble complexes. It was postulated that *in vivo*, tannin and protein may form not only insoluble complexes with corresponding antinutritional effects, but also soluble complexes, the effects of which are unknown. The formation of soluble tannin-protein complexes may result in increased absorption and metabolism of tannin.

Several authors have suggested that the main effect of tannins may be an erosion of the intestinal mucosa (Bernays *et al*, 1989; Kumar and Vaithiyanathan, 1990). It is thought that tannins bind to the gut epithelium and will then act as an irritant (Bernays *et al*, 1989) and they also reduce the absorption of nutrients from the gut (McLeod, 1974; Kumar and Singh, 1984) by reducing its permeability. A reduction in permeability of the gut wall will produce signals of physical distension, which is an important feedback mechanism in the ruminant for controlling feed intake (Kumar and Vaithiyanathan, 1990). Previously, an increase in faecal nitrogen with increasing dietary tannin levels was used as one of the main lines of evidence for digestion inhibition by tannins (Bernays *et al*, 1989). However, this situation is changing. Robbins *et al* (1987a) declare that faecal nitrogen is not a precise indicator of dry matter digestibility or in fact any dietary parameter and should not be used in ecological studies. Bernays *et al* (1989) indicate that elevated faecal nitrogen may be due to a combination of increased mucus and intestinal cell debris caused by erosion by tannin.

A final negative effect caused by tannins in the ruminant diet is a disrupted mineral balance. Sodium may be deficient, caused by increased secretion of mucus and cell damage as a result of tannin ingestion (Freeland *et al*, 1985a, 1985b). Iron may also become deficient because it is able to form insoluble complexes with tannins (Freeland *et al*, 1985a, 1985b).

Microbial toxicity

As well as the direct physiological effects described in the preceding section, tannins can adversely affect rumen function by inhibiting the growth and metabolism of rumen microorganisms. This may be the most important effect of tannins since the productivity of ruminants is critically dependent on the metabolic activity of the overall microbial population. Many authors have described microbial inhibition by tannins (Henis *et al*, 1964; Tagari *et al*, 1965; Makkar *et al*, 1988). Inhibition has been measured as reduction in growth rate or as a function of a relevant biochemical parameter such as cellulose degradation or proteolysis. Bae *et al* (1993) reported that condensed tannins of *Lotus corniculatus* inhibited extracellular endoglucanase activity of *Fibrobacter succinogenes* at a tannin concentration of 100μg/ml. The

same authors have reported complete inhibition of extracellular endoglucanase activity of four species of anaerobic rumen fungi by the same condensed tannin preparation at a level of 300μg/ml (McAllister *et al*, 1994). Growth and proteolysis of *Butyrivibrio fibrisolvens* and *Streptococcus bovis* were reduced by condensed tannins of *Onobrychis viciifolia* (< 600μg/ml) (Jones *et al* , 1994). Growth of *Streptococcus bovis* was inhibited by tannic acid at a concentration less than 5mg/ml (Brooker *et al*, 1994).

The effect of tannin structure on microbial toxicity is largely unknown (Scalbert, 1991). Most studies have been conducted with crude tannin extracts hence until experiments are carried out with defined tannin structures, no general conclusions can be drawn. In addition, the mechanisms of tannin toxicity are unclear, although 3 possibilities have been proposed. Firstly, tannins can inhibit microbes via enzyme inhibition and substrate deprivation. The reactivity of tannins towards proteins and to a lesser extent, carbohydrates, may lead to complex~~ation~~ing of extracellular microbial enzymes and substrates. Secondly, tannins may affect microbial growth via cell wall synthesis. Several authors have reported changes in bacterial cell morphology induced by tannins. Most often, the bacteria increase in size without dividing (Henis *et al*, 1964; McAllister *et al*, 1994). Finally, tannins may inhibit microbial growth by complexing metal ions (Scalbert, 1991). Bacterial growth is highly dependent on the metal ion status of the environment. As described previously, tannins can form insoluble complexes with many metal ions such as ferric and cupric (Kennedy and Powell, 1985) thus it is possible that they reduce the availability of essential metal ions for microorganisms.

The overall conclusion with respect to the effects of tannins upon ruminant nutrition is that there appears to be a threshold of dose, above which negative effects on protein digestion and nutrient absorption become apparent. This dose will vary depending on the animal, the overall diet it is consuming and the type of tannin, especially whether it is hydrolysable or not.

1.2.4 Microbial defences against tannins

Although it has been shown that tannins as a broad class of chemical compounds can be toxic to microorganisms, not all microbes are affected to the same extent. Many microorganisms are able to grow and develop on materials high in tannin, for example certain woods (eg *Quercus* spp., oak), and it is believed that these organisms have evolved defence mechanisms to detoxify the tannins. One such mechanism is the secretion of polymers that have a high affinity for tannins. These polymers form complexes with the tannin thus rendering it unable to bind with extracellular enzymes which are essential for the growth of the organism. For example, Chiquette *et al* (1988) reported a glycocalyx surrounding cells of the rumen anaerobe *Prevotella ruminicola* which was only present when cells were grown in the presence of a high tannin-containing variety of birdsfoot trefoil (*Lotus corniculatus*). O'Donovan and Brooker (1995) have recently reported the tannin-inducible production of an exopolysaccharide (EPS) by the tannin-resistant rumen bacterium *Streptococcus caprinus*, and suggest a protective role for this EPS.

A second mechanism by which microorganisms may detoxify tannins is by the production of metal ion sequestering molecules called siderophores. Many bacteria and fungi produce siderophores however their role in tannin detoxification has only recently been proposed (Scalbert, 1991). It is thought that because tannins may inhibit microbial growth by depleting iron supplies, bacteria and fungi may increase production of siderophores in their presence. To date, no experiments have been conducted to test this theory.

A third microbial tannin-detoxification mechanism is that of actual tannin biodegradation. Little is known about tannin biodegradation pathways in microorganisms. To date, most research has focused on aerobic systems and no data exists on the anaerobic degradation of tannins. Microbial degradation of condensed tannins, either aerobically or anaerobically, has never been adequately demonstrated. Although there have been several reports of microorganisms able to grow on condensed tannins as a unique carbon source (Deschamps, 1985; Deschamps and Leulliette, 1985; Lewis and Starkey, 1969; Makkar *et al*, 1994), the results need to be

interpreted with caution. Most studies have used relatively crude extracts as the source of tannin and often this has been combined with an undefined medium. In addition, microbial growth has often been used as the sole indicator of tannin biodegradation. In future, purified condensed tannins of known molecular structure should be used in growth experiments with truly defined media and tannin degradation should be verified using independent techniques such as NMR spectroscopy to identify breakdown products.

In contrast to condensed tannins, microbial degradation of hydrolysable tannins, particularly the gallotannins, is reasonably well characterised. It has been known since the turn of this century that several fungi of the ~~genii~~ genera *Aspergillus* and *Penicillium* produce an enzyme, tannin acylhydrolase, which allows them to grow aerobically on hydrolysable tannins (Knudson, 1913 as cited by Scalbert, 1991). Tannin acylhydrolase has also been described in a strain of the yeast Candida (Aoki *et al*, 1976) and most recently in the fungi *Trichoderma viridae* and *Fusarium solanii* (Bajpai and Patil, 1996). The enzyme catalyses the hydrolysis of ester bonds between a phenolic acid and an alcohol. The alcohol in the ester may be methanol, glucose, gluconic acid, gallic acid (in which case the ester bond is a depside) or a flavanol (Scalbert, 1991; Haslam and Stangroom, 1966; Aoki *et al*, 1976). The gallic acid released from the hydrolysable tannins may accumulate or it may be further degraded depending on the oxygen supply in the growth medium.

1.2.5 Reducing the negative effects of tannins

1.2.5.1 Natural defences

Some animals have developed defence mechanisms to counter the negative effects of ingested tannins. One such mechanism is the production of substances that preferentially bind with tannins. The saliva of several species (rats, mice, humans and deer) has been shown to contain proteins rich in proline, which as described in Section 2.2 of this chapter have a very high affinity for tannins (Robbins *et al*, 1987a; Austin *et al*, 1989). Austin *et al* (1989) proposed that browsing species such as deer are more often exposed to tannins than grazing species such as cattle and as a result they have developed saliva containing tannin-binding proteins. Tannin-

binding proteins have not been demonstrated in saliva from sheep or cattle (Austin *et al*, 1989). Tannin-binding proteins may benefit the animal by either reducing the negative effects of tannins on protein digestion or by reducing the toxic effects of tannins by preventing their binding to the intestinal epithelium.

1.2.5.2 Traditional strategies

Preferential binding by tannins to certain substances has been exploited by several authors to improve the intake of some tannin-containing feeds (eg Pritchard *et al*, 1988). Most of this work has focused on the use of polyethylene glycol (PEG) which has been shown to bind preferentially with condensed tannins in the rumen, preventing formation of insoluble tannin-protein complexes (Pritchard *et al*, 1988; Pritchard *et al*, 1992; Waghorn *et al*, 1990; Jones and Mangan, 1977). Furthermore, it has been shown that addition of PEG to a condensed tannin-protein complex will reverse the complex and the tannin will bind with the PEG, releasing the protein into solution (Jones and Mangan, 1977).

The addition of PEG to tannin-rich fodder as a means of reducing the negative effects of the tannins is both impractical and uneconomical. In Australia, sheep and cattle are exposed to tannins when grazing open rangelands and consequently, neither management practices designed to limit intake nor application of PEG to forage are very practical. Under some circumstances, such as drought-feeding animals with cut forage, PEG treatment of the tannin-containing supplement may be practically possible. However, the primary objective of drought -feeding is usually to keep the afflicted animals alive in order to reduce financial losses; hence any benefits gained by PEG treatment would be far outweighed by the financial costs of its use.

Several authors have improved the intake of tannin-containing forages (eg mulga, *Acacia aneura*) by sheep by supplementing them with molasses (McMeniman, 1976; Entwistle and Baird, 1976). Subsequent research revealed that the improved performance could be replicated by supplementing sheep with the mineral content of 50g molasses/day (Hoey *et al*, 1976). It

was further revealed that sulphur had the greatest impact and it is now routine practice for mineral supplements to be provided to sheep grazing mulga.

In northern New South Wales and southern and central Queensland, lopped mulga (*Acacia aneura*) is often fed to cattle and sheep as a supplement, often for a period of several months. In this situation, time of cutting can be an important factor in tannin toxicity. Several authors have reported that tannin content is highest in new season's growth and decreases with age of the leaves (Kumar and Vaithiyanathan, 1990; Provenza *et al*, 1990). Furthermore, the level of tannins in plants has been shown to be related to conditions of low soil fertility (Barry and Manley, 1986; Lowther *et al*, 1987), high light intensity, high temperatures and drought (Ahn *et al*, 1989). Hence time of harvesting and soil conditions prior to harvest could be considered in a bid to reduce the negative effects of ingested tannins upon ruminants. However, in many parts of the world space for grazing animals is limited, and lopped, tannin-containing forage is often fed to ruminants year-round. Consequently, time of cutting is not likely to be a controllable factor here.

1.2.5.3 New approaches

There is currently world-wide interest in using previously unutilised or under-utilised plants as feed resources for ruminants. Unfortunately, while many potential candidates exhibit suitable protein content they also contain antinutritive factors such as tannins which make them unsuitable. If however, the antinutritive properties could somehow be overcome, such plants (eg *Calliandra calothrysus*, *Codariocalyx gyroides*, *Gliricidia sepium*) could become valuable feed sources.

In Australia, interest in fodder trees has centred largely on native species, particularly the acacias, and such species (eg mulga) are important sources of feed in times of drought or prolonged dry seasons. However, despite a crude protein content of 10-14%, mulga is avoided by sheep and cattle due to its high tannin content (11-14% DM). However, it has been observed that feral goats and camels and native species such as kangaroos are able to efficiently

utilise mulga as a feed source and this has led to the proposal that these animals may contain a microbial population in their gastrointestinal tract capable of conferring tolerance to ingested tannins. There is now much interest in isolating anaerobic microorganisms that may play a role in the apparent detoxification of ingested tannins, with the long-term aim of using these microorganisms to confer tannin-tolerance to domestic ruminants thereby improving the utilisation of tannin-rich feeds.

The successful transfer of mimosine tolerance from Hawaiian goats to Australian cattle by Jones and Megarrity (1986) has created a precedent which has been the inspiration for this approach. The shrub legume *Leucaena leucocephala* was introduced into Australia to provide valuable protein in the dry season for cattle production in the sub-tropics. However, *Leucaena* proved to be toxic to cattle due to the non-protein amino acid mimosine being broken down to the goitrogenic 3-hydroxy-4-1(H)-pyridone (3,4-DHP) in the rumen. In Indonesia and Hawaii, goats and cattle consume *Leucaena* with no ill-effects due to microbial degradation of 3,4-DHP to non-toxic compounds in the rumen. When the rumen contents from these animals was transferred to Australian ruminants, apparently stable mimosine tolerance was transferred and spread naturally throughout the test population. A Gram-negative, rod-shaped bacterium that degrades 3,4-DHP, its isomer 2,3-DHP, but not mimosine has since been isolated by Allison *et al* and named *Synergistes jonesii* (Allison *et al*, 1990).

Preliminary studies involving the mass transfer of rumen fluid from feral goats to domestic sheep resulted in an improved utilisation of tannin-containing mulga (*Acacia aneura*) (Miller and Brooker, 1995). Matthew *et al* (1991) isolated a novel *Streptococcus* species from the rumen of feral goats that is highly resistant to tannins (Brooker *et al*, 1994). This organism is not closely related to *Streptococcus bovis* and it has been named *Streptococcus caprinus*, to reflect its origin from the goat (Brooker *et al*, 1994). The presence of this organism appears to be correlated with a diet containing tannins as it is not present in domestic ruminants consuming tannin-free diets (Brooker *et al*, 1994). In addition, when this organism was transferred to sheep and cattle, its population in the rumen was highest whilst the animals were consuming

tannins and dropped significantly when tannin was removed from their diet (Miller and Brooker, 1995). At present, this bacterium's mode of resistance to tannins has not been established but preliminary evidence suggests it may be via the tannin-inducible production of an exopolysaccharide which acts as a protective barrier around the bacterium. It is not yet clear whether *S. caprinus* modifies tannins in any way. Other authors have reported the isolation of possibly the same or a closely related species of bacteria from the rumen of a goat fed *Desmodium ovalifolium* (up to 17% condensed tannins) (Nelson *et al*, 1995) and the cecum of koalas (Osawa, 1990). Osawa has also reported the isolation of Gram-negative bacilli from the faeces and caecal-wall of koalas which are capable of degrading tannin-protein complexes (Osawa, 1992). Most recently, Bhat *et al* (1996) have reported the isolation of a tannin-protein complex-degrading fungus from the faeces of cattle fed oak (*Quercus incana*) leaves.

In addition to bacteria that are resistant to tannins or are able to modify tannins or tannin-protein complexes; several studies have revealed bacteria that are able to degrade phenolic monomers anaerobically. Many of these bacteria have been isolated from the rumen, for example *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986), and species of *Coprococcus* (Tsai and Jones, 1975; Patel *et al*, 1981). *Eubacterium oxidoreducens* is a strictly anaerobic bacterium that degrades gallate (3,4,5-trihydroxybenzoate), phloroglucinol (1,3,5-trihydroxybenzene) and pyrogallol (3,4,5-trihydroxybenzene) to acetate and butyrate in the presence of formate and hydrogen as electron donors. *Coprococcus* sp. $Pe_1 5$ is another strict anaerobe that degrades phloroglucinol to acetate and carbon dioxide; the initial step being the reduction of the aromatic ring catalysed by a phloroglucinol reductase and NADPH as the electron donor (Patel *et al*, 1981). Brune and Schink (1992) have reported the characterisation of a non-ruminal strict anaerobe, *Pelobacter acidigallici*, which is also capable of degrading several trihydroxybenzene derivatives to acetate.

1.3 Objectives of this study

Rumen microorganisms could confer tannin-tolerance to their hosts either by being unaffected by, resistant to or capable of degrading tannins. Given the chemical diversity of tannins and the

diversity of their effects upon microorganisms, it is likely that both these mechanisms prevail in the rumen. Furthermore, unlike the detoxification of mimosine, it is likely that tannin-detoxification is the result of a consortium of microorganisms.

Therefore, this research project set out to identify new bacteria that play a role in the detoxification of tannins in feral goats. More specifically, the project aimed to:

- identify and characterise tannin-resistant or tannin-degrading bacteria from feral goats;
- determine the mechanism of tannin-resistance or tannin-degradation;
- examine the molecular mechanisms controlling tannin-resistance or tannin degradation in these organisms.

CHAPTER 2 Materials and Methods

For ease of reading, all the experimental procedures used in this thesis are presented here on a chapter by chapter basis. The recipes for media, buffers and solutions used are included in an Appendix. Unless otherwise indicated, routine molecular biological techniques were as described in either Current Protocols in Molecular Biology by Ausubel *et al* (1989) or Molecular Cloning: A Laboratory Manual by Sambrook *et al* (1990).

2.1 Experimental procedures used in Chapter 3

2.1.1 Preparation of anaerobic media

Broth

Anaerobic broth media were prepared by mixing all components in a glass Schott bottle, adjusting to the correct pH and then boiling in a microwave oven for approximately 20 seconds. The bottle was then capped and placed inside a Coy anaerobic hood with an internal atmosphere of 95% v/v CO_2/ 5% v/v H_2. Anaerobic conditions were maintained with a palladium catalyst and circulator unit. Once inside the hood, the lid of the bottle was loosened and the bottle was left inside the hood for a period of at least 2 hours during which time gas exchange occurred and the media became anaerobic. Aliquots of media were then dispensed into 15ml Hungate tubes which had been stored in the anaerobic atmosphere for at least 15 hours. The tubes were then capped, removed from the anaerobic hood and sterilised by autoclaving for 15 minutes at 121°C and 15psi.

Plates

Nutrient plates for anaerobic bacteria were prepared by adding agar (1.5% w/v) to media before autoclaving. Plates were poured in a laminar-flow cabinet once the medium had cooled to approximately 45°C. Once the agar medium had set, the plates were transferred to the Coy anaerobic hood and left there overnight to allow the medium to become anaerobic via gas exchange.

2.1.2 Bacterial enrichment

A crimp-top bottle containing 100ml of enrichment medium (modified M10 (mM10), Appendix) supplemented with 1.0g (wet weight) of crushed, fresh *Acacia aneura* leaves was inoculated with a 1.0ml aliquot of crude goat rumen fluid collected from a feral goat browsing *Acacia* sp. in the Arkaroola region of South Australia. The medium was incubated at 39°C for 5 days after which a second bottle of enrichment medium supplemented with 0.5 g/100ml of *Acacia* condensed tannin extract (see section 2.1.7) was inoculated with a 1/500 dilution of the primary enrichment. This culture was incubated at 39°C for two days after which the enrichment was repeated twice more using enrichment medium containing 1.5 g/100ml *Acacia* condensed tannin extract. Throughout the incubations, bacterial growth and diversity was monitored by examining wet-mounts of enrichment aliquots under the light microscope. Bacteria were isolated from the final enrichment culture by plating onto mM10 medium supplemented with 0.5% cellobiose, glucose and starch in the absence of tannin. Individual isolates were stored at -80°C in mBHI (Appendix) medium containing 20% (w/v) glycerol.

2.1.3 Fermentation tests

Fermentation of various carbon sources by individual bacterial isolates was determined in NB medium (Appendix) supplemented with the test compound to a level of 0.5% w/v. A single bacterial colony was picked off an mBHI plate after overnight incubation and resuspended in an aliquot of Anaerobic Dilution Solution (Appendix). Duplicate Hungate tubes containing 5.0ml of NB medium were inoculated with 10µl of this bacterial suspension and then incubated at 39°C overnight. Growth was determined by measuring the A_{600} of the cultures in a spectrophotometer (LKB Biochrom NOVASPEC, Cambridge England) that had been modified to accept 15ml Hungate tubes.

2.1.4 VFA Analysis

Sample preparation

A Hungate tube containing 10.0ml of mBHI medium was inoculated with a single colony of bacteria from an mBHI plate and incubated at 39°C overnight. Duplicate 1.0ml aliquots were

removed and placed in 1.5ml microfuge tubes and centrifuged at 13000rpm in a microcentrifuge for 3 minutes. The supernatants were transferred to clean microfuge tubes and 0.20ml of protein precipitant solution (Appendix) added to each. The solutions were mixed, 0.20ml of internal standard solution (Appendix) was added, then centrifuged as before. The supernatant was then removed and stored at -20°C until assayed.

Sample analysis

A Shimadzu Gas Chromatograph (GC-14A) combined with Delta Data System (SGE Analytical Products) was used to determine the VFA content of the spent-culture medium. The column used was an SGE BP21 (0.5µm film, 25.0m x 0.53 mm ID filled with polyethylene glycol, Part no. 054474). The start temperature was 100°C which was increased at the rate of 9°C per minute to a maximum of 150°C. The injector temperature was 240°C while the detector temperature was 280°C. The sample injection size was 0.2µl. The concentration of component acids was calculated by comparing the ratio of acid peak area to internal standard peak area with the corresponding ratios measured on standard VFA mixtures.

2.1.5 Gram Staining (Ogimoto and Imai, 1984)

Gram staining was performed using Hucker's modified procedure as described in Ogimoto and Imai (1984).

2.1.6 DNA-DNA Hybridisation

DNA was isolated from 10ml cultures of the bacteria of interest by the standard procedure for bacteria described in Ausubel *et al* (1989). Sheared DNA (200ng) from *S. ruminantium* K2, *S. ruminantium* S23, *Propionibacterium* and *Streptococcus bovis* 2B were blotted to a piece of Hybond N^+ membrane (Amersham) according to routine protocols (Ausubel *et al*, 1989). The DNA was alkali-fixed to the membrane and then prehybridised in aqueous prehybridisation solution (Appendix) for 4 hours at 65°C. Sheared K2 DNA (200ng) was labelled with α^{32}P-dCTP using a random priming procedure according to the instructions of the manufacturer (Amersham). This was then hybridised to the DNA bound to the membrane for 17 hours at

65°C. The membrane was washed twice for 15 minutes each in 2X Standard Saline Citrate (SSC)/0.1% w/v sodium dodecyl sulphate (SDS) at room temperature, once in 0.5X SSC/0.1% w/v SDS for 30 minutes at room temperature and finally once in 0.5X SSC/0.1% w/v SDS for 15 minutes at 42°C. (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate) The membrane was then autoradiographed for 4 hours after which time the X-ray film was developed and the radioactivity in each spot determined by cutting out each spot from the nylon membrane and counting in a liquid scintillation counter (Beckman Instruments). Percent homology was determined by expressing CPM (test hybridisation)/CPM (homologous hybridisation control).

2.1.7 *Acacia* condensed tannin crude extract

An extract of *Acacia aneura* condensed tannins was prepared by P. Martin, Queensland Department of Primary Industries, Brisbane, Queensland, by extracting crushed *Acacia* leaves with 70% v/v acetone:water according to the method of Broadhurst and Jones (1978).

2.2 Experimental procedures used in Chapter 4

2.2.1 Growth on tannins

Filter-sterilised stock solutions of tannic acid (Sigma, Cat. No. T0125) and *Acacia* condensed tannin crude extract in Anaerobic Dilution Solution were prepared in an atmosphere of 95% CO_2, 5% H_2 immediately prior to use. Aliquots of these solutions were added to Hungate culture tubes containing 5.0ml of sterile defined NB medium to a final concentration of 0.1, 0.5 and 1.0% (w/v). The tubes were inoculated with washed cells prepared from an overnight culture of the bacterium grown in mBHI broth and suspended in Anaerobic Dilution Solution. After 24 hours incubation at 39°C, growth was quantified by plating serial dilutions of the cultures onto mBHI plates and determining the viable cell counts.

These values were subtracted from a viable cell count made immediately following inoculation to provide the net change in bacterial numbers.

2.2.2 Paper Chromatography

Gelatin extraction of tannic acid

A 10ml solution of 10% w/v tannic acid in H_2O was freshly prepared and to this was added an equal volume of a 2% w/v gelatin (Sigma) solution. The resulting tannic acid-gelatin precipitate was pelleted by centrifugation at 12100x *g* for 15 minutes at 4°C in a JA20 rotor in a Beckman J2-HS centrifuge. The supernatant was removed and re-extracted with an equal volume of 2% w/v gelatin. This process was repeated twice more until no visible tannic acid-gelatin precipitate formed. To remove any uncomplexed gelatin, the resulting solution was extracted once with Tris-buffered phenol pH 8.0 (Ausubel *et al*, 1989), once with phenol:chloroform 1:1 v/v and then finally with chloroform alone. The combined supernatants were then dried down in a SpeediVac, resuspended in 1.0ml H_2O and stored at 4°C.

Chromatography of tannic acid and detection of reducing sugars

Paper chromatography was carried out using the ascending-solvent technique in a glass chromatography tank. Samples were loaded along an origin line marked in pencil 20mm from the bottom edge of a piece of Whatman No.1 filter paper cut to dimensions 167x210mm. Two different solvents, A and B (Appendix), were used separately. The solvents were placed in the chromatography tank at least 40 minutes before starting development in order to allow the atmosphere inside the tank to become completely saturated with solvent. Samples were loaded onto the paper in multiple 2.0µl aliquots using a Gilson micropipettor, drying the spots with a hair-drier between each application. Standard sugar solutions (glucose, galactose and ribose, 1.0% w/v) were included as controls. Development of each chromatogram proceeded until the solvent reached the upper edge of the paper. The paper was then removed from the tank and allowed to air-dry in a fume-hood. Reducing sugars were detected by spraying the paper with aniline/phthalate reagent (Appendix), drying it with a hair-drier and then heating it in an oven at 105 °C for 10 minutes. Reducing sugars appeared as reddish brown spots. The papers were also placed on a UV Transilluminator (UltraViolet Products Inc., California, USA) and photographed with Polaroid instant black and white film (Type 667) to allow detection of both

reducing sugars and phenolic acids. Reducing sugars fluoresce under UV light and appear on film as white spots while phenolic compounds absorb UV light and appear as dark spots.

2.2.3 Purification of condensed tannin from *Acacia* by Sephadex LH20 chromatography

Acacia leaves (50g wet weight) were ground under liquid nitrogen in a mortar and pestle and then washed four times with 300ml of diethyl ether containing 0.1% w/v ascorbic acid (Sigma), in order to remove fats, essential oils and most of the chlorophyll. The ether was removed by vacuum-filtration through a sintered-glass funnel. The sediment containing flavonoids was then placed in a conical flask with 400ml of methanol (AR grade)/0.1% w/v ascorbic acid. The flask was covered with aluminium foil to exclude light and left in a fume-hood overnight. A glass column of dimensions 380x25mm was packed with Sephadex LH20 (Pharmacia) gel equilibrated in 100% methanol. The flavanol extract was separated from the solid leaf material by vacuum-filtration through a sintered-glass funnel and then loaded onto the column. The column was washed with methanol until the A_{280} of the eluate reached a constant minimum value thereby removing most of the non-tannin flavonoids from the gel. Condensed tannins bound to the gel were eluted with 50:50 acetone:water and collected as a single fraction. The condensed tannins were then crystallised by freeze-drying. To prevent oxidation, crystallised tannins were stored in 1.5ml screw-cap Sarstedt tubes inside a Coy anaerobic chamber with an internal gas atmosphere of 95% N_2, 5% H_2.

2.2.4 Detection of gallic acid in growth medium

Hungate tubes or 50ml crimp-top bottles of anaerobic NB medium containing either 1.0 or 2.0% w/v tannic acid as sole energy source were prepared. Media were inoculated with a suspension of K2 cells and incubated at 39°C for a period of 30 hours. Uninoculated media containing tannic acid were included as controls. At regular intervals, 0.1ml aliquots were removed from each tube inside the Coy anaerobic hood using a sterile syringe and 25G needle. Each aliquot was transferred to a glass tube (9x75mm) containing 0.15ml of methanolic rhodanine solution (Appendix). After exactly 5 minutes, 0.10ml of 0.5N KOH (Appendix)

was added and after a further 2.5 minutes the mixture was diluted to 2.5ml with RO H_2O. The A_{520} was measured 5-10 minutes later using a Shimadzu UV-160A spectrophotometer with quartz glass cuvettes (1.0cm path length). A standard curve was prepared using gallic acid standards prepared by diluting gallic acid stock solution (Appendix) in NB medium. Gallic acid assays were conducted in duplicate for each tube at each sampling time.

2.3 Experimental procedures used in Chapter 5

2.3.1 Preparation of crude cell lysate

A 100ml culture of K2 cells in mBHI medium was grown overnight. The culture was then transferred to a centrifuge tube and spun at 8,700x*g* for 15 minutes at 4°C in a Beckman JA-10 rotor. The supernatant was discarded and the cell pellet was washed once with 0.1 M sodium phosphate buffer pH 5.8. The washed cells were then resuspended in 10ml of the same buffer and the cells were lysed by two passages through a French pressure cell at 20,000 lb/in^2 and 3°C.

2.3.2 Incubation of crude lysates with tannic acid

Two 2.0ml aliquots of crude lysate prepared as above were placed in separate 10ml disposable yellow-capped tubes. One aliquot was boiled for 15 minutes and then cooled on ice. Aliquots of a 10% w/v tannic acid solution prepared in phosphate buffer (pH 5.8) were added to each tube to a final concentration of 0.1% w/v and the tubes were then incubated in a 37°C water bath. Triplicate 0.1ml aliquots were removed at various time intervals and the concentration of gallic acid in each aliquot was determined as follows:

Each aliquot was transferred to a glass tube (9x75mm) containing 0.15ml of methanolic rhodanine solution (Appendix). After exactly 5 minutes, 0.10ml of 0.5N KOH was added and after a further 2.5 minutes the mixture was diluted to 2.5ml with RO H_2O. The A_{520} was measured 5-10 minutes later using a Shimadzu UV-160A spectrophotometer with quartz glass cuvettes (1.0cm path length). A standard curve was prepared using gallic acid (Sigma) standards prepared in phosphate buffer pH 5.8.

2.3.3 Preparation of cell-free extracts

Bacterial cultures in triplicate (10ml, mBHI) supplemented with 0.2% w/v gallic acid, 0.2% w/v tannic acid or 0.1% w/v gallic acid methyl ester (GAME, Sigma) were centrifuged and the cell pellet was washed once with 0.1 M sodium phosphate buffer pH 6.6 and suspended in 3.0 ml of the same buffer. Glass beads (0.05g, 106 µm, Sigma) and polyvinyl pyrrolidone (0.010g, Calbiochem) were added to the cell suspension and the mixture was sonicated at 200W using a Branson Sonifier 450 (USA) at 6 cycles of 15 seconds duration with cooling on ice-water for 60 seconds between cycles. Lysates were centrifuged at 25000x *g* at 4°C and the supernatant was filtered through a 0.22 µm filter (Millex-GS, Millipore). The cell-free extract was stored in the dark at 4°C. Protein concentration was determined using Bradford's reagent as described in Ausubel *et al* (1989) with bovine serum albumin (BSA) Fraction V (Sigma) as the standard.

2.3.4 Tannin acylhydrolase assay

Cell-free extracts were diluted in 0.1 M sodium phosphate buffer pH 6.6 to a protein concentration of 0.02 -0.15 mg/ml and pre-incubated for 5 minutes at 37°C prior to initiating the assay. A 5.0 mg/ml stock solution of GAME dissolved in 0.1 M sodium phosphate buffer pH 6.8 was pre-incubated under the same conditions. The enzyme reaction was initiated by addition of substrate. When the pH optimum was determined, 5 different 0.1 M sodium phosphate buffers were used to generate a pH range from 5.8 - 8.0. A zero time 0.1 ml aliquot was immediately removed to a glass tube (9 x 75 mm) immersed in a bath of liquid nitrogen to stop the reaction. Aliquots (0.1 ml) were removed from the reaction at 1.0 min intervals over 5.0 minutes and snap frozen in liquid nitrogen. The concentration of gallic acid in each aliquot was determined as described above in section 2.3.2. The reaction rate, in µmoles of gallic acid produced min^{-1} mg $protein^{-1}$, was calculated from the slope of the graph. For determination of K_m and V_{max}, enzyme incubations were conducted in duplicate for each substrate concentration and the mean initial rates of reaction were calculated.

2.3.5 ρ-nitrophenyl acetate esterase assay (Hespell and O'Bryan-Shah, 1988)

The assay mixture contained 1.0ml of 0.1 M sodium phosphate buffer pH 7.0 in 1 mM dithiothreitol, 5.0µl of 0.25 M ρ-nitrophenyl acetate (Sigma) dissolved in 100% dimethylformamide (Sigma), and 100µl of cell-free extract. The assay was initiated by addition of cell-free extract and the mixture was incubated at 37°C for 15 minutes. The A_{450} was measured immediately and a standard curve was prepared using ρ-nitrophenyl phosphate (Sigma).

2.3.6 α-napthyl acetate esterase assay (Hespell and O'Bryan-Shah, 1988)

The assay mixture contained 0.5ml of 0.2 M sodium phosphate buffer pH 7.0, 1.75ml RO H_2O, 0.15ml of 5.0 mM α-napthyl acetate ester (Sigma) dissolved in 100% methanol, and 100µl cell-free extract. The assay was initiated by the addition of the cell-free extract and was incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 0.5ml Fast Garnet GBC (5 mg/ml in 10% w/v SDS). The reaction was then incubated at room temperature for 15 minutes and then the A_{560} was determined. A standard curve was prepared using α-napthol (Sigma).

2.3.7 4-methylumbelliferyl acetate esterase assay (McDermid *et al*, 1990)

The assay mixture contained 10µl of 10 mM 4-methylumbelliferyl acetate (Sigma) dissolved in 100% methanol, 10µl of 0.5 M potassium phosphate buffer pH 6.8, and cell-free extract to a total volume of 100µl. The reaction was initiated by the addition of cell-free extract and was incubated at 37°C for 10 minutes. The reaction was terminated by diluting with 2.9ml H_2O and the fluorescence was measured at an excitation wavelength of 330nm and an emission wavelength of 445nm. A standard curve was prepared from 4-methylumbelliferone (Sigma).

2.3.8 Tributyrin (glyceryl tributyrate) esterase assay

Anaerobic mBHI agar plates containing 1.0% v/v tributyrin (ICN Biomedicals) were inoculated with K2 cells and incubated at 39°C for two days, after which they were examined for zones of hydrolysis surrounding the colonies. Aliquots of cell-free extract were also tested for

tributyrin-hydrolysing activity by spotting onto mBHI/1.0% w/v tributyrin agar plates and incubating at 39°C for up to 2 hours.

2.3.9 Phenylmethylsulphonyl fluoride (PMSF) inhibition assay

Aliquots of cell-free extract were incubated with and without PMSF (Sigma) (1.0 and 5.0 mM) at 37°C for a period of 30 minutes. The residual tannin acylhydrolase activity was then determined as described in section 2.3.4.

2.4 Experimental procedures used in Chapter 6

2.4.1 Determination of specific activity

Cell-free extracts

Cell-free extracts were prepared as described in section 2.3.3. Specific activity of tannin acylhydrolase in cell-free extracts was determined using the assay described in section 2.3.4. Assays were conducted at 37°C for 5 minutes using the substrate GAME at a concentration of 0.2% w/v.

Whole-cell suspensions

Bacterial cultures (10ml) in mBHI medium supplemented with 0.2% w/v tannic acid, 0.2% w/v gallic acid or 0.1% w/v GAME were centrifuged and the cell pellet washed once with sodium phosphate buffer pH 6.8. The cell pellets were then resuspended in 2.0ml of the same buffer. To determine specific activity, 50µl cell suspension was added to a 1.5ml microcentrifuge tube containing 900µl sodium phosphate buffer pH 6.8. The reaction mixes were preincubated at 37°C for 5 minutes and were initiated by adding 600µl of 0.5% w/v GAME dissolved in phosphate buffer pH 6.8. Immediately after addition of substrate, a 0.1ml aliquot was removed and transferred to a glass tube immersed in liquid nitrogen. Subsequent 0.1ml aliquots were removed every 2 minutes for 20 minutes. The concentration of gallic acid in each aliquot was determined using the assay described in section 2.2.4. The number of cells present in each

reaction was determined by direct microscopic count using a haemocytometer and light microscope. Specific activity was expressed as μmoles gallic acid produced min^{-1} $cell^{-1}$.

2.4.2 Gel electrophoresis and analytical isoelectric focusing

Gel electrophoresis was performed in the presence of SDS as described in Ausubel *et al* (1989) with 4% w/v stacking and 10% separating gels, unless indicated. Analytical isoelectric focusing (IEF) was performed in 5% polyacrylamide (Sigma) gels incorporating 2.5% v/v Pharmalyte (Pharmacia) using a Pharmacia FBE 3000 flat-bed apparatus. The anode and cathode solutions were 0.5 M acetic acid and 0.5 M sodium hydroxide respectively. Both types of gels were stained for protein using Coomassie Blue R250 (Sigma) as described in Ausubel *et al* (1989).

2.4.3 Zymogram analyses

Tannin acylhydrolase

Duplicate aliquots of cell-free extracts were fractionated by gel electrophoresis or analytical IEF. Following electrophoresis, the gel was divided in half and proteins in one half were localised by staining with Coomassie Blue R250. The other half of the gel was washed at room temperature three times for 30 minutes per wash in a buffer containing 1 mM Tris-HCl, pH 7.5, 20% propan-2-ol and 5 mM β-mercaptoethanol, in order to remove the SDS. The gel was then washed at 4°C in sterile water for 1 hour followed by phosphate buffer pH 6.8 for 1 hour. A 0.8% w/v agarose (Sigma) gel (0.75 mm thickness) containing 0.1% w/v GAME in 0.1 M sodium phosphate buffer pH 6.8 was overlaid onto the polyacrylamide gel and the sandwich was incubated for 1 hour at 37°C. Analytical IEF gels were divided in half and the proteins in one half were stained with Coomassie Blue while the other half gel was washed three times in distilled water for 15 minutes each in order to remove the carrier ampholytes. The gel was then overlaid with an agarose gel as above. After incubation, both types of gel sandwiches were flooded with 0.67% methanolic rhodanine solution (Appendix) for 10 minutes and then with 0.5N KOH, in order to develop the purple-coloured rhodanine-gallate complex.

Methylumbelliferyl acetate esterase

Duplicate aliquots of Triton X-100 (BDH) extracts (see 2.4.4) were fractionated by SDS-PAGE. Following electrophoresis the gel was divided and the proteins in one half were stained with Coomassie Blue R250 as described. The other half-gel was treated as above to remove the SDS and allow the proteins to renature. The buffer was removed and the gel was placed onto a glass plate on top of a UV Transilluminator. The gel was then flooded with a 1.0 mM solution of methylumbelliferyl acetate in 10% ethanol/90% sodium phosphate buffer pH 6.8 and incubated at room temperature. Methylumbelliferone, the product of methylumbelliferyl acetate hydrolysis, was detected after excitation with UV light. The fluorescence was recorded on Polaroid Type 667 instant black and white film using a Polaroid MP4 land camera equipped with a 530nm barrier filter.

2.4.4 Triton X-100 extraction of tannin acylhydrolase activity

Experiment 1

A 10ml culture of K2 cells in mBHI/0.2% w/v gallic acid was grown to an A_{600} of 0.8 and then the culture was centrifuged and the supernatant was discarded. The pellet was washed once in RO H_2O and then the cell suspension was divided into half. One half of the cells were resuspended in 1.0ml of RO H_20 and the other half were resuspended in 1.0ml of 1.0% w/v Triton X-100 in RO H_2O. Both cells suspensions were shaken gently on a rocking platform for 2 hours at room temperature. The suspensions were then centrifuged in a microcentrifuge on high speed for 10 minutes. Aliquots were tested for tannin acylhydrolase and methylumbelliferyl acetate esterase activity (Chapter 6 section 6.2.3 and 6.2.4) and were run on a 10% SDS-PAGE gel.

Experiment 2

Two 10ml cultures of K2 in mBHI/0.2% w/v gallic acid were grown to an A_{600} of 0.8. The cultures were centrifuged and the cell pellets were washed once in 0.1 M sodium phosphate buffer pH 6.8. The cell pellets were resuspended in 1.0ml of 1.0% Triton X-100 in phosphate buffer and then these 2 cell suspensions were combined. The cell suspension was shaken

gently on a rocking platform for 2 hours at room temperature. Duplicate 50µl aliquots were removed at 0, 10, 20, 30, 60 and 120 minute intervals and the proteins in these aliquots were acetone precipitated as follows: The cells in each aliquot were pelleted by brief centrifugation in a microcentrifuge and the supernatant removed to a tube containing 8-volumes of acetone. The proteins were left to precipitate at -20°C overnight. The samples were then centrifuged at 12,100x*g* for 10 minutes at 4°C and the acetone was removed. The protein pellet was then resuspended in 85% v/v acetone and left at -20°C for at least 1 hour. Following centrifugation as above, the supernatant was removed and the protein pellet was allowed to partially dry, after which it was redissolved in 50µl phosphate buffer pH 6.8. The protein concentration of each sample was determined using Bradford's reagent as described by Ausubel *et al* (1989). Aliquots of each sample were fractionated on a 12% SDS-PAGE gel.

Experiment 3

Bacterial cultures (10ml) in mBHI medium supplemented with 0.2% w/v tannic acid, 0.2% w/v gallic acid or 0.1% w/v GAME were centrifuged and the cell pellet was washed once with 0.1 M sodium phosphate buffer pH 6.8. The cell pellets were then resuspended in 1.0ml of 1.0% Triton X-100 in 0.1 M phosphate buffer pH 6.8 and extracted for 2 hours as described above. The proteins in the supernatant were acetone precipitated as above and then the specific activity of tannin acylhydrolase determined as described in section 2.3.4.

2.4.5 Tannin acylhydrolase activity in isolates of *S. ruminantium*

Glycerol stocks of each bacterial strain were streaked onto anaerobic mBHI agar plates and incubated anaerobically overnight. A single, large colony of each strain was picked off the plate and resuspended in 100µl of 0.1 M sodium phosphate buffer pH 6.8. These cell suspensions were removed from the anaerobic hood and each was divided into two 50µl aliquots. One aliquot of each strain was boiled for 10 minutes and then cooled to room temperature to serve as a negative control. To each cell suspension was added 50µl of 0.5% w/v GAME dissolved in 0.1 M phosphate buffer pH 6.8 and the tubes were incubated at 37°C for up to 2 hours. Following incubation, methanolic rhodanine (Appendix) and KOH were added to each tube as

described in sections 2.2.4 and 2.3.4, in order to detect gallic acid release from GAME. Tubes were visually assessed for production of purple coloration which was scored as presence of tannin acylhydrolase activity.

2.4.6 Growth of isolates on tannic acid as a sole energy source

Single colonies of each bacterial strain were inoculated into anaerobic defined NB medium containing 1.0% w/v tannic acid as sole energy source. Bacteria were also inoculated into defined NB medium containing 0.8% w/v mannitol as sole energy source which served as positive controls. All tubes were incubated anaerobically at 39°C overnight. Growth was scored by visual assessment of aliquots of cultures under the light microscope.

2.5 Experimental procedures used in Chapter 7

2.5.1 Construction of plasmid libraries

Genomic DNA was isolated from a 100ml mBHI culture of *S. ruminantium* K2 by a routine procedure described in Ausubel *et al* (1989). Aliquots of this DNA were digested with a restriction endonuclease and then ligated to an appropriately restriction endonuclease-digested plasmid vector according to standard procedures (Ausubel *et al*, 1989). The following combinations of insert and vector DNA were tested: *Sau* 3AI-partially digested K2 DNA ligated to *Bam* HI-digested pGEM-3Z (Promega); *Eco* RI-partially or completely digested K2 DNA ligated to *Eco* RI-digested pGEM-3Z; *Sau* 3AI-partially digested K2 DNA ligated to *Bam* HI-digested pBR322. Ligations were ethanol precipitated (Ausubel *et al*, 1989) and then transformed into electrocompetent *E. coli* cells via electroporation (Ausubel *et al*, 1989). The following *E. coli* strains were tested and prepared for electroporation as described in Ausubel *et al* (1989): ER2267 (New England Biolabs); XL-1 Blue (Stratagene); DH5α. Electroporation conditions were: 12.5 keV/cm voltage, 25 μF capacitance and 200 ohms resistance. Transformed cells were placed into 1.0ml SOC (Appendix) and allowed to express for 1 hour at 37°C with gentle shaking. Cells electrotransformed with vectors containing the *lac* Z gene were plated onto LB (Appendix) agar plates containing ampicillin (Sigma, 50 μg/ml), X-gal

(Promega, 40 μg/ml) and isopropylthiogalactoside (IPTG, Boehringer Mannheim, 40 μg/ml) and incubated at 37°C overnight. Cells transformed with pBR322 were plated onto LB agar plates containing 50 μg/ml ampicillin. Libraries were stored at -80°C in SOC containing 18% v/v glycerol.

2.5.2 Construction of bacteriophage libraries

K2 genomic DNA was partially digested with *Eco* RI and ligated to either λgt11 (Promega) or λDASH II (Stratagene) *Eco* RI-digested, dephosphorylated arms. Ligations were packaged using a GigaPack XL packaging kit according to the instructions of the manufacturer (Stratagene). Packaged reactions were titred using the appropriate *E. coli* host strain (Y1090r$^-$, λgt11 host; LE392, λDASH II host) and then the libraries were amplified for long-term storage according to the Stratagene protocol. Libraries were stored at 4°C in SM (Appendix) containing a few drops of chloroform and also at -80°C in SM containing 7% v/v dimethyl sulphoxide (DMSO, BDH).

2.5.3 Screening of libraries for expression of tannin acylhydrolase activity

Aliquots of plasmid libraries were plated onto LB agar plates containing 50 μg/ml ampicillin and 40 μg/ml IPTG and incubated overnight at 37°C. The bacterial cells on the agar plates were then permeabilised with chloroform by placing the plates in a glass bell-jar containing a chloroform-saturated atmosphere for 20 minutes. The colonies on the plates were then overlaid with a thin layer of 0.8% w/v agarose containing 0.1% w/v GAME. The plates were then incubated for 30 minutes at 37°C and then were flooded with methanolic rhodanine solution (Appendix) for 10 minutes followed by 0.5 N KOH (Appendix).

Aliquots of bacteriophage libraries were plated using LB top and bottom agar as described in Ausubel *et al* (1989). Plates bearing λgt11 plaques contained 40μg/ml IPTG to induce synthesis of *lac*Z-fusion proteins. Plaques were screened for tannin acylhydrolase activity in essentially the same manner as bacterial colonies except that it was not necessary to treat plaques with chloroform.

2.5.4 Cloning of a tannin-inducible promoter

Alu I-digested and *Hae* III-digested K2 DNA was ligated into the *Sma* I site of the prokaryotic promoter selection vector pKK232-8 (Pharmacia). Products of the ligation reactions were ethanol precipitated and then electroporated into *E. coli* ER2267 cells. After incubating the transformants at 37°C for 1 hour, the entire bacterial populations were plated onto LB agar plates containing 25 µg/ml chloramphenicol and incubated at 37°C overnight. Each resulting colony was patched onto LB plates containing 25, 50, 100, 200 and 400 µg/ml chloramphenicol and also onto a second set of LB plates containing these same concentrations of chloramphenicol plus 0.2% w/v tannic acid. All plates were incubated aerobically at 37°C overnight and then examined for colonies that displayed an altered resistance to chloramphenicol in the presence of tannic acid.

2.5.5 Isolation of tannin acylhydrolase for immunisations

Tannin acylhydrolase for the primary immunisation and the first boost immunisation was isolated as follows: A cell-free extract derived from *S. ruminantium* K2 cells grown in mBHI/0.1% w/v GAME was prepared by sonication as described in section 2.3.3 and then fractionated on polyacrylamide IEF gels (pH 5-8) as described in section 2.4.2. After staining with Coomassie Blue R250 the protein band corresponding to P_i 7.0 was excised and loaded onto a 10% SDS-PAGE gel. Following electrophoresis and staining with Coomassie Blue, the 60kDa band corresponding to the tannin acylhydrolase was excised.

Material for the second and subsequent boost immunisations was isolated on a preparative scale as follows: A cell-free extract was fractionated by preparative isoelectric focusing (pH 5-8) in Ultrodex gel (Pharmacia) using a flat-bed apparatus (FBE 3000, Pharmacia) according to the Pharmacia instruction booklet. The region of gel corresponding to pH 7.0 was removed and packed into a 2.5ml syringe and the proteins contained within the resin were eluted with 0.1 M sodium phosphate buffer pH 6.8. Aliquots of this protein sample, designated fraction #24, were then fractionated on a 1.5mm thick, 10% SDS-PAGE preparative gel (Protein IIXi,

BioRad). The gel was stained with copper chloride according to the procedure described in Harlow and Lane (1988) and protein corresponding to the tannin acylhydrolase was excised.

2.5.6 Preparation of immunogen, immunisations and test bleeds

Polyacrylamide gel slices containing protein for immunisations were macerated with a sterile scalpel and mixed with paraffin oil in a ratio of approximately 1:1 v/v. This mixture was then emulsified by continually passing back and forth between 2 glass syringes connected by a double-ended 21G needle. New Zealand White rabbits were immunised subcutaneously at multiple sites over the back and hindquarters as described in Harlow and Lane (1988). The immunisation schedule used is described in Table 2.1.

Table 2.1 Immunisation schedule

Time	Procedure	Amount of immunogen
day 0	primary immunisation, 4 of 4 rabbits	10µg protein/rabbit
	test bleed to provide preimmune sera	
day 30	1st boost, 4 of 4 rabbits	5µg protein/rabbit
day 54	test bleed	
day 64	2nd boost, 2 of 4 rabbits	40µg protein/rabbit
day 75	test bleed	
day 90	3rd boost, 2 of 4 rabbits	20µg protein/rabbit
day 104	test bleed	

Test bleeds were taken from the marginal ear vein at the intervals shown and were processed to provide preimmune/immune sera as described in Ausubel *et al* (1989).

2.5.7 Immunoblotting and immunodetection

Antisera were analysed for the presence of tannin acylhydrolase-specific antibodies by immunoblot. Cell-free extracts derived from *S. ruminantium* K2 cells grown in mBHI/GAME were fractionated by electrophoresis in 10% SDS-PAGE gels. Both prestained and unstained protein standards were included in each gel. Following electrophoresis, gels were equilibrated in transfer buffer (Towbin buffer, Appendix) for 30 minutes. Proteins in the gels were then transferred to PVDF membranes (Immobilon-P, Millipore) using the tank transfer system (Mini-Trans Blot, BioRad) as described in Ausubel *et al* (1989). Proteins were electroblotted at 100V for 30 minutes with cooling supplied by the ice block integral to the BioRad apparatus. After transfer, membranes were reversibly stained with Ponceau S (Ajax Chemicals) (Ausubel *et al*, 1989) and the position of the molecular weight standards was marked with indelible ink. The membranes were then cut into appropriate-sized strips between the protein tracks in order to provide the required number of samples for immunoblotting with preimmune and immune sera. The membrane strips were then soaked in water until all the Ponceau S stain had been removed.

PVDF membranes were immunoblotted according to the procedure described in Ausubel *et al* (1989). Briefly, membranes were sealed in individual plastic bags and blocked for a minimum of 2 hours in blocking buffer (TNT/5% w/v skim milk powder, Appendix) at room temperature. They were then incubated with the test antisera (1/50 or 1/100 dilution) in TNT/1% w/v skim milk powder at room temperature for 2 hours. Membranes were washed 4 times with TNT (Appendix) at room temperature for 10 minutes each time. They were then incubated with the secondary antibody-enzyme conjugate (1/30,000 dilution, goat-anti rabbit IgG-alkaline phosphatase conjugate, Sigma Cat. No. A3812) in TNT/1% w/v skim milk powder for 1 hour at room temperature. Membranes were washed 4 times as before and then the colour developed using bromochloroindoyl phosphate/nitroblue tetrazolium (BCIP, Boehringer Mannheim/NBT, Sigma) as described in Ausubel *et al* (1989).

2.5.8 N-terminal protein sequencing

An aliquot of fraction #24 (see section 2.5.5) containing approximately 30µg of total protein was fractionated on a 10% SDS-PAGE gel according to the protocol for microsequencing described in Ausubel *et al* (1989). Following electrophoresis the proteins in the gel were transferred to a PVDF membrane (Immobilon-P, Millipore) as described above. The proteins on the membrane were then stained with a modification of the Coomassie Blue stain, which contained 0.10% Coomassie Blue R250 in 40% methanol. The membrane was then destained in 50% methanol. Acetic acid was not included in the stain or destain because it may cause blockage of the amino terminus.

After staining and destaining, the band corresponding to the tannin acylhydrolase was excised from the membrane using a sterile scalpel. The protein contained within this membrane slice was then subjected to N-terminal sequencing via Edman degradation by the Department of Biochemistry, The University of Adelaide, using an Applied Biosystems 475A protein sequencer.

2.5.9 Southern transfers and hybridisations with a synthetic degenerate oligonucleotide

Aliquots of K2 genomic DNA were digested with a variety of restriction endonucleases and then fractionated in 0.8% w/v agarose gels according to standard protocols (Ausubel *et al*, 1989). DNA was then transferred to nylon membranes (Hybond N^+, Amersham) by the downward capillary, alkaline-transfer technique described in Ausubel *et al* (1989). The synthetic degenerate oligonucleotide DO1 (100 pmoles, see 7.2.4) was end-labelled with $\gamma^{32}P$ (ATP) using T4 Polynucleotide kinase (New England Biolabs) according to the procedure of Ausubel *et al* (1989). Membranes were prehybridised in glass hybridisation bottles (Hybaid) containing 5ml prehybridisation solution (Appendix) for at least 1 hour at 34°C according to standard procedures (see Ausubel *et al*, 1989; Sambrook *et al*, 1989). Hybridisation was conducted in 5ml hybridisation solution (Appendix) at the temperatures indicated in the text (Chapter 7 section 7.2.4) for a period of approximately 16 hours. Following hybridisation,

membranes were initially washed at room temperature in 6X SSC/0.1% SDS (Appendix), and then in the same solution at gradually higher temperature until the counts detected by a hand-held mini-monitor fell to almost background level. Washed membranes were then wrapped in plastic "cling-film" and autoradiographed according to standard practice. Exposed autoradiographs were developed by hand using Phenisol X-Ray Developer (Ilford) and Hypam Rapid Fixer (Ilford).

2.5.10 Bacteriophage library screening with a degenerate oligonucleotide

Aliquots of the λgt11 library were plated onto 135mm diameter LB plates as described in Ausubel *et al* (1989). Plaques were transferred to Hybond N^+ filter discs (132mm diameter, Amersham) according to the instructions of the manufacturer. The phage-bearing filters were then prehybridised and hybridised at 40°C with end-labelled DO1 as described above and then autoradiographed. Hybridisation signals on the X-ray films were aligned with plaques on the plates and an area of 5mm diameter surrounding the positive plaques was removed from the plates using the end of a sterile glass pasteur pipette. These agar plugs were then transferred to 1.0ml phage dilution buffer (SM, Appendix) and the phage particles were allowed to elute out of the plugs for several hours at 4°C. Aliquots of eluted phage were then subjected to a second round of hybridisation with DO1 as before.

2.5.11 PCR using DO1 and M13 primers

K2 genomic DNA was completely digested with *Sau* 3AI and ligated into the *Bam* HI site of the plasmid vector pBluescript SK (250ng, Stratagene). Three separate ligations were performed, containing vector to insert DNA in the mass ratios of 1:2, 1:1 and 1:0.5. After overnight incubation at 12°C, the products of each ligation were ethanol precipitated and resuspended in 18µl of sterile water. Each ligation was then divided into 6 equal-sized aliquots, to be used as template for a PCR amplification containing either DO1 and M13 reverse primer or DO1 and M13 forward primer and one of three $MgCl_2$ concentrations; 0, 1.5 and 3.0 mM. The experimental design is represented schematically in Figure 7.10. Reactions containing religated

pBluescript and also uncut pBluescript amplified with M13 forward and reverse primers in the absence of DO1 were included as controls.

Reaction mixtures contained the following in a total volume of 50µl: 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3) (PCR Buffer II, Perkin Elmer); 44 pmoles DO1; 30 pmoles either M13 forward or reverse primer; 0.2 mM dNTPs; 0, 1.5 or 3.0 mM $MgCl_2$; 1 Unit Taq Polymerase (AmpliTaq, Perkin Elmer); 2.0µl ligation reaction. Reactions were cycled in a Perkin Elmer thermocycler using the following conditions: 30 seconds denaturation at 94°C; 30 seconds primer annealing at 40°C; 60 seconds extension at 72°C for 30 cycles. This program was followed by a 10-minute extension period at 72°C, following which the reactions were held at 4°C until analysis. Aliquots representing 10% of the reaction volume were analysed by electrophoresis in 1.5% agarose gels.

2.5.12 In-gel proteolysis to obtain a peptide sequence

Five aliquots of fraction #24, each containing approximately 20µg total protein, were fractionated on a large 10% SDS-PAGE gel as described in Ausubel *et al* (1989). The gel was stained with Coomassie Blue R250 and then destained according to standard protocols. The band corresponding to tannin acylhydrolase was excised from each track using a sterile scalpel, being careful to avoid excess gel material. The gel slices were then pooled. An identical sized piece of non-protein containing gel was excised and included as a "blank". Both samples of gel slices were cut into 2mm-square pieces and then placed in separate Eppendorf tubes and covered with sterile water for 10 minutes. The liquid was then removed and discarded. The gel pieces were then covered with a solution of acetonitrile (HPLC grade, BDH):water 1:1 v/v and allowed to stand for 20 minutes. This wash was repeated until no more blue colour leached out of the gel. The gel pieces were then covered with 100% acetonitrile for just enough time to allow the volume of gel to be decreased to about 1/4 of its original volume. The remaining liquid was then removed and 0.1 M Tris-HCl pH 8.5 was added to just cover the gel pieces once they had rehydrated, followed by 5µl of 45 mM dithiothreitol (DTT). The samples were incubated at 50°C for 20 minutes, cooled to room temperature and 5µl of 100mM

iodoacetamide were added. The samples were incubated in the dark at room temperature for 20 minutes. Endoproteinase Lys C (1.0µg; sequencing grade, Cat. No.1420 429; Boehringer Mannheim), reconstituted according to the manufacturers instructions, was added to each sample and then enough Tris buffer was added to just cover the gel pieces. The samples were then incubated overnight at 37°C. The supernatants were removed and set aside, and then 100µl of 70% v/v trifluoroacetic acid (TFA, sequencing grade, Cat. No. T1647, Sigma) was added to each sample. The samples were incubated at 37°C for 30 minutes after which time the supernatants were removed and added to the first supernatant. This TFA extraction was then repeated. A further 2 extractions were performed as before with 200µl 1:1 TFA:acetonitrile and the supernatants from these extractions were added to the previous pooled extracts. The combined extractions were then dried completely in a SpeediVac vacuum centrifuge, after which the pellets were redissolved in 150µl of water containing 0.06% v/v TFA.

Each sample was run on an HPLC (Hewlett-Packard 1090LC Series II) at 40°C using a Vydac Protein and Peptide 250 x 2.1 mm C-18 column (Cat. No. 218TP52) with a guard column. Absorbances were read on diode arrays at 214nm and 280nm. The column was washed through with HPLC Buffer B (0.05% v/v TFA in acetonitrile) before being fully equilibrated with HPLC Buffer A (0.05% v/v TFA in water). The peptides in the samples were separated in a linear gradient of 0-70% acetonitrile over 70 minutes at a flow rate of 0.15 ml/minute, according to the following program:

Time (minutes)	% Buffer B	Flow rate (ml/min)
0-5	1.0	0.15
5-75	1.0-70.0	0.15
75-76	70.0-98.0	0.15
76-80	98.0-1.0	0.15
80-87	1.0	0.15

Peaks were collected manually. One peak was submitted to the Nucleic Acid and Protein Chemistry Unit of the Department of Plant Science, University of Adelaide for N-terminal sequencing.

2.5.13 Degenerate oligonucleotide primed (DOP)-PCR

Degenerate oligonucleotide primed PCR (DOP-PCR) was performed using DO1 and DO2 primers with *S. ruminantium* K2 chromosomal DNA as template. Reaction mixes contained the following in a total volume of 25µl: 1X PCR buffer (PCR Buffer II, Perkin Elmer); 44 pmoles DO1; 88 pmoles DO2; 0.2 mM dNTPs; 3.0 mM $MgCl_2$; 0.5U Taq Polymerase (AmpliTaq, Perkin Elmer); 5.0, 0.5 or 0.05µg K2 DNA. Reactions were cycled in a Perkin Elmer thermocycler using the following conditions: 30 seconds denaturation at 94°C; 30 seconds primer annealing at 37°C; 60 seconds extension at 72°C for 5 cycles; followed by 30 seconds denaturation at 94°C; 30 seconds primer annealing at 45°C; 60 seconds extension at 72°C for 30 cycles. This program was followed by a 10-minute extension period at 72°C, following which the reactions were held at 4°C until analysis. Aliquots representing 10% of the reaction volume were analysed by electrophoresis in 1.2% agarose gels.

2.5.14 Cloning of PCR products

PCR products were cloned by ethanol precipitating a 10µl aliquot of the PCR reaction (Ausubel *et al*, 1989) and resuspending the pellet in 5µl of sterile water. These samples were then ligated to 32ng of pBluescript digested with the blunt-end-generating restriction endonuclease *Hinc* II, as described in Ausubel *et al* (1989). Ligation reactions were ethanol precipitated and the pellets were resuspended in 10µl of sterile water. Electrocompetent *E. coli* DH5α cells were transformed with the resuspended ligation reactions by electroporation using a BioRad Gene Pulser electroporator as described in section 2.5.1. Transformed cells were expressed in 1.0ml SOC for 1 hour at 37°C and then aliquots were plated onto LB agar plates containing ampicillin, IPTG and X-gal as described in section 2.5.1. White-coloured colonies were picked and grown overnight at 37°C in 2.0ml LB broth containing 50 µg/ml ampicillin. Plasmids were isolated by the alkaline-lysis miniprep technique described in Ausubel *et al* (1989). Aliquots of

plasmid minipreps were analysed by double-digestion with the restriction endonucleases *Cla* I and *Xho* I followed by agarose gel electrophoresis.

CHAPTER 3 Bacterial enrichment and identification.

3.1 Introduction

As stated in Chapter 1, feral goats in Australia are able to subsist on tannin-rich forages such as *Acacia* sp. which are poorly digested by domestic cattle and sheep. Although tannins are known to inhibit ruminant digestion, the precise mechanisms by which this is achieved have not been proven. One of the most important effects of tannins may be the inhibition of microbial growth and metabolism in the rumen. It has been proposed that the apparent detoxification of ingested tannins in feral goats may be due to the action of novel rumen microorganisms. These microbes may confer tannin-tolerance upon their host by either being resistant to the tannins or by chemically modifying the tannins and thereby negating their inhibitory properties.

To date, the focus of research in this field has been centred on the ability of tannins to form insoluble complexes with proteins. This focus has been reflected in earlier attempts to isolate microorganisms that may be involved in the detoxification of tannins. Previous authors have isolated such microbes by plating dilutions of rumen, caecal or faecal samples onto agar media containing a tannin-protein precipitate and selecting those organisms which appear to hydrolyse this precipitate as evidenced by clear zones in an otherwise turbid medium. Although this experimental approach has resulted in the isolation of a tannin-resistant *Streptococcus* (Osawa, 1990), other, perhaps more important microorganisms may be overlooked because they fail to grow on such a medium. Furthermore, the link between ability to grow and form clearing zones on a tannin-protein complexed medium and a role in the detoxification of tannins has not yet been proven.

Therefore, an alternative strategy was adopted in this research project. Rumen fluid from feral goats browsing tannin-rich *Acacia* in the arid pastoral zone of South Australia was enriched for microorganisms which are able to grow in a liquid medium containing tannins. In addition, rather than use the commonly-used hydrolysable tannin, tannic acid, an extract of *Acacia* condensed tannin was employed as the selective pressure. It was believed that this tannin

preparation would be more appropriate as it was derived from the plants eaten by the feral goats.

3.2 Results

3.2.1 Bacterial isolation -selection with condensed tannins

In an attempt to isolate microorganisms that may play a role in the detoxification of tannins in feral goats organisms were selected using an enrichment-culture technique.

The primary enrichment culture was initiated using crushed *Acacia* leaves as the available growth substrate and selection pressure. After 5 days incubation this culture contained innumerable different bacterial types and also many active protozoa. An aliquot of this culture was then transferred to fresh enrichment medium, this time containing 0.5% (w/v) of a crude extract of *Acacia* condensed tannin (see Chapter 2 for description of preparation) instead of crushed leaves, in order to increase the selection pressure. Microscopic examination of an aliquot of this second culture after 2 days incubation showed that there were still many different cell types present; although the protozoa appeared not to have been carried over. The selection pressure was then increased by transferring an aliquot of the second culture to fresh medium containing 1.5% (w/v) of the *Acacia* crude tannin extract. After 2 days incubation there was a dramatic reduction in the number of bacteria able to grow in this level of crude condensed tannin. Four morphologically distinct types were apparent and these were maintained in a subsequent final culture at this same level of tannin. A morphological description of these is as follows; 1) medium sized, motile curved rods; 2) small, non motile chains of cocci; 3) large, non motile single cocci and 4) non motile filamentous bacteria.

3.2.2 Isolation and identification of motile curved rods

In order to allow further characterisation of one isolate, the motile curved rods were isolated from the final enrichment culture on M10 agar medium which contained the 3 soluble sugars glucose, cellobiose and starch but lacked tannin. Subsequently it was discovered that this bacterium also grew well on mBHI medium (broth and agar) and consequently this became the

medium for routine maintenance of this organism due to its simplicity of preparation. This bacterium was arbitrarily designated "isolate K2".

Microscopic examination of wet-mounts and Gram stained cells revealed this organism to be a Gram negative, motile, curved rod (Figure 3.1). In order to identify this bacterium, it was first tested for its ability to ferment a variety of carbohydrates. The results appear in Table 3.1.

The bacterium's carbohydrate fermentation profile was not sufficient to make an unequivocal identification. Consequently, the profile of volatile fatty acids produced by the organism when grown on glucose as an energy source was determined. GC analysis of the VFAs produced by K2 is shown in Figure 3.2 and shows that the major acids produced are propionate and acetate (10.54 ± 0.08 mM and 8.76 ± 0.28 mM, respectively). These values correspond to approximately 52% and 43% of the total VFAs, respectively. The peak on the chromatogram between the acetate and propionate peaks is an artifact which was present in all samples including the standards.

The morphology, VFA production profile and carbohydrate fermentation profile suggest that K2 belongs to the genus and species *Selenomonas ruminantium. Selenomonas ruminantium* has been divided into 3 subspecies: *lactilytica*, *ruminantium* and *bryanti* (Bergey's Manual, 1984). Members of the subspecies *lactilytica* are differentiated from the other two subspecies based on their ability to ferment lactate as a sole energy source. As can be seen in Table 3.1, K2 is unable to ferment lactate and hence does not fit into this group. Members of the subspecies *ruminantium* can be differentiated from *bryanti* by their ability to produce H_2S from cysteine. When K2 was grown in H_2S-test medium, a black precipitate was produced, indicating the production of H_2S. Based on these results, K2 was identified as a strain of *Selenomonas ruminantium* subspecies *ruminantium* . Further confirmation of the identity of K2, at least to the species level, was provided by DNA-DNA hybridisation with the previously characterised *S. ruminantium* strain S23 (Zhang *et al*, 1991). When sheared, genomic DNA from K2 was radiolabelled and hybridised to sheared, genomic DNA from S23, the mean DNA

homology from triplicate hybridisations was found to be 75%. In contrast, homology to control DNA from *Propionibacterium acnes* (a known propionate producer) and *Streptococcus bovis* was less than 1%. K2 was also independently identified as *Selenomonas ruminantium* by the Clinical Microbiology Department of the Institute of Medical and Veterinary Science in Adelaide (Table 3.2) using the API bacterial identification system.

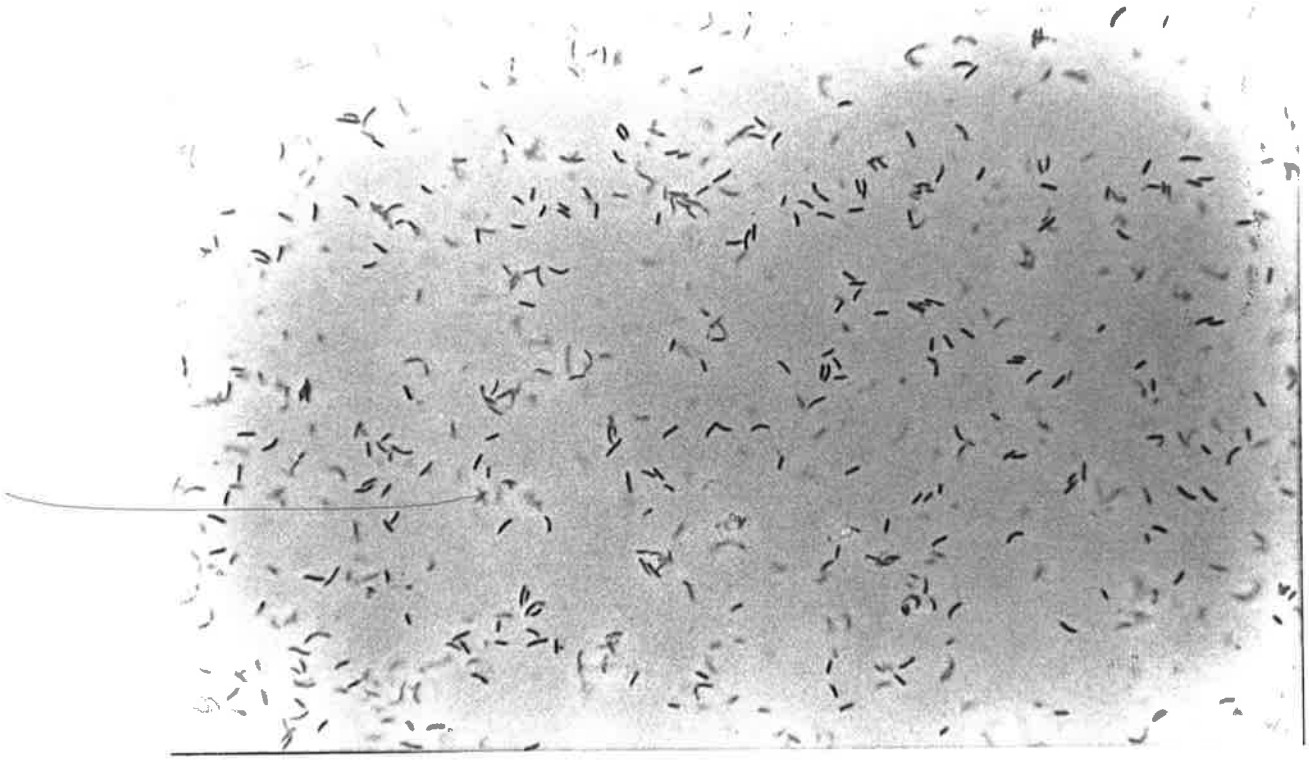


Figure 3.1 Photomicrograph of isolate K2 grown in mBHI broth medium. (400x magnification, phase contrast)

Table 3.1 Fermentation of carbohydrates by isolate K2

Substrate[1]	Growth
arabinose	+
cellobiose	+
dextrin	-
fructose	+
galactose	+
glucose	+
glycerol	-
lactate	-
lactose	+
maltose	+
mannitol	+
mannose	+
raffinose	+
rhamnose	-
ribose	+
starch	+
sorbitol	+
sucrose	+
trehalose	-
xylose	-

1. Fermentation tests were conducted in NB medium supplemented with the test substrate to a final concentration of 0.5% w/v as described in Chapter 2.

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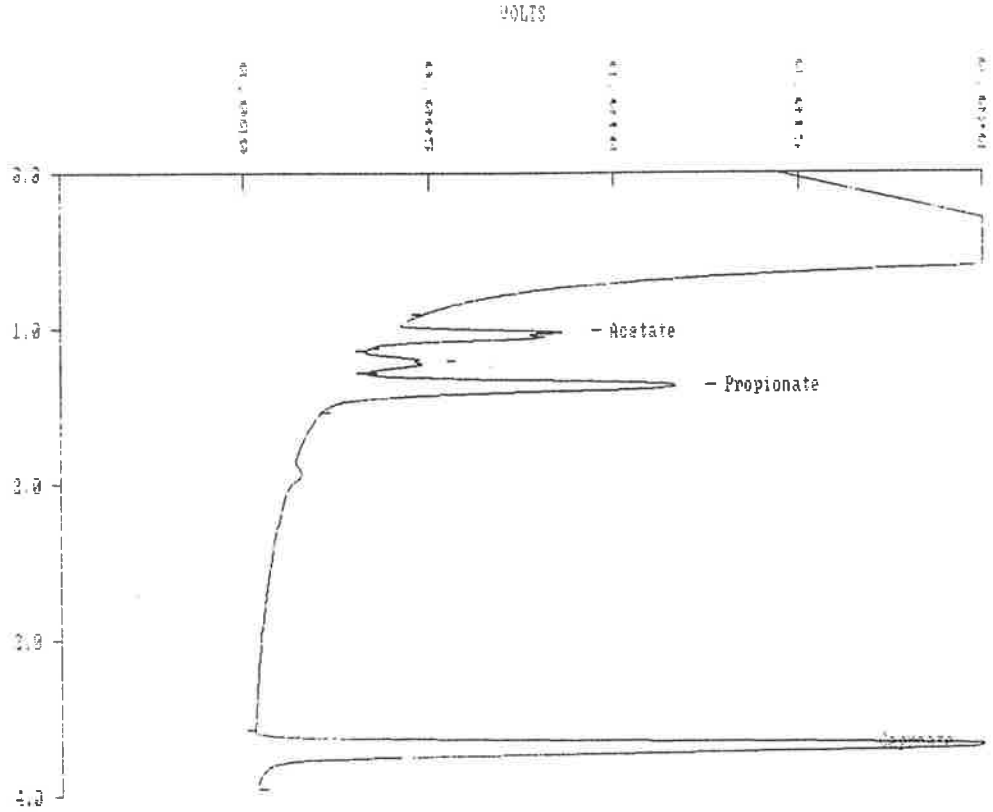
Figure 3.2 Gas chromatography of Volatile Fatty Acids (VFAs) produced by isolate K2 grown on glucose.



PEAK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCENT
1	1	Acetate	1.0	4.2	4.66	18.76	10.3
2	2		1.2	4.7	1.72	8.19	4.5
3	3	Propionate	1.4	5.0	9.09	49.30	27.0
4	-	Caproate	3.7	4.8	20.84	106.57	58.3
					36.32	182.82	100.0

Table 3.2 Characterisation of *S.ruminantium* K2 by the IMVS

Test	Result
Gram Stain	Gram negative bacillus, medium sized, curved
Growth	Strict anaerobe
Oxidase	Negative
Catalase	Negative
Motility	Positive
Haemolysis	β on sheep blood agar
Indole	Negative
Bile Growth	Positive
Esculin hydrolysis	Positive
Resistant to:	Vancomycin (5μg)
	Colistin Sulphate (5μg)
	Kanamycin (1000μg)
Nitrate	Negative
Gas Chromatography:	
Non Volatile	Major : Lactic
	Minor: Succinic, Malonic
Volatile	Major: Propionic, Acetic
	Minor: Butyric

3.3 Discussion

An enrichment-culture approach was adopted to select for organisms capable of growing in the presence of high levels of tannin for two reasons. Firstly, this allowed for the use of a liquid medium which was desirable because it best represents the environment available for microbial growth within the rumen. Secondly, since the relative population sizes of such bacteria within the rumen were unknown, an enrichment approach allowed the detection of bacteria that may have been present in very low numbers. M10 medium was chosen as the basis of the enrichment medium because this medium was originally designed as a general-purpose, non-selective medium for the enumeration of rumen bacteria and one which did not require the addition of sterile, clarified rumen fluid (Caldwell and Bryant, 1966). This medium was adapted for the experiment by leaving out the soluble carbohydrates glucose, cellobiose and starch and replacing these with a crude, condensed tannin extract prepared from the plants eaten by the feral goats which were the source of the rumen fluid.

Of the four morphologically-distinct bacteria resulting from the enrichment experiment, the chains of cocci were possibly the previously isolated and characterised *Streptococcus caprinus*, because they formed clear zones when plated on tannic acid-treated mBHI agar plates and they were the same size and shape. Such Gram positive, tannic acid-protein complex-clearing cocci have been isolated from a number of animal species that consume tannin-rich plants including feral goats and camels (Brooker *et al*, 1994), koalas, kangaroos and ring-tail possums (Osawa and Sly, 1992) and domestic goats (Nelson *et al*, 1995). The possible re-isolation of *Strep. caprinus* in this enrichment experiment suggests that firstly, the enrichment strategy worked, and secondly, confirms their positive selection in the presence of tannins.

Isolate K2 was chosen as the first to be identified and characterised merely because it was motile and had a distinct shape; features which were hoped would facilitate its identification. The other four isolates were stored for future work and were not characterised further. K2 was identified as a strain of *Selenomonas ruminantium* subspecies *ruminantium* on the basis of carbon source fermentation, VFA production, morphology and DNA homology. According to

Wayne *et al* (1987), bacteria are generally regarded as being of the same species if they exhibit DNA homology approximately ≥70%. *S. ruminantium* is a common ruminal inhabitant and is believed to be the major propionate producer in the rumen. Although not cellulolytic, *S. ruminantium* is able to ferment a large range of soluble sugars and is thus often present in large numbers in the rumen (Caldwell and Bryant, 1966). *S. ruminantium* subspecies *lactilytica* is believed to play an important role in lactate utilisation within in the rumen (Gilmour *et al*, 1996). The genetic diversity within *S. ruminantium* appears to be very great (Zhang *et al*, 1991), which may explain why this species has never previously been described in relation to tannin-resistance.

In conclusion, this chapter has described the isolation of an anaerobic bacterium from feral goat rumen fluid which is capable of growing in the presence of a high concentration of *Acacia* condensed tannin. This bacterium was identified as a strain of *Selenomonas ruminantium* subspecies *ruminantium*. Since this bacterium was isolated by an enrichment process, no conclusions can be drawn as to its population size in the feral goat rumen. Consequently, its significance in the rumen and its role, if any, in tannin bioremediation are unknown. Future experiments will aim to clarify these issues.

CHAPTER 4 Characterisation of *S. ruminantium* subsp. *ruminantium* strain K2

4.1 Introduction

In the previous chapter, experiments were described detailing the enrichment of bacteria from feral goat rumen fluid using condensed tannins. One bacterium was selected for further study and was identified as a strain of *S. ruminantium*. In this chapter, this isolate's ability to grow in medium containing tannins is examined in more detail.

The medium used for the isolation of K2 was not truly defined in the sense that it contained yeast extract and protein hydrolysate. Consequently, although no carbohydrates were added to this medium, it cannot be concluded that the organisms isolated were fermenting the condensed tannin. Therefore, in order to distinguish between tannin-resistance and actual tannin degradation, growth experiments were conducted using a defined medium with tannins as the only available energy source. The distinction between tannin-resistance and tannin biodegradation has important implications on the possible role this bacterium may play in the bioremediation of tannins in the feral goat. Tannin-resistance may indicate that K2 is another example of a bacterium able to function normally in the presence of tannins and therefore perhaps contributes to the goats' ability to thrive on a tannin-rich diet by providing a source of microbial protein. Alternatively, in addition to implying inherent tannin resistance, actual growth on tannins would provide evidence for the exciting possibility that some rumen bacteria are capable of degrading tannins and therefore contribute to the goats' ability to digest tannin-rich feeds by removing tannin from the rumen.

4.2 Results

4.2.1 Growth on tannic acid and *Acacia* condensed tannin crude extract

The defined medium of Nili and Brooker (1995) was used to test if K2 could grow on either tannic acid or the *Acacia* condensed tannin crude extract initially used in its isolation, as a sole

which represent the net change in bacterial numbers

source of fermentable carbon. The results (Figure 4.1) show that growth occurred on both substrates and in a dose-dependent manner.

Three additional commercial preparations of tannic acid were tested as substrates for growth of K2 (Table 4.1) The number of cells/ml media after 48 hours incubation was determined for each culture by direct cell counts using a haemocytometer and light microscope. A tube containing 1.0% glucose was used as a positive control. Once again, the results represent the net change in bacterial numbers.

Table 4.1 Growth of K2 in different batches of tannic acid

Substrate	cells/ml
Tannic acid (May and Baker, batch # G28151)	$8.9 \times 10^6 \pm 2.7 \times 10^6$
Tannic acid (BDH, batch # 249K18722511)	$4.0 \times 10^7 \pm 1.0 \times 10^7$
Tannic acid (Sigma, batch # 11F-0559)	$5.6 \times 10^6 \pm 1.4 \times 10^6$
Glucose	$8.5 \times 10^8 \pm 2.0 \times 10^8$

All substrates were used at 1.0% w/v as sole energy source in 6.0ml NB medium.

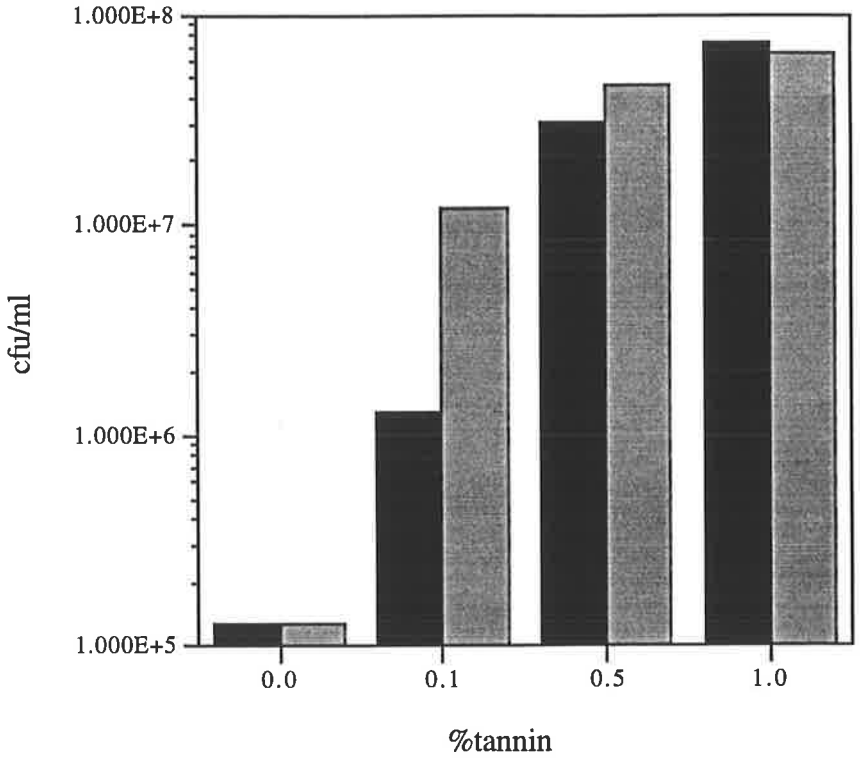


Figure 4.1 Growth of *S. ruminantium* K2 in defined medium containing tannins as sole energy source.

■ tannic acid

■ *Acacia* condensed tannin crude extract

4.2.2 Free glucose content of tannic acid

As described in Chapter 1, tannic acid is composed of various esters of gallic acid and glucose. Since commercial supplies of tannic acid are known to contain varying quantities of free ie. unesterified, gallic acid (Hagerman and Butler, 1989; and this chapter), it follows that they may also contain free glucose which may have contributed to the growth of K2 in defined medium containing tannic acid. Consequently, tannic acid was examined for the presence of free glucose.

Tannic acid was fractionated using paper chromatography and then tested for the presence of reducing sugars by spraying the chromatogram with aniline/phthalate reagent. There were two main reasons for this approach. Firstly, methods based on the enzymatic detection of glucose, such as the use of glucose oxidase, were not possible because tannic acid inactivates glucose oxidase by precipitation and also masks the spectrophotometric detection of the reaction products. Secondly, paper chromatography is a simple technique and the necessary materials and equipment were available in the laboratory.

In the first experiment, 4.0µl of a 20% solution of tannic acid was fractionated using a butanol:ethanol:water 4:2:2.2 solvent system (solvent B). In lanes 1 and 2 (Figure 4.2A) a broad trail of UV-absorbing phenolics is apparent, indicating the wide variety of phenolic esters present in tannic acid. Within the phenolic "trail" in each lane (1 and 2), three zones of higher UV-absorption are evident, at R_f 0.12, 0.47 and 0.67. This may indicate that this sample of tannic acid contains a predominance of perhaps 3 phenolics. When this chromatogram was sprayed with aniline/phthalate reagent and viewed under natural light (ie. white) to detect reducing sugars, only the lanes which contained control sugars produced characteristic coloured spots (lanes 2, 3 and 4). Hence, if the tannic acid in lane 1 contained any free glucose it was at a level undetectable by this test. The same conclusion was drawn when the sprayed chromatogram was viewed under UV light (Figure 4.2B). Under UV light, reducing sugars treated with aniline/phthalate fluoresce. Strong fluorescence was only observed in the control lanes 2, 3 and 4. Slight fluorescence may be visible in lane 1, level with the glucose controls

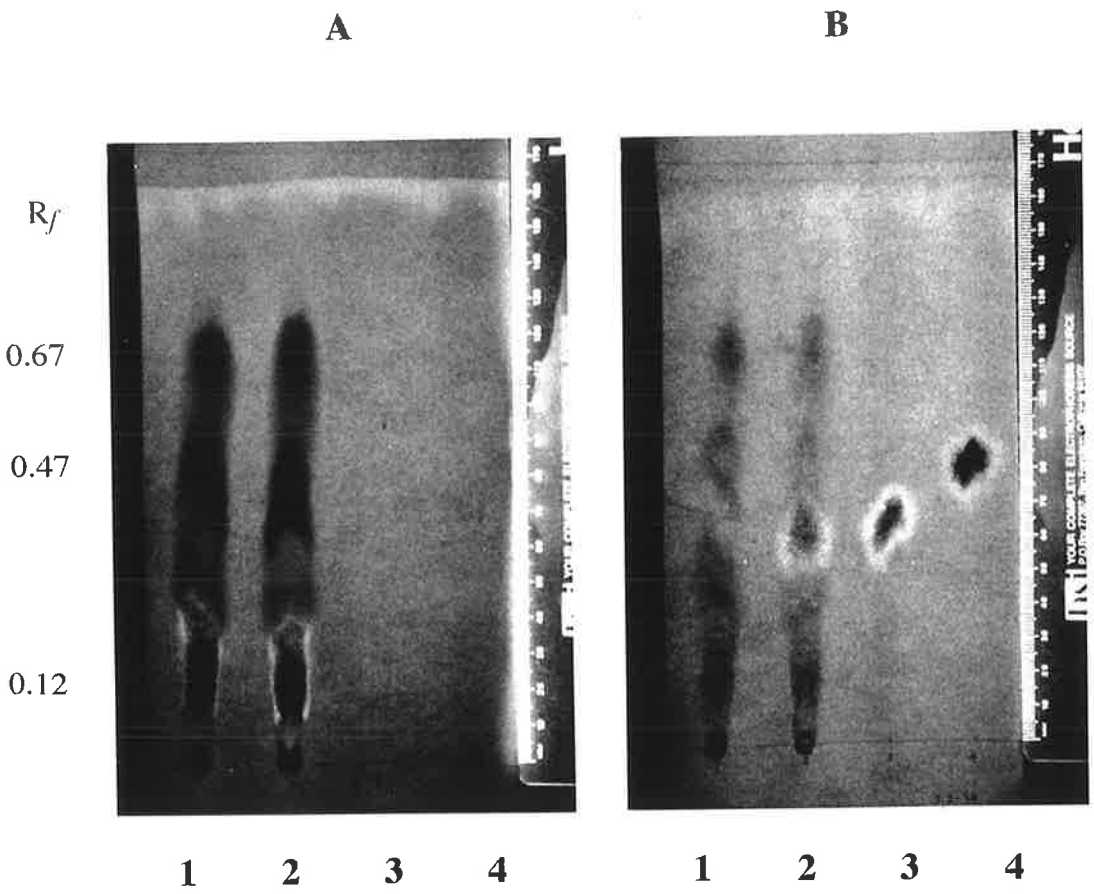


Figure 4.2 Paper chromatography of tannic acid developed with Solvent B.

A. Chromatogram photographed under UV light before spraying with aniline/phthalate.

B. Chromatogram photographed under UV light after spraying with aniline/phthalate.

Lane 1, 4.0µl of 20% tannic acid; lane 2, 4.0µl of 20% tannic acid + 4.0µl of 1% glucose standard; lane 3, 4.0µl of 1% glucose standard; lane 4, 4.0µl of 1% ribose standard.

(lanes 2 and 3), however it is not clear if this is actual fluorescence or a non-UV-absorbing zone adjacent to the intense UV-absorbing zone with R_f 0.47. There also appears to be no dark-coloured centre to this spot as shown by the controls which further suggests that it may not be indicative of glucose.

Due to restrictions in the amount of tannic acid that could be loaded onto a chromatogram, an attempt was made to increase the proportion of free glucose that may be in tannic acid by removing as much of the high molecular-weight esters as possible. Hopefully this would increase the detection limit by allowing more sample to be loaded. Consequently, a sample of tannic acid was extracted several times with gelatin in an attempt to remove the high molecular-weight esters by protein precipitation. Aliquots of this gelatin-extracted tannic acid were then fractionated by paper chromatography and sprayed with aniline/phthalate to detect reducing sugars (Figures 4.3 and 4.4). The paper chromatograms shown in Figures 4.3 and 4.4 differ only in the amount of gelatin-extracted tannic acid sample loaded. Two solvent systems were used for each experiment in order to vary the fractionation conditions. A general observation from Figures 4.3 and 4.4 is that there appears to be less UV-absorbing material present in this sample than in the un-modified tannic acid, especially near the origin line of the chromatograms. Since the equivalent of 2.5x and 5x the amount of tannic acid was loaded in Figures 4.3 and 4.4 respectively, compared to Figure 4.2, this result indicates that the gelatin extraction of tannic acid did indeed remove a substantial amount of phenolic esters. In Figure 4.3B, a faint fluorescent spot is visible in lane 5 level with the glucose control in lane 6. An equivalent spot is not evident in lane 1 (Figure 4.3A). In Figure 4.4 (A and B), a broad fluorescent trail is evident in lanes 1 and 5, possibly indicating the presence of reducing sugars in the gelatin-extracted tannic acid. It is not clear if these spots are due to free glucose because they appear as a continuous "trail" above and below the glucose standard. These spots may be due to gallic acid-glucose esters that retain a reducing aldehyde group on glucose carbon 6, i.e. mono-, di-, tri-, quaternary- galloyl glucose esters.

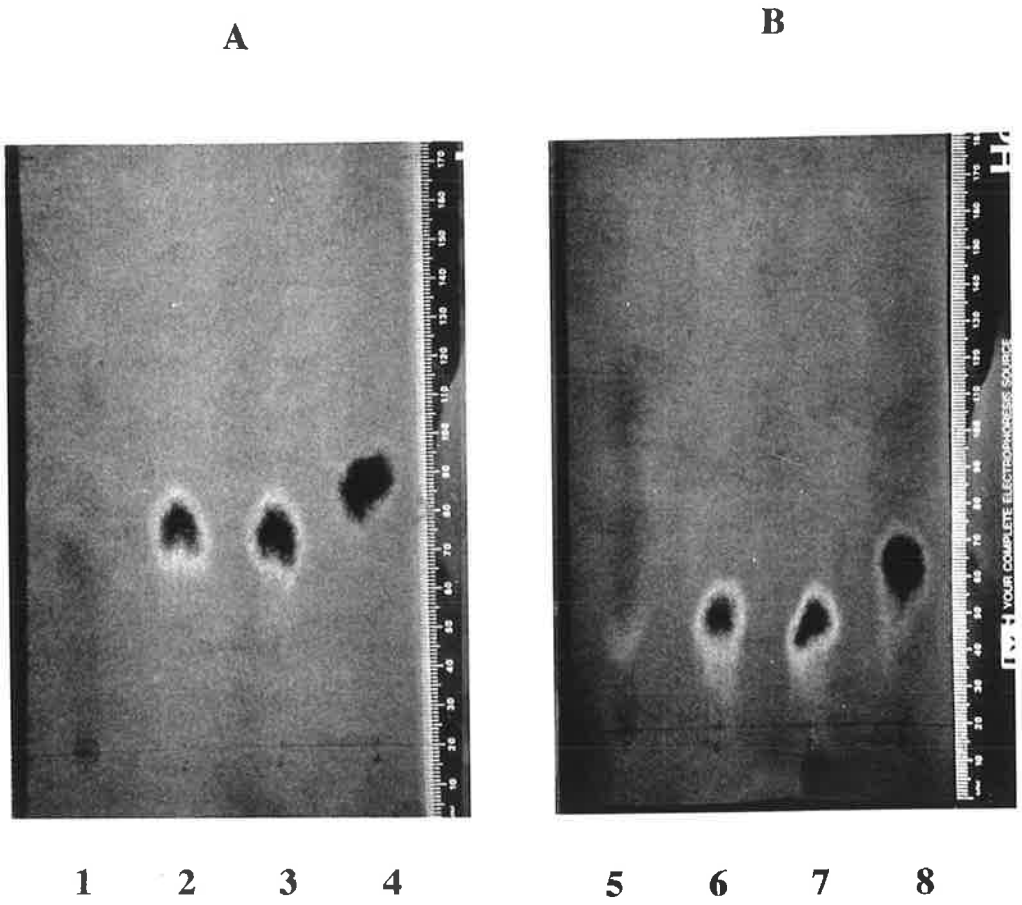


Figure 4.3 Paper chromatography of gelatin-extracted tannic acid.

A. Chromatogram developed with Solvent A and photographed under UV light after spraying with aniline/phthalate.

B. Chromatogram developed with Solvent B and photographed under UV light after spraying with aniline/phthalate.

Lanes 1 and 5, 2.0µl of gelatin-extracted tannic acid; lanes 2 and 6, 4.0µl of 1% glucose standard; lanes 3 and 7, 4.0µl of 1% galactose standard; lanes 4 and 8, 4.0µl of 1% ribose standard.

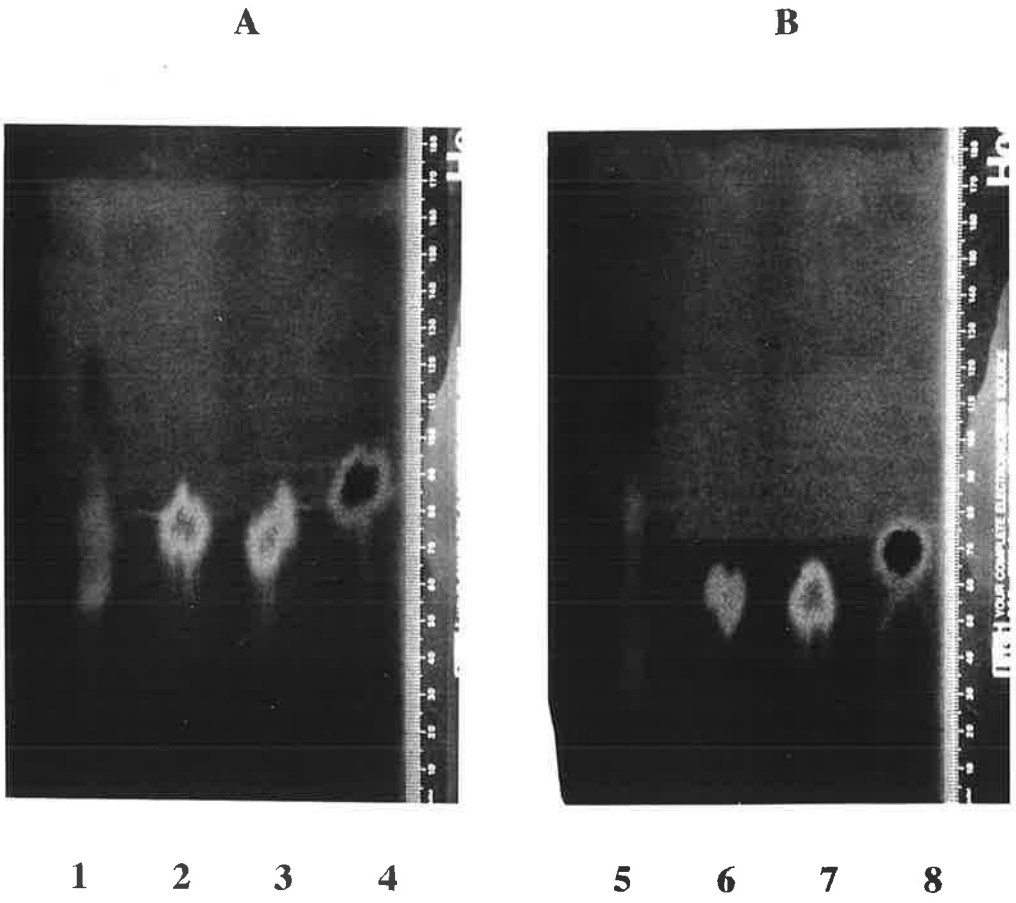


Figure 4.4 Paper chromatography of gelatin-extracted tannic acid.

A. Chromatogram developed with Solvent A and photographed under UV light after spraying with aniline/phthalate.

B. Chromatogram developed with Solvent B and photographed under UV light after spraying with aniline/phthalate.

Lanes 1 and 5, 4.0µl of gelatin-extracted tannic acid; lanes 2 and 6, 4.0µl of 1% glucose standard; lanes 3 and 7, 4.0µl of 1% galactose standard; lanes 4 and 8, 4.0µl of 1% ribose standard.

Based on the intensity of fluorescence of the spot in lane 1 Figure 4.3B, a rough estimate of the unesterified glucose content of tannic acid was made, assuming that this fluorescence was in fact due to glucose. Hence it was calculated that tannic acid may contain approximately 0.2% (w/w) unesterified glucose. By extension, when K2 was observed to grow in defined medium on 1.0% tannic acid as a sole energy source, this medium could have contained 0.002% free glucose. K2 was therefore tested for its ability to grow on such a low level of glucose as sole energy source in defined NB medium. No growth occurred at the levels 0.0%, 0.0005%, 0.002%, 0.004% and 0.01%; growth only occurred in the 0.5% control tube.

4.2.3 Growth on monophenolic compounds and purified *Acacia* condensed tannin

A variety of low-molecular weight phenolic compounds were tested for their ability to support growth of K2 in defined medium (Table 4.2), using the method described in Chapter 2 section 2.1.3. K2 was unable to ferment any of these compounds as a sole energy source, at the tested level of 0.5% w/v.

Table 4.2 Fermentation of monophenolics by K2

Substrate	Growth
benzoic acid	-
catechin	-
trans-cinnamic acid	-
ρ-coumaric acid	-
ferulic acid	-
gallic acid (3,4,5-trihydroxybenzoic acid)	-
gallic acid methyl ester	-
ρ-hydroxybenzoic acid	-
phloroglucinol (1,3,5-trihydroxybenzene)	-
vanillic acid	-

A small amount of pure condensed tannin was isolated from 50g of *Acacia* leaves (obtained from the same region of South Australia as the feral goat rumen fluid), by the method of Van Hoven and Furstenburg (1992) as detailed in Chapter 2 section 2.2.3. Chromatography on a Sephadex LH20 column removed all non-tannin phenolics from the plant extract and the high molecular-weight condensed tannins bound to the column. These were eluted from the column in 50% aqueous acetone and collected in one large fraction of approximately 70ml volume. The solvent was removed by freeze-drying, leaving approximately 250mg of rust-brown coloured tannin crystals. A sample of this pure condensed tannin was tested for its ability to support growth of K2. K2 was unable to grow on this pure condensed tannin as a sole energy source.

4.2.4 Release of gallic acid from tannic acid into growth medium during growth of *S. ruminantium* K2

Having established that K2 could not ferment gallic acid nor did it appear to be growing on unesterified glucose present in tannic acid, it became obvious that this organism must have been modifying the gallic acid-glucose esters in order for it to gain energy for growth. It was therefore hypothesised that K2 was breaking the ester bonds between gallic acid and the glucose moieties, thereby releasing glucose which could be used as a growth substrate. If this were true, it was predicted that an increase in the gallic acid concentration of the medium would be detected during growth of K2 on tannic acid. To this end, the rhodanine method of Inoue and Hagerman (1988) was adapted to measure free gallic acid in medium at various time intervals over the course of incubation.

In the first experiment (Figure 4.5), K2 was grown in Hungate tubes in 6.0ml defined NB medium containing 2.0% tannic acid as sole energy source. Two tubes were inoculated and two were left uninoculated to serve as controls. All four tubes were incubated at 39°C. There are several points to note in Figure 4.5. Firstly, it can be seen that at time 0, both the inoculated and the uninoculated tubes show a measurable level of gallic acid (approximately 0.8

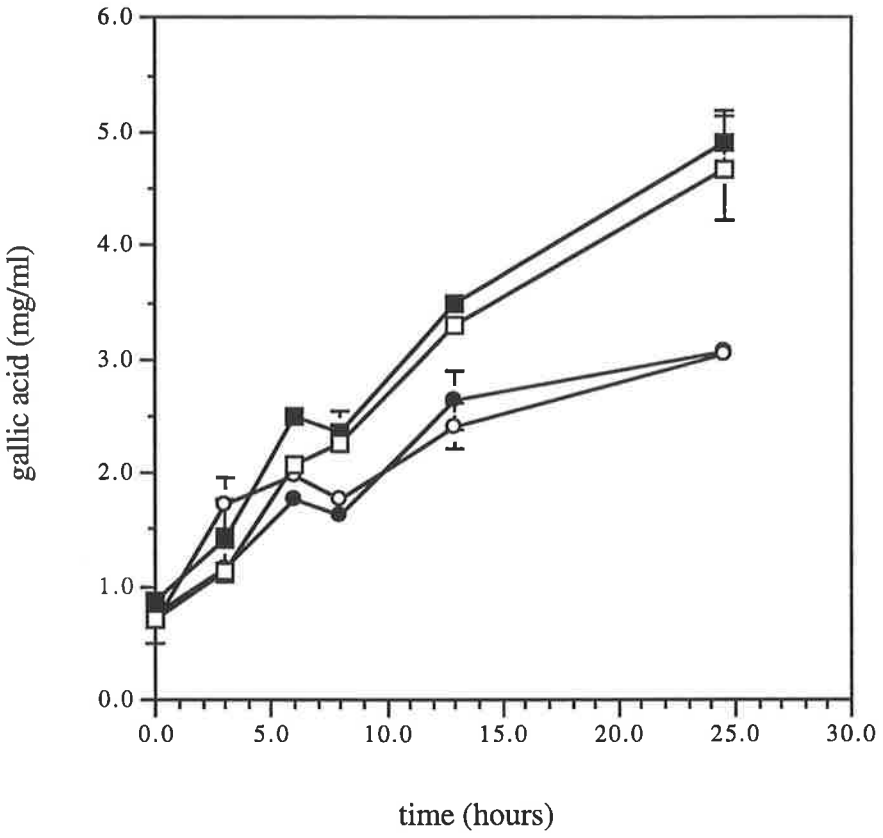


Figure 4.5 Release of gallic acid from tannic acid into medium during growth of K2 on 2.0% w/v tannic acid as sole energy source. (6.0ml culture volume) Values are means ± standard deviation of duplicate gallic acid measurements.

—□— Inoculated, replicate 1

—■— Inoculated, replicate 2

—○— Uninoculated control, replicate 1

—●— Uninoculated control, replicate 2

mg/ml or 4.7 mM). Since the rhodanine assay only detects free gallic acid, ie. esterified gallic acid does not react (Inoue and Hagerman, 1988), this result represents the background level of gallic acid present in this batch of tannic acid. Secondly, it is evident that there has been a release of gallic acid from tannic acid in the uninoculated control tubes. In these tubes, the gallic acid concentration has increased from approx. 0.8 mg/ml at the start of the incubation to approx. 3.0 mg/ml after 24.5 hours incubation. Finally, the level of gallic acid in the medium in the inoculated tubes was approx. 1.7-fold higher than the uninoculated controls at the end of the incubation period.

In the second experiment (Figure 4.6), K2 was grown in medium containing 1.0% tannic acid as sole energy source. The results are very similar to Figure 4.5 and show that gallic acid was released from tannic acid into the medium in both the inoculated and uninoculated treatments. In the uninoculated control tubes, the gallic acid concentration increased from approx. 0.7 mg/ml to approx. 1.5 mg/ml, over a 30 hour period. In contrast, in the inoculated tubes, the gallic acid concentration increased from approx. 0.7 mg/ml to approx. 2.6 mg/ml over the same time span. This is approximately the same relative release of gallic acid in the inoculated tubes compared to the uninoculated as was observed in the experiment illustrated in Figure 4.5, in which the cells were grown on twice the tannic acid concentration. In this experiment (Figure 4.6), the release of gallic acid from tannic acid appears to have reached its maximum after 25 hours incubation, in both the inoculated and uninoculated treatments. The tubes in Figure 4.5 were incubated for only 24.5 hours hence it is not known if a "plateau" effect would have been observed in this case.

In the third experiment, the effect of increasing the culture volume was examined. K2 was grown in crimp-top bottles containing 50ml media including 1.0% tannic acid as sole energy source. Growth of K2 was also determined by direct cell counts of aliquots removed from the culture at various time intervals. From the graph (Figure 4.7) it can be seen that once again there has been a release of gallic acid from tannic acid in both the inoculated and uninoculated treatments. In the uninoculated control, the gallic acid concentration appears to possibly reach a

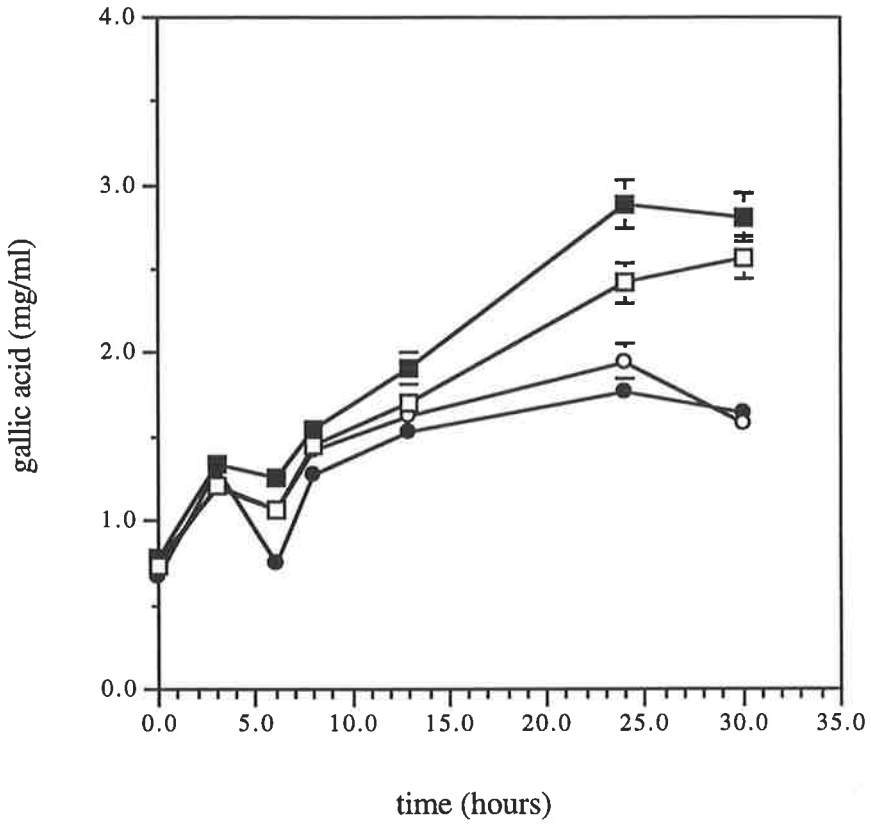


Figure 4.6 Release of gallic acid from tannic acid into medium during growth of K2 on 1.0% w/v tannic acid as sole energy source. (6.0ml culture volume) Values are means ± standard deviation of duplicate gallic acid measurements.

—□— Inoculated, replicate 1

—■— Inoculated, replicate 2

—○— Uninoculated control, replicate 1

—●— Uninoculated control, replicate 2

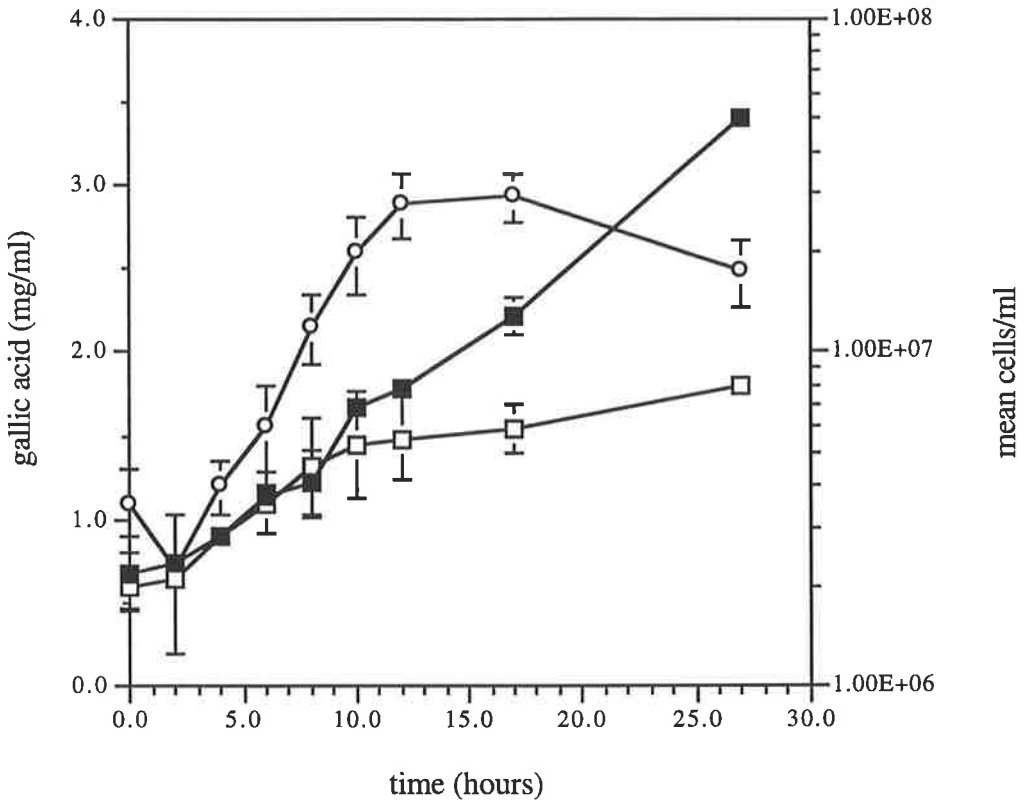


Figure 4.7 Release of gallic acid from tannic acid into medium during growth of K2 on 1.0% w/v tannic acid as sole energy source. (50.0ml culture volume) Values are means ± standard deviation of duplicate gallic acid measurements.

—■— gallic acid (mg/ml) inoculated

—□— gallic acid (mg/ml) uninoculated

—○— *S. ruminantium* K2 (cells/ml)

plateau at approx. 1.8 mg/ml after 27 hours incubation. In contrast, the gallic acid concentration in the inoculated culture appears to be still increasing at 27 hours and has reached a level of approx. 3.4 mg/ml. The apparent spontaneous release of gallic acid from tannic in uninoculated media and the relative difference of gallic acid release between the inoculated and the uninoculated treatments are consistent with the two previous experiments.

For the first time in this experiment, growth of K2 was determined at each sampling time. In Figure 4.7, it can be seen that K2 grew to a cell density of approx. 3 x 10^7 cells/ml and this growth occurred in the first 12 hours of incubation. This level of growth on tannic acid as the sole energy source is consistent with all previous results.

4.2.5 Growth on agar media containing tannic acid

Strep. caprinus , a previously characterised tannin-resistant bacterium (Brooker *et al*, 1994) isolated from the rumen of feral goats browsing tannin-rich *Acacia* , has been shown to produce zones of clearing surrounding colonies grown on mBHI agar medium containing 0.5% w/v tannic acid. When *S. ruminantium* strain K2 was plated onto mBHI/0.5% tannic acid agar, no growth occurred. This result was in direct conflict with experiments whereby K2 was readily cultured in mBHI/0.5% tannic acid broth. Consequently, an experiment was set-up to further investigate this apparent inhibition of growth on solid media containing tannic acid.

Anaerobic mBHI agar plates containing different concentrations of tannic acid were prepared as described in Chapter 2. Plates were inoculated with a suspension of cells and incubated overnight at 39°C in a Coy anaerobic hood. Growth was assessed after 24 hours incubation. The results (Table 4.3) indicate inhibition of growth of K2 at all levels of tannic acid tested.

Table 4.3 Growth of *S. ruminantium* K2 on solid media containing tannic acid

	% tannic acid								
Bacterial strain	0	0.25	0.5	1.0	1.5	2.0	2.5	3.0	3.5
Streptococcus caprinus 2.2	+	+	+	+	+	-	-	-	-
Selenomonas ruminantium K2	+	-	-	-	-	-	-	-	-

\+ growth observed

\- no growth observed

4.3 Discussion

This is the first report of an anaerobic bacterium capable of growing on a hydrolysable tannin as a sole energy source. To my knowledge, Lewis and Starkey (1969) provide the only other unequivocal report of bacterial growth on a hydrolysable tannin; in this case aerobic growth by a member of the genus *Achromobacter*. One of the problems associated with this field of study is the lack of availability of pure tannins, both hydrolysable and condensed. Tannic acid, a hydrolysable tannin, is the only commercially-available tannin. This means that many authors are forced to use crude preparations of tannins which makes the interpretation of results difficult. Hence although growth of K2 did occur from the *Acacia* condensed tannin crude extract, because of its crude nature it was decided to concentrate all subsequent studies on tannic acid alone. In addition, tannic acid was chosen because its structure has been well characterised. The fact that K2 could not utilise pure *Acacia* condensed tannin as a sole energy source validates this decision, and suggests that non-tannin components of the crude extract were responsible for the observed growth.

The accumulation of gallic acid in the growth medium during growth of *S. ruminantium* K2 on tannic acid as the sole energy source suggests that the gallic acid-glucose ester bonds were being hydrolysed. Since K2 was unable to ferment gallic acid or any other phenolic monomers, and there did not appear to be sufficient free glucose present in tannic acid to provide growth, K2 was probably deriving its carbon from the glucose released upon hydrolysis. No author has previously quantified bacterial growth on a hydrolysable tannin hence the results presented here cannot be compared directly. However, it is evident that growth on tannic acid, at least at the highest level tested (1.0%), is not optimal since markedly higher cell densities are observed when glucose is the sole energy source. This result may suggest that only a subset of the gallic acid-glucose esters are hydrolysed by the bacterium, or it may simply reflect the relatively low concentration of esterified glucose present in a sample of tannic acid. It has been estimated by Salunkhe *et al* (1989) that tannic acid contains on average, one glucose molecule esterified to 9-10 gallic acid residues. Hence when tannic acid was included in media as the sole energy source at the level of 1.0%, if this was completely

hydrolysed by K2 it would only yield approximately 0.1% glucose. However, this estimation is complicated by the fact that commercial supplies of tannic acid vary in composition both between batches from the same manufacturer and between different manufacturers. Since the distribution of the different-sized esters varies from batch to batch of tannic acid, it is difficult to establish a generalised molar ratio of glucose to gallic acid. Hence in order to predict the theoretical cell yield from a given amount of tannic acid, this ratio would need to be empirically determined for each batch.

From the results presented in this chapter, it is evident that tannic acid is unstable in solution, even under anaerobic conditions and neutral pH. This result agrees with the findings of Osawa and Walsh (1993). These authors showed that a 1.0% solution of tannic acid (pH 6.5) contained 5.5 mM free gallic acid and that when this solution was incubated anaerobically for a period of three days, a further 12 mM gallic acid was released. In the experiments presented here, a 1.0% tannic acid solution contained 4.1 mM free gallic acid and yielded a further 6.5 mM gallic acid after approximately 24 hours incubation.

The growth of K2 occurred in the first twelve hours of incubation and yet during this period there was little difference in gallic acid concentration in this culture compared to the uninoculated control. These facts, combined with the apparent instability of tannic acid in solution, may tempt one to suggest that the growth observed was simply a consequence of the non-specific hydrolysis of tannic acid. However, a number of points argue against this scenario. Firstly, if this were so, one would not expect the gallic acid concentration to differ between the inoculated and uninoculated treatments at any stage of the incubation. Acid hydrolysis of tannic acid caused by a pH decrease arising from VFA production could not explain the observed difference because evidence suggests that very little hydrolysis of tannic acid occurs anaerobically below pH 5 (Osawa and Walsh, 1993). Secondly, if the growth was due to non-specific hydrolysis, any glucose-fermenting, tannin-resistant bacterium should produce the same growth result in medium containing tannic acid as the sole energy source. This was not the case when the tannin-resistant bacterium *Streptococcus caprinus* 2.2 was

tested. Finally, the increase in gallic acid concentration in the uninoculated media does not necessarily mean that completely-unesterified glucose was made available, because gallic acid could be released from depside as well as ester bonds. It is not known if particular ester or depside bonds are more readily hydrolysed than others and hence further work needs to be conducted to address this question.

Several authors have isolated tannin-resistant microorganisms by plating rumen or faecal samples onto rich media treated with tannic acid (Brooker *et al*, 1994; Osawa, 1990 and Bhat *et al,* 1996) Two types of organisms are detected by this approach: those that merely grow on the plates and those that grow and produce clear zones in the turbid tannic acid-protein complexed media. The significance of these clear zones in relation to tannin-resistance is unknown however some authors have claimed they represent enzymatic hydrolysis of the tannin-protein complexes. No experimental evidence supports this claim. *S. ruminantium* K2 grew readily in rich broth medium (mBHI) containing 0.5% tannic acid yet was incapable of growing on the same medium incorporating 1.5% agar. This result is difficult to explain and may suggest that there is a fundamental difference in the toxicity of tannic acid (or a component thereof) when in solution compared to when incorporated in solid media. Alternatively, the growth inhibition may be an example of nutrient-deprivation caused by the tannin-protein precipitate contained in the agar medium. When tannic acid is added to broth media, a tannin-protein precipitate forms immediately and settles to the bottom of the culture tube within a matter of seconds, leaving a less-turbid supernatant. However, when tannic acid is incorporated into media containing agar, the medium is mixed immediately prior to pouring into petri dishes and consequently the tannin-protein precipitate becomes evenly distributed throughout the solidified medium. It is therefore possible that essential nutrients are "trapped" within the medium and consequently K2 is unable to grow. This issue could perhaps be answered by testing for growth on plates containing tannic acid and varying agar concentrations, to determine if growth can occur at low agar concentrations which would result in a less dense matrix.

Conclusion

In this chapter, *S. ruminantium* K2 was shown to grow on a hydrolysable tannin (tannic acid) as a sole energy source. During this growth, gallic acid accumulated in the growth medium, indicating that the organism was obtaining its energy from glucose released from the hydrolysis of gallic acid-glucose esters.

CHAPTER 5. Enzyme characterisation.

5.1 Introduction

In the previous chapter it was shown that *S. ruminantium* K2 was capable of growing on a hydrolysable tannin as a sole energy source and evidence was provided that this was achieved via the hydrolysis of gallic acid-glucose esters. The cleavage of gallic acid-glucose ester bonds is a reaction known to be catalysed by an enzyme called a tannin acylhydrolase (EC 3.1.1.20). This enzyme has previously only been described in aerobic fungi and yeast of the ~~genii~~ genera *Aspergillus* and *Penicillium* (Knudson, 1913 as cited by Scalbert, 1991), *Candida* (Aoki *et al*, 1976) *Fusarium* and *Trichoderma*. (Bajpai and Patil, 1996). Fungal and yeast tannin acylhydrolases are secreted and catalyse the hydrolysis of ester bonds between a phenolic acid and an alcohol. As described in Chapter 1, the nature of the alcohol group in the ester has little influence on activity, and may be methanol, glucose, gluconic acid, gallic acid, quinic acid and other related alicyclic compounds, flavanols or a terpene. The carboxylic acid however must be benzylic and must belong to a phenolic acid bearing two hydroxyl groups in *ortho* position such as in gallic or protocatechuic acid (Scalbert, 1991). These fungi are able to grow on hydrolysable tannins such as tannic acid as sole energy source because the expression of tannin acylhydrolase results in the depolymerisation of the tannin and the subsequent release of the component carbohydrate and phenolic acids. As stated in Chapter 1, the gallic acid that is released may accumulate in the growth medium or it may be further degraded to carbon dioxide by enzymes other than the tannin acylhydrolase. Since the fungi degrade the phenolic ring of gallic acid aerobically, the fate of the gallic acid depends largely on the oxygen status of the medium.

The experiments described in this chapter were conducted to provide unequivocal evidence of the hydrolysis of tannic acid by the bacterium *S. ruminantium* K2 and to demonstrate the enzymatic basis by which this is achieved. In addition, these experiments were conducted to characterise this novel enzyme so that its properties could be compared to fungal tannin acylhydrolases and its role in K2 and significance to tannin detoxification could be determined.

5.2 Results

5.2.1 Enzymatic hydrolysis of tannic acid by a crude cell lysate.

The presence of tannin acylhydrolase activity in K2 was determined by measuring the hydrolysis of tannic acid by a crude cell lysate. Gallic acid was only detected when tannic acid was incubated with crude cell lysate (Figure 5.1). Control lysates that were boiled for 10 minutes before addition of tannic acid did not release gallic acid.

5.2.2 Development and optimisation of an enzyme assay.

Having demonstrated what appeared to be the enzymatic hydrolysis of tannic acid by *S. ruminantium* K2, an enzyme assay was developed which would allow characterisation of the enzyme responsible. This assay was based on the previously described rhodanine assay and measured the release of gallic acid from gallic acid methyl ester (GAME). Figure 5.2 shows the absorption spectra of the gallic acid-rhodanine and GAME-rhodanine complexes. At a wavelength of 520nm there was some absorbance due to the substrate however this was compensated for by blanking all assays on buffer + GAME.

In a preliminary cell-fractionation experiment, activity was detected in cell-free extracts and none was detected in concentrated spent growth medium. Hence all assays described here were carried out using cell-free extracts. In addition, the activity of the enzyme was measured in aerobic assays because no difference in the rate of hydrolysis of GAME was detected under either anaerobic or aerobic conditions (data not shown).

In cell-free extracts prepared from K2 cells grown in medium containing 0.2% w/v tannic acid, gallic acid release from GAME was linear with time for at least 30 minutes (Figure 5.3) and with protein up to an assay concentration of at least 70 μg/ml (Figure 5.4). In order to determine whether the rhodanine assay was influenced by pH, gallic acid standard curves were

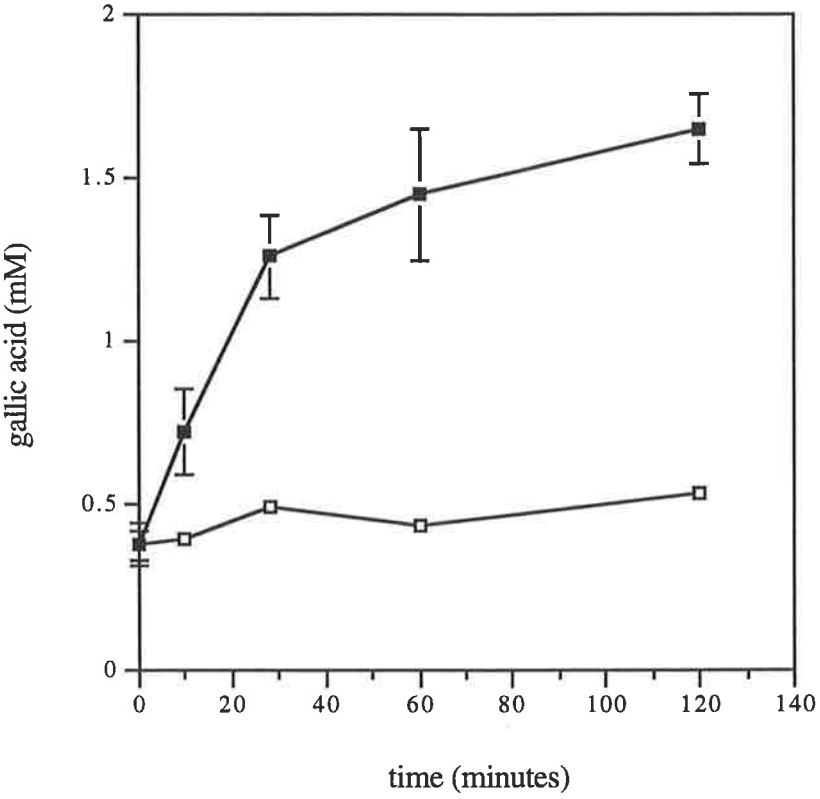


Figure 5.1 Enzymatic hydrolysis of tannic acid by a crude cell lysate of *S. ruminantium* K2. (Values are means ± standard deviation of triplicate gallic acid measurements).

crude cell lysate

boiled crude cell lysate

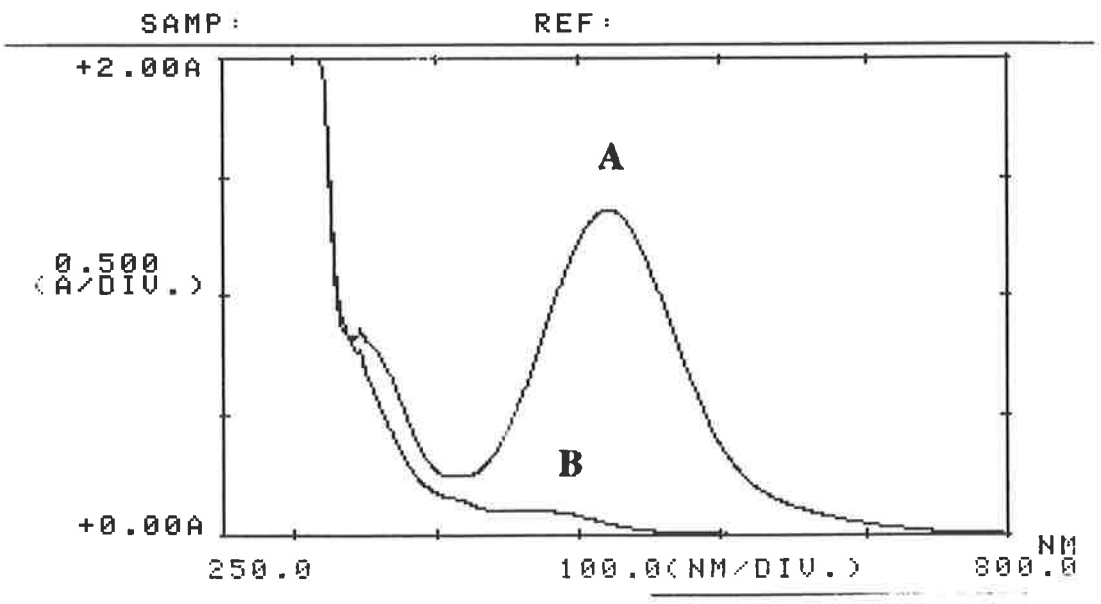


Figure 5.2 Absorption spectra of gallic acid-rhodanine (A) and GAME-rhodanine (B).

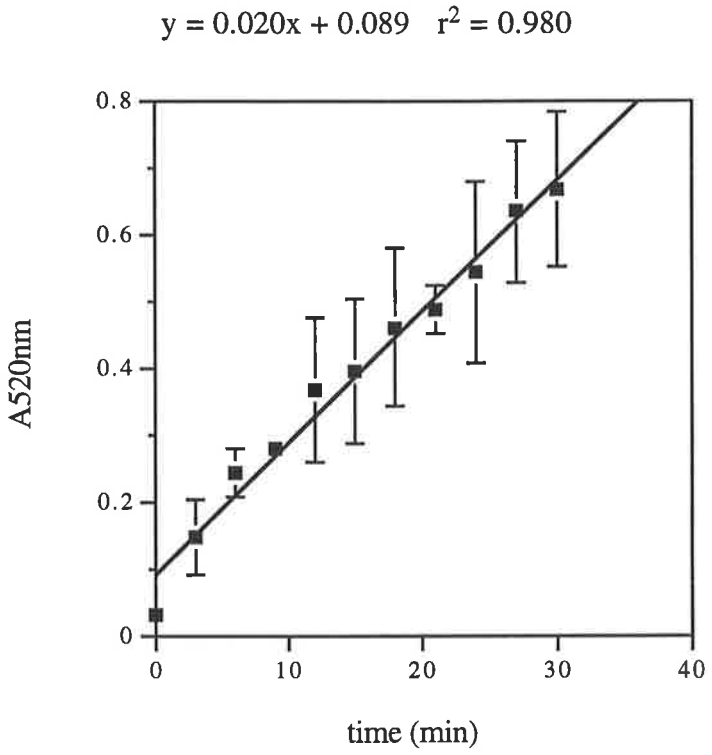


Figure 5.3 Time course of enzyme reaction.

The reaction mix contained 1.1 mM GAME and 0.09 mg/ml protein. (Values are means ± standard deviation of duplicate samplings).

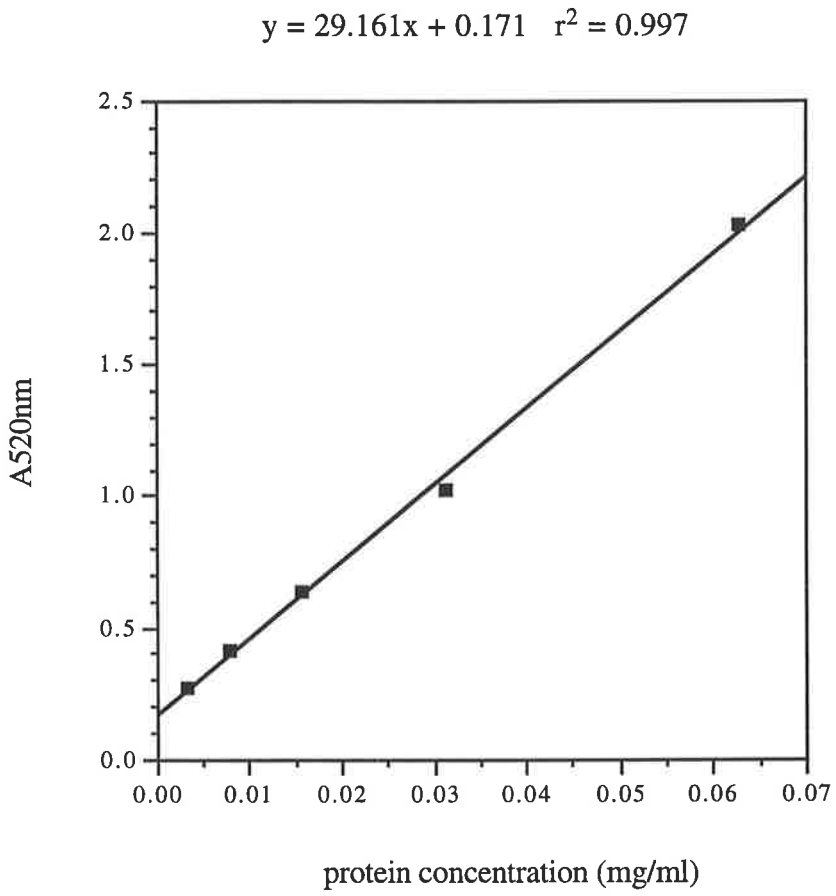


Figure 5.4 Relationship between assay protein concentration and gallic acid production.

The reaction mixes contained 10.9 mM GAME and were incubated at 37°C for 5 minutes.

prepared for each pH buffer. The regression lines fitted to these data are described in Table 5.1 and show that there is little influence of pH on A_{520} values. A standard curve illustrating the linear relationship between the concentration of gallic acid and the absorbance of gallic acid-rhodanine complex at 520nm is presented in Figure 5.5. The effect of pH and temperature on the rate of GAME hydrolysis by cell-free extracts were then determined. As can be seen from Figures 5.6 and 5.7, the enzyme exhibited a pH optimum of 7 and an optimum assay temperature of between 30 and 40°C.

Table 5.1 Linear regression equations of gallic acid standards prepared in different pH buffers

pH 5.8	y = 1.096x - 0.021	$r^2 = 0.998$
pH 6.2	y = 1.087x - 0.007	$r^2 = 0.992$
pH 6.6	y = 1.098x + 0.0.018	$r^2 = 0.997$
pH 7.0	y = 1.059x - 0.051	$r^2 = 0.995$
pH 8.0	y = 1.071x - 0.048	$r^2 = 0.994$

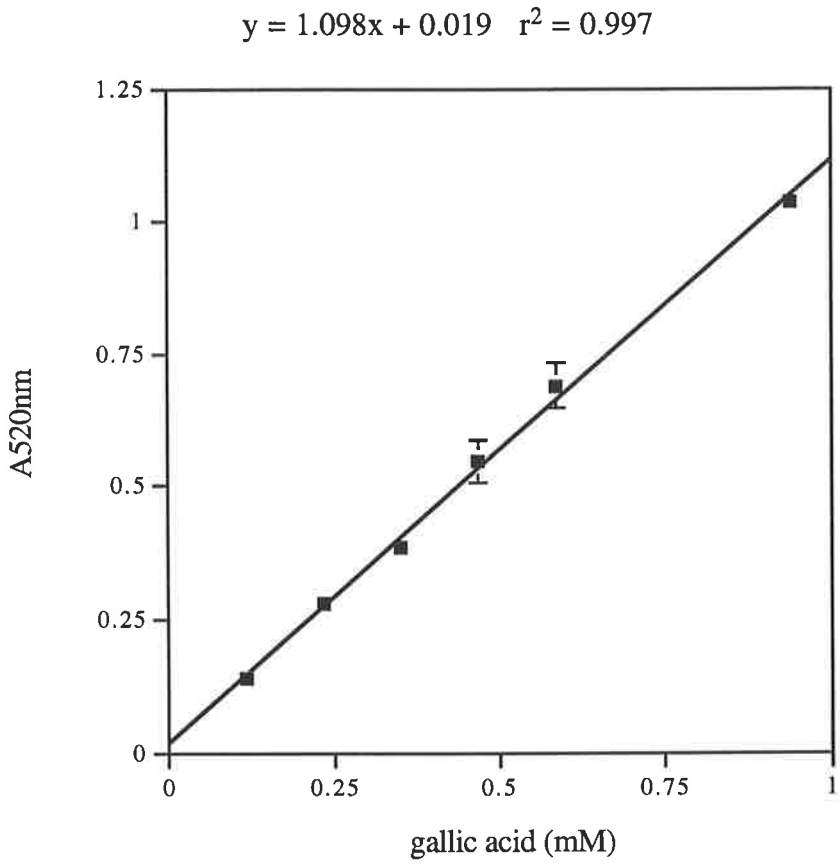


Figure 5.5 Relationship between gallic acid concentration and absorbance of gallic acid-rhodanine complex at 520nm. (Values are means ± standard deviation of triplicate gallic acid measurements).

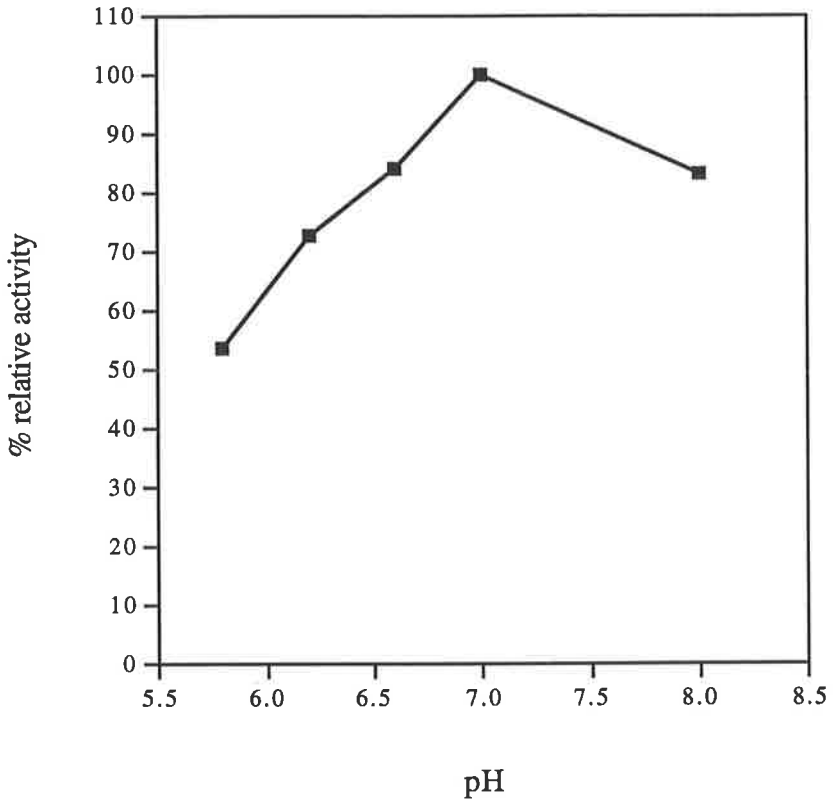


Figure 5.6 pH optimum of K2 tannin acylhydrolase.

The activity at each pH value is expressed as a percentage of the activity at pH 7.0.

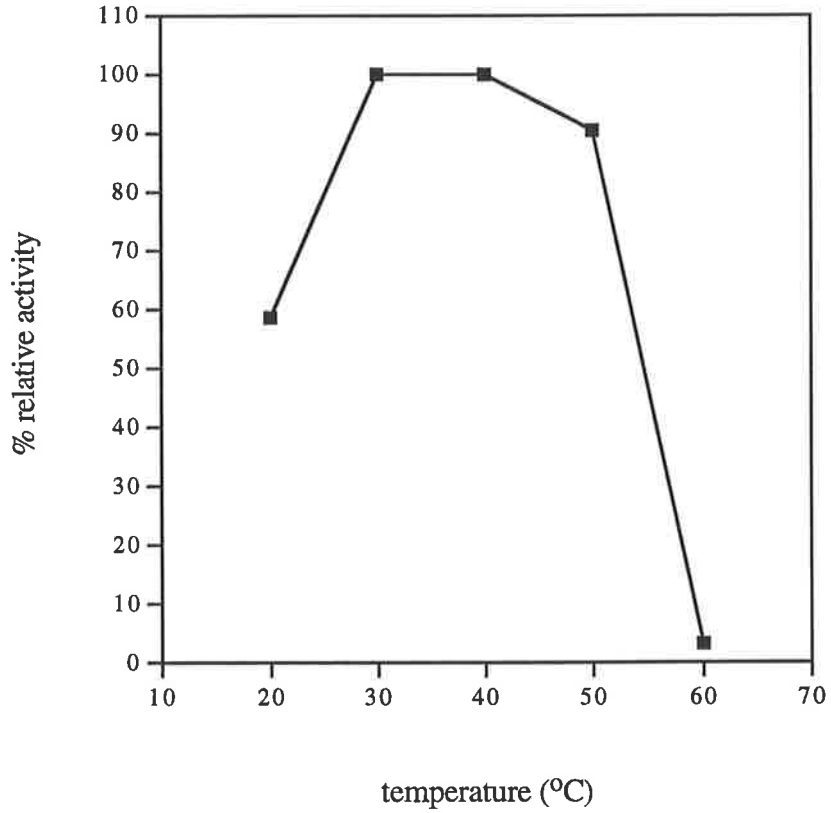


Figure 5.7 Temperature optimum of K2 tannin acylhydrolase.

The activity at each temperature is expressed as a percentage of the activity at 30°C.

5.2.3 Determination of K_m and V_{max}.

Using the optimum assay conditions, the K_m and V_{max} for tannin acylhydrolase were determined using the following procedure. The relationship between substrate concentration and initial rate of reaction was determined (Figure 5.8) and showed that with increasing substrate (GAME) concentration, the initial rate of reaction increased up to a certain concentration, beyond which there was no further increase in rate. The slopes of the lines in Figure 5.8 provided the initial rates of reaction (V, µmoles gallic acid produced/min/mg protein). The reciprocal of the initial rate of reaction (1/V) was plotted against the reciprocal of the substrate concentration (1/S) to give a Lineweaver-Burk plot (Figure 5.9). The K_m and V_{max} were calculated from this plot as follows:

Lineweaver-Burk plot has equation $y = 0.182x + 159816.052$

$$\mathbf{V_{max}} = \frac{1}{\text{y intercept}}$$

$$= \frac{1}{159816.052} = \mathbf{6.3 \pm 0.5\ \mu moles\ gallic\ acid\ produced/min/mg\ protein}$$

and $\mathbf{K_m} = V_{max} \times \text{slope}$

$= 6.3 \times 0.182$

$= 1.15$ µmoles GAME/ 700µl reaction

$= 1.6 \times 10^{-3}$ moles GAME/ litre

$= \mathbf{1.6 \pm 0.2\ mM\ GAME}$

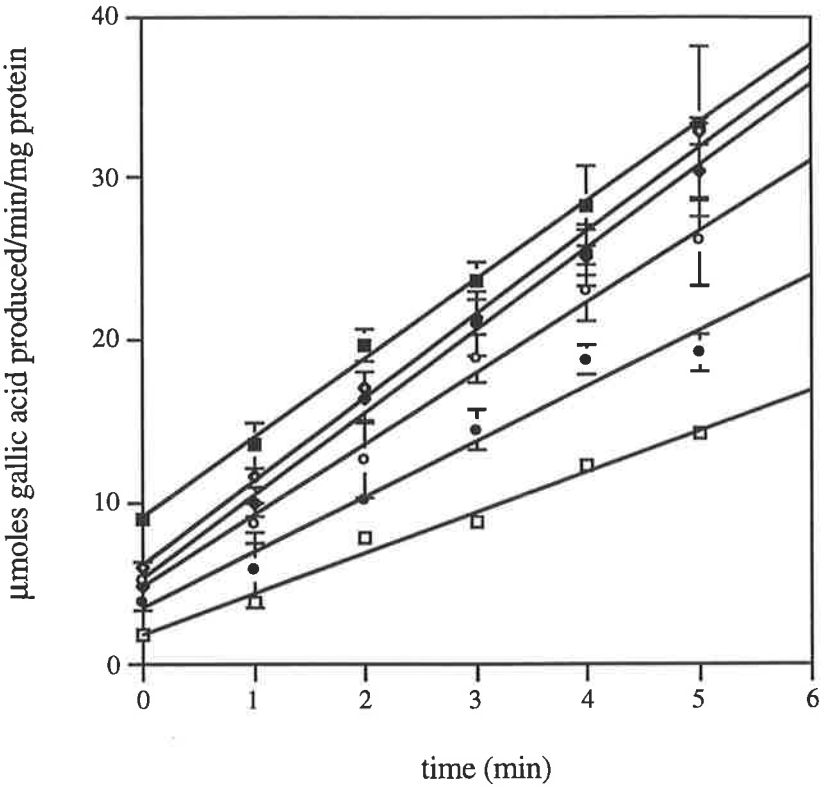


Figure 5.8 Time course of reaction with increasing substrate (GAME) concentration.

- ▫ 1.1 mM
- • 2.2 mM
- ○ 3.3 mM
- ◆ 4.4 mM
- ◇ 5.4 mM
- ■ 10.9 mM

All reactions were incubated at 37°C and contained 0.022 mg/ml protein in a total volume of 700µl. Incubations were conducted in duplicate for each substrate concentration; plotted values are the mean ± standard deviation.

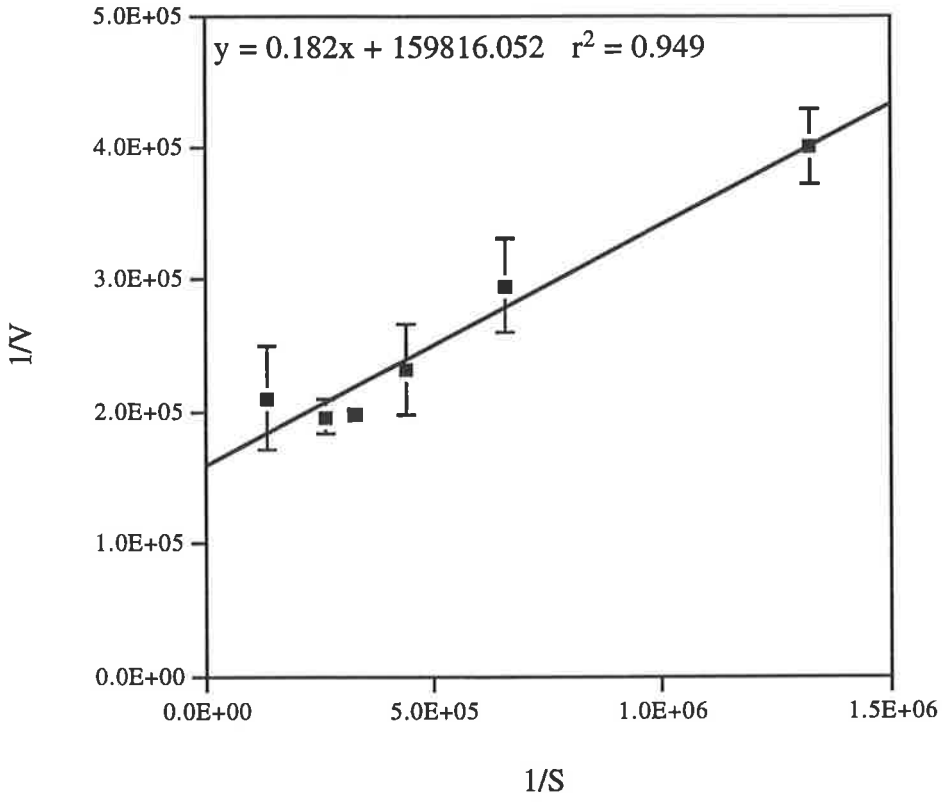


Figure 5.9 Lineweaver-Burk plot.

5.2.4 Substrate specificity

In order to determine if this enzyme was a tannin acylhydrolase or a non-specific esterase, cell-free extracts were tested for the ability to hydrolyse a range of esters. Cell-free extracts were able to hydrolyse GAME and gallic acid lauryl ester but were unable to hydrolyse α-napthyl acetate, ρ-nitrophenyl acetate, methylumbelliferyl acetate or glyceryl tributyrate (tributyrin). In addition, the enzyme displayed 83 and 81% residual activity when preincubated with 1 and 5 mM phenylmethylsulphonylfluoride (PMSF), respectively.

5.3 Discussion

The hydrolysis of a tannic acid solution by the crude cell extract over a 2 hour period is indicative of a tannin acylhydrolase (EC 3.1.1.20). As noted in the introduction to this chapter, such activity has previously been described in aerobic fungal and yeast systems. This is the first report of tannin acylhydrolase-like activity in an anaerobic bacterium and may be the first experimental evidence of such activity in any bacterium. The significance of tannin acylhydrolase and its distribution amongst rumen bacteria will be discussed further in Chapters 7 and 9.

In order to be able to characterise the enzyme responsible for the hydrolysis of tannic acid, an enzyme assay needed to be developed. The first requirement of this assay was a defined substrate since tannic acid was not suitable because of its variable composition. Gallic acid methyl ester was chosen as a defined substrate because it is readily available, chemically pure and is the simplest phenolic ester known to be hydrolysed by previously characterised tannin acylhydrolases.

Several tannin acylhydrolase assays have been previously described and used to characterise the fungal and yeast enzymes. These methods have either been based on autotitration of the acid liberated from tannic acid or spectrophotometric detection of small differences in UV absorption of gallic acid and GAME or tannic acid. However, all these methods have shortfalls. Autotitration requires specialist equipment and has been shown to be considerably inaccurate (Gutfreund, 1965). Spectroscopic methods relying on the detection of differences in UV absorption between product and substrate have also proved to be unreliable, mainly because the absorption differences are so small. Haslam and Tanner (1970) took these deficiencies into consideration and developed a new assay based on the spectrophotometric detection of ρ-nitrophenol resulting from the hydrolysis of ρ-nitrophenol esters of gallic acid and 3-*O*-methylgallic acid. Unfortunately however, these substrates are not commercially available and hence need to be specially synthesised. Since the rhodanine procedure of Inoue and Hagerman (1988) had already been found to provide an easy and accurate measure of gallic acid released

from tannic acid by both growing cells and by a crude cell lysate, it seemed logical to continue using this method to measure gallic acid liberated from GAME by cell-free extracts.

The pH and temperature optima of the K2 tannin acylhydrolase are consistent with an enzyme derived from a rumen bacterium that grows in an environment of neutral pH and 39°C temperature. Not surprisingly this pH optimum differs from that previously described for fungal and yeast enzymes. A crude preparation of *Aspergillus niger* tannin acylhydrolase exhibited a broad pH optimum of 4-5 and more detailed analysis revealed that this broad optimum was composed of two optima of pH 4.2 and pH 4.8 (Haslam and Tanner, 1970). These authors suggested that such results correspond to the pH optima of different galloyl esterases and supported previous work by Rogers *et al* (1961) who observed that purification of tannin acylhydrolase by ion-exchange chromatography resulted in the enzyme being eluted in four different fractions each with comparable specificities. A more detailed analysis of the K2 cell-free extracts would need to be conducted to determine whether they also exhibit multiple galloyl esterases of differing pH optima.

Iibuchi *et al* (1968) determined the pH optimum of *Aspergillus oryzae* tannin acylhydrolase to be 5.5 and in this case, multiple pH optima were not detected. Both species of *Aspergillus* tannin acylhydrolases exhibited broad temperature optima of 30-40°C, which is in agreement with my results. The only other tannin acylhydrolase to have been characterised is that of the yeast *Candida* sp. K-1 (Aoki *et al*, 1976). This enzyme exhibited a pH optimum of 6.0 and a temperature optimum of 50°C.

Haslam and Tanner (1970) are the only authors to have published K_m and V_{max} data for tannin acylhydrolase. These authors found for ρ-nitrophenyl gallate a K_m of 3.91×10^{-5} mol/l and a V_{max} of 21.1×10^{-5} mol/l/hr. For ρ-nitrophenyl-3-*O*-methylgallate, the K_m was 8.2×10^{-5} mol/l and the V_{max} was 16.9×10^{-5} mol/l/hr. Comparison with K2 tannin acylhydrolase is not possible because different substrates were used. In the future, it may be productive to

determine the K_m/V_{max} for ρ-nitrophenyl gallate esters using the Haslam and Tanner assay and purified K2 tannin acylhydrolase which would allow direct comparison with the fungal enzyme.

One of the questions raised by this work is that of classification of the K2 enzyme; specifically whether the enzyme is a true tannin acylhydrolase or a non-specific esterase. Esterases have been classified as either A-esterases (EC 3.1.1.2) or B-esterases (EC 3.1.1.1) based on their behaviour toward organophosphorus compounds such as diisopropylfluorophosphate (DIFP), although this grouping is ambiguous and the subject of constant debate and revision. In general, A-esterases are not inhibited by organophosphorus compounds and hydrolyse them as substrates (Krisch, 1971). A-esterases are also called arylesterases because they hydrolyse esters of aromatic alcohols and carboxylic acids, such as phenyl acetate and α-napthyl acetate. B-esterases (formerly known as non-specific esterases) belong to the group of serine hydrolases which includes esterases, proteases and lipases because they contain a serine residue at their active site. The hydrolytic mechanism of serine hydrolases generally involves a similar catalytic triad, consisting of a nucleophilic serine that acts in conjunction with a histidine and an aspartic acid residue (Tesch *et al*, 1996) Consequently, B-esterases are inhibited by compounds such as DIFP and PMSF which bind to the active serine residue irreversibly. Unfortunately, many B-esterases have also been shown to hydrolyse aromatic esters and hence the distinction between A- and B- esterases is misleading.

Currently, very little is known about esterase production by rumen bacteria, however esterase activity has been reported in *S. ruminantium* strains HD4 and D (Hespell and O'Bryan-Shah, 1988). These strains exhibited predominantly ρ-nitrophenyl acetate- and butyrate- esterase activity although low levels of α-napthyl acetate-, butyrate- and caprylate- esterase were also recorded. No activity towards these esters was shown by K2 cell-free extracts[1] . The K2 putative tannin acylhydrolase was not inhibited significantly by preincubation with PMSF; hence, it is concluded that a serine residue is not essential for catalysis. Previous evidence

[1] Although some methylumbelliferyl acetate esterase activity was measured, subsequent experiments demonstrated that a different enzyme from the K2 tannin acylhydrolase was responsible for this activity. The methodology and experimental evidence in support of this conclusion will be presented in the next chapter.

indicated that preincubating purified serine hydrolases with 1 mM PMSF resulted in a reduction of activity of at least 90% (Tesch *et al*, 1996; Choi *et al*, 1990; Krisch, 1971). These results suggest that the K2 enzyme may not be a non-specific esterase nor an arylesterase and perhaps may be specific for esters of gallic acid and alcohols.

The hydrolysis of gallic acid lauryl ester by cell-free extracts of K2 is interesting because this activity could be regarded as lipase activity. Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) preferentially act on emulsified substrates with long-chain fatty acids and as mentioned above, share the same hydrolytic mechanism as that of serine esterases and serine proteases. No kinetic assays were conducted using gallic acid lauryl ester so comment cannot be made on the relative rates of hydrolysis of this substrate versus GAME. Consequently, it is not possible to say whether the enzyme responsible displays any preference for esters of a certain chain-length. In addition, it cannot be concluded that the same enzyme is responsible for the hydrolysis of both galloyl esters. Further work needs to be conducted to resolve these issues and the most appropriate methods to achieve this will be discussed in Chapter 8.

As indicated in the introduction to this chapter, fungal tannin acylhydrolases display specificity for esters containing gallic acid. In order to unequivocally classify the K2 enzyme as a true tannin acylhydrolase, similar specificity for gallic acid esters would need to be demonstrated. This could be achieved by demonstrating a lack of hydrolysis of closely related phenolic acid esters, such as benzoic acid-, caffeic acid-, ferulic acid-, ρ-coumaric acid- and cinnamic acid-methyl esters. Unfortunately, many of these esters are not commercially available and would need to be synthesised-to-order. Also, a new enzyme assay would need to be developed to measure their hydrolysis (or lack thereof) since the rhodanine assay would not be suitable because it is specific for gallic acid.

Conclusion

In conclusion, an enzymatic basis has been demonstrated for the hydrolysis of tannic acid by the bacterium *S. ruminantium* K2. An assay has been developed which has allowed the

characterisation of this enzyme and from the results of this characterisation it can be concluded that this enzyme does not appear to be a non-specific esterase nor an arylesterase and may represent a new, bacterial tannin acylhydrolase.

CHAPTER 6 Enzyme size, isoelectric point, regulation of activity and distribution among Selenomonads.

6.1 Introduction

From the results presented in Chapter 5 it was concluded that the enzyme responsible for the hydrolysis of tannic acid produced by *S. ruminantium* K2 may be a novel bacterial tannin acylhydrolase. Research by Haslam and Stangroom (1966) and Ötük and Deschamps (1983) has shown that tannin acylhydrolases produced by fungi are induced by GAME and tannic acid but not by simple phenols such as gallic acid, salicylic acid or salicylic acid methyl ester. In this chapter the activity of the K2 tannin acylhydrolase is examined in cells grown in media containing various phenolic compounds in order to determine whether this enzyme is regulated in a similar way. In addition, experiments are conducted to determine the enzyme's size, isoelectric point and possible cellular location. The results of these experiments may help to further determine the significance of this enzyme with respect to both its metabolic role in K2 and its possible role in tannin detoxification in the feral goat.

Finally, research to date has concentrated on the characterisation of tannin acylhydrolase activity in a single strain of *S. ruminantium*. As yet, no information has been provided on whether or not other strains of *S. ruminantium* display this activity. As described in Chapter 3, *S. ruminantium* contains three subspecies; *ruminantium*, *lactilytica* and *bryanti* and DNA fingerprinting studies suggest there are a large number of apparently different strains within these subspecies (Zhang *et al*, 1991). Information on the distribution of this enzyme may help to suggest a possible role in tannin detoxification and also perhaps explain why this enzyme activity has not previously been reported. Hence, in the final experiment in this chapter, a range of isolates of *S. ruminantium* are examined for tannin acylhydrolase activity.

6.2 Results

6.2.1 Regulation of tannin acylhydrolase activity by phenolic compounds.

The specific activity of the tannin acylhydrolase was measured in cell-free extracts prepared from K2 cells grown in rich medium containing various phenolic compounds. Table 6.1 shows that there is a measurable level of tannin acylhydrolase activity in cells grown in the absence of phenolics (0.1 μmoles gallic acid produced/min/mg protein), however the activity is increased 35-fold in cells grown in the presence of tannic acid and 16-fold in the presence of GAME. Tannin acylhydrolase activity was not increased above the basal level in cells grown in the presence of ferulic acid or catechin.

Table 6.1 Specific activity of tannin acylhydrolase in cell-free extracts prepared from *S. ruminantium* K2 grown in the presence of phenolic compounds.

Medium*	Specific activity (μmoles gallic acid produced/min/mg protein)
mBHI	0.13 ± 0.02
mBHI/FA	0.12 ± 0.03
mBHI/C	0.18 ± 0.04
mBHI/TA	4.52 ± 1.07
mBHI/GAME	2.10 ± 0.20
mBHI/GA	0.42 ± 0.10

* mBHI, modified Brain Heart Infusion medium; mBHI/FA, mBHI medium supplemented with 0.2% w/v ferulic acid; mBHI/C, mBHI medium supplemented with 0.2% w/v catechin; mBHI/TA, mBHI medium supplemented with 0.2% w/v tannic acid; mBHI/GAME, mBHI medium supplemented with 0.1% w/v GAME; mBHI/GA, mBHI medium supplemented with 0.2% w/v gallic acid. Values represent means ± standard deviations of triplicate cell-free extracts.

Due to unforeseen circumstances, several different probes were used on the sonicator which was used to prepare the cell-free extracts during the course of this project. Consequently, problems were encountered making cell-free extracts of consistent specific activity. Therefore, in order to confirm the data which suggested an upregulation of enzyme activity in cells grown in the presence of certain phenolics, experiments were conducted to measure the tannin acylhydrolase activity in whole cells. In suspensions of washed K2 cells at densities of 5×10^4 - 3×10^6 cells/ml, gallic acid production from GAME was linear for at least 20 minutes. Table 6.2 shows that the tannin acylhydrolase activity increased 27-, 19-, and 55-fold in cells grown in the presence of gallic acid, GAME and tannic acid respectively compared to cells grown in rich medium containing no phenolic compounds. These data confirm the results obtained from cell-free extracts.

Table 6.2 Specific activity of tannin acylhydrolase in whole-cell suspensions prepared from *S. ruminantium* K2 grown in the presence of phenolic compounds.

Medium*	Specific activity (pmoles gallic acid produced/min/cell)
mBHI	$0.02 \pm 5.6 \times 10^{-5}$
mBHI/TA	1.10 ± 0.76
mBHI/GAME	$0.38 \pm 7.1 \times 10^{-3}$
mBHI/GA	0.54 ± 0.13

* see Table 6.1 for description of media. Values represent means ± standard deviations of duplicate cell suspensions.

6.2.2 Determination of enzyme size and isoelectric point

In order to further characterise the tannin acylhydrolase, a zymogram was developed using GAME as the substrate and employing rhodanine to detect the liberated gallic acid. In an attempt to determine the molecular size of the enzyme, cell-free extracts were fractionated on a 10% SDS-PAGE gel and then examined directly for activity using the zymogram technique. Figure 6.1 reveals that tannin acylhydrolase activity was localised to a single polypeptide of approximately 60kDa in size. In addition, the zymogram qualitatively confirms the data presented in section 6.2.1; that is, cell-free extracts derived from cells grown in the presence of gallic acid, tannic acid and GAME display much higher tannin acylhydrolase activity than extracts derived from cells grown in the absence of these phenolic compounds.

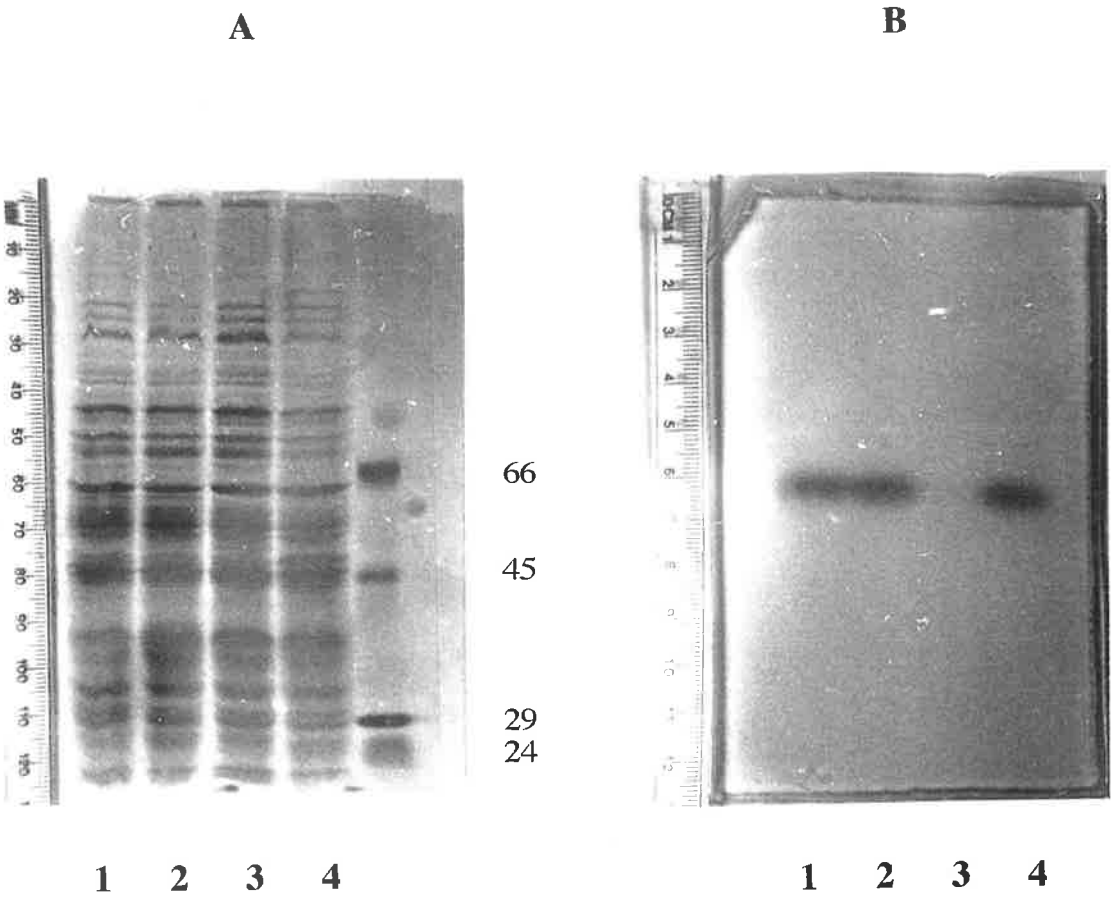


Figure 6.1 SDS-PAGE and zymogram analysis of tannin acylhydrolase activity in cell-free extracts of *S. ruminantium* K2. (10% acrylamide gel)

Crude protein was derived from cell-free extracts of cells grown in medium containing; lane 1, 0.2% w/v gallic acid; lane 2, 0.1% w/v GAME; lane 3, no phenolics; lane 4, 0.2% w/v tannic acid. MW, molecular weight markers (kDa) are indicated on the right. A, Coomassie Blue R250-stained gel; B, zymogram gel.

In order to determine the approximate isoelectric point of the K2 tannin acylhydrolase, cell-free extracts were fractionated by isoelectric focusing in polyacrylamide gels and then examined for activity by zymogram. Initially, the broadest possible pH gradient (pH 3-10) was used to separate the proteins in the cell-free extracts. There are several points to note from Figure 6.2. Firstly, from the Coomassie-stained gel it is evident that the majority of proteins in the cell-free extracts have an acidic isoelectric point (approximately between pH 4-5). Secondly, from the zymogram tannin acylhydrolase demonstrated an isoelectric point well above the majority of cell extract proteins and close to pH 7. Finally, there may be multiple zones of activity in the cell extract derived from cells grown in medium containing gallic acid.

The approximate isoelectric point of the K2 tannin acylhydrolase was confirmed by conducting isoelectric focusing and zymogram analysis over a narrower pH range. Figure 6.3 shows that the tannin acylhydrolase activity was localised to a region of approximately pH 7.0-7.1. It is also apparent from Figure 6.3 that there may be two proteins of differing isoelectric points displaying tannin acylhydrolase activity; although it appears that one protein is either more abundant than the other or is more active upon the substrate GAME.

In general, the isoelectric focusing/zymogram studies also qualitatively confirm the upregulation of tannin acylhydrolase activity in cells grown in medium containing gallic acid, tannic acid and GAME. It should be noted that different cell-free extracts were used in each of the experiments presented in Table 6.1, Figure 6.1 and Figure 6.2 and hence any anomalies in the results reflect the problems encountered making cell-free extracts with consistent specific activities by sonication.

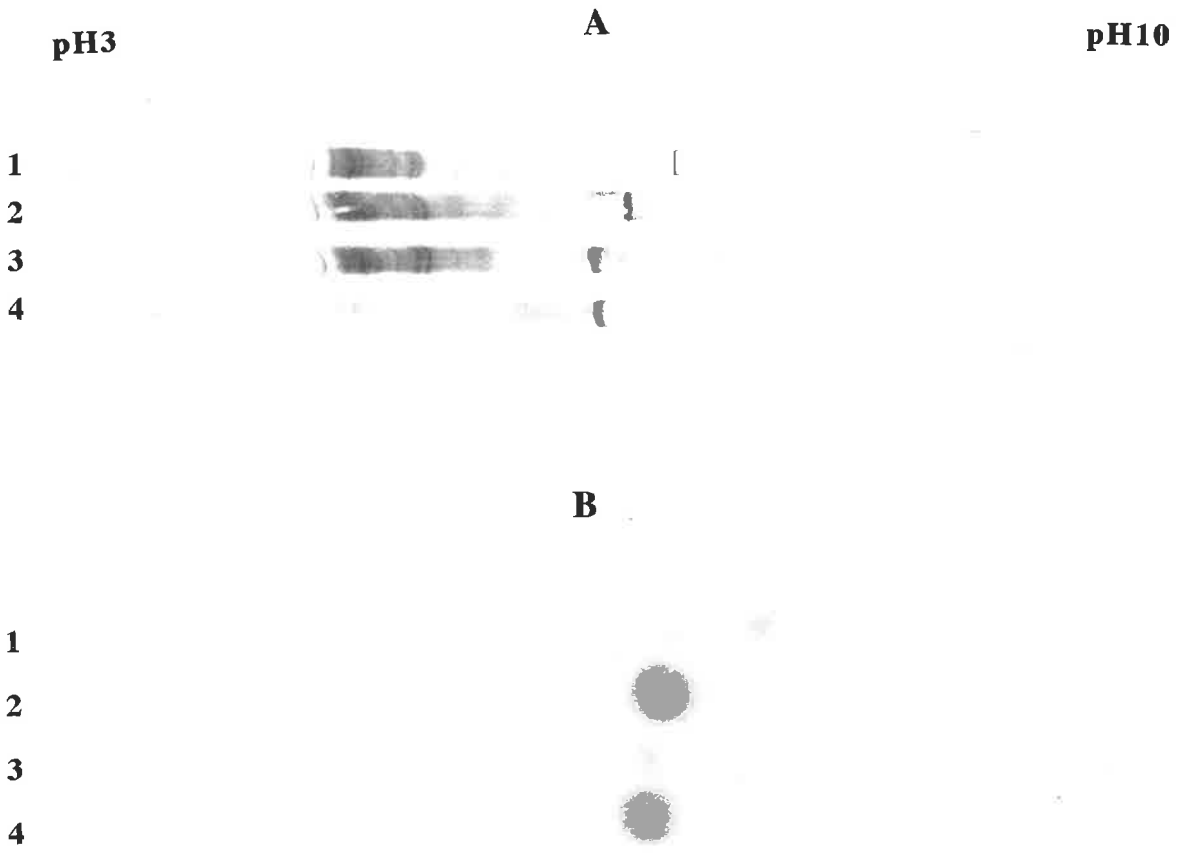


Figure 6.2 Analytical isoelectric focusing (pH 3-10) and zymogram analysis of tannin acylhydrolase activity in cell-free extracts of *S. ruminantium* K2.

Crude protein was derived from cell-free extracts of cells grown in medium containing; lane 1, 0.2% w/v gallic acid; lane 2, 0.1% w/v GAME; lane 3, no phenolics; lane 4, 0.2% w/v tannic acid. A, Coomassie Blue R250-stained gel; B, zymogram gel.

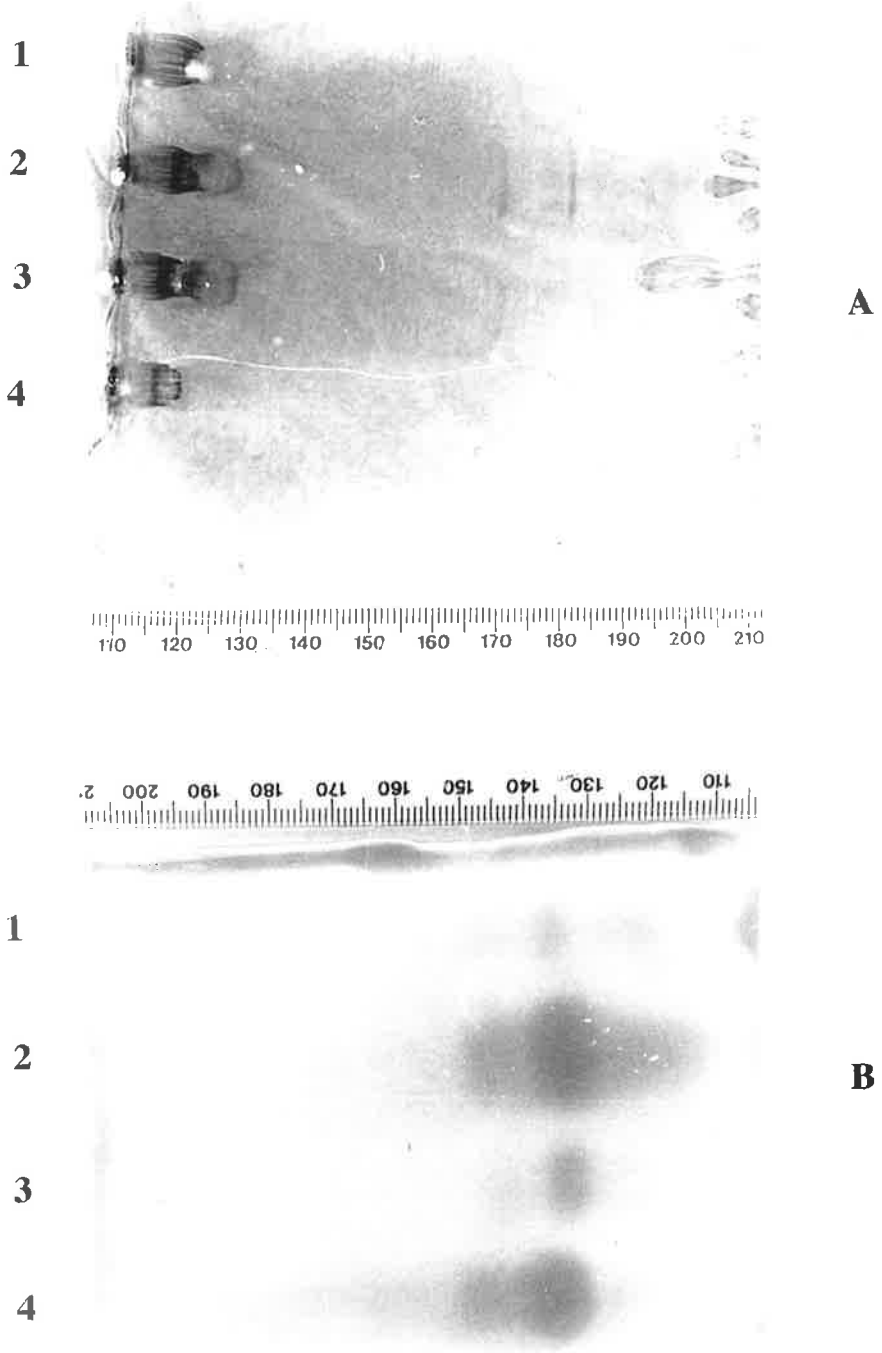


Figure 6.3 Analytical isoelectric focusing (pH 5-8) and zymogram analysis of tannin acylhydrolase activity in cell-free extracts of *S. ruminantium* K2.

Crude protein was derived from cell-free extracts of cells grown in medium containing; lane 1, 0.2% w/v gallic acid; lane 2, 0.1% w/v GAME; lane 3, no phenolics; lane 4, 0.2% w/v tannic acid. A, Coomassie Blue R250-stained gel; B, zymogram gel.

6.2.3 Extraction of tannin acylhydrolase by Triton X-100

Haroun Shah recently reported what appeared to be membrane vesicles in oral strains of *Selenomonas* (*pers. comm*, Shah, 1995) implicated in periodontal disease. These membrane vesicles appeared to be derived from "blebs" in the outer membrane and were shown to contain proteases important for cell-invasion. Furthermore, the enzymes contained within these vesicles were easily released by the non-ionic detergent Triton X-100. Therefore, *S. ruminantium* strain K2 was examined for the presence of Triton X-100-extractable tannin acylhydrolase.

When washed K2 cells were suspended in 1.0% Triton X-100 for a period of 2 hours at room temperature, the supernatant displayed high tannin acylhydrolase activity. In contrast, the supernatant from a corresponding aliquot of cells suspended in water without detergent displayed no tannin acylhydrolase activity (data not shown). When aliquots of each supernatant were run on a 10% SDS-PAGE gel, bands of stained protein were only visible in the Triton-extracted sample (Figure 6.4).

To further characterise the extraction of tannin acylhydrolase by Triton X-100, the effect of extraction time on protein yield and composition was examined. Unfortunately, the presence of Triton X-100 in the extracts interfered with the measurement of protein using Bradford's reagent. Consequently, the proteins in each sample were acetone-precipitated and then resuspended in sodium phosphate buffer in order to remove the Triton X-100. In a 2 hour period, maximum protein yield was not reached (Figure 6.5). Aliquots of each sample taken at each time point were run on a 12% SDS-PAGE gel in order to assess the effect of extraction duration on protein composition (Figure 6.6). It is clear that over the 2 hour extraction period, while the absolute amount of protein extracted increased with time, there was no difference in the types of proteins extracted in the first 10 minutes compared to 2 hours of extraction. This result suggests that the Triton X-100 exerted the same effect over time and did not selectively release any particular proteins as time increased.

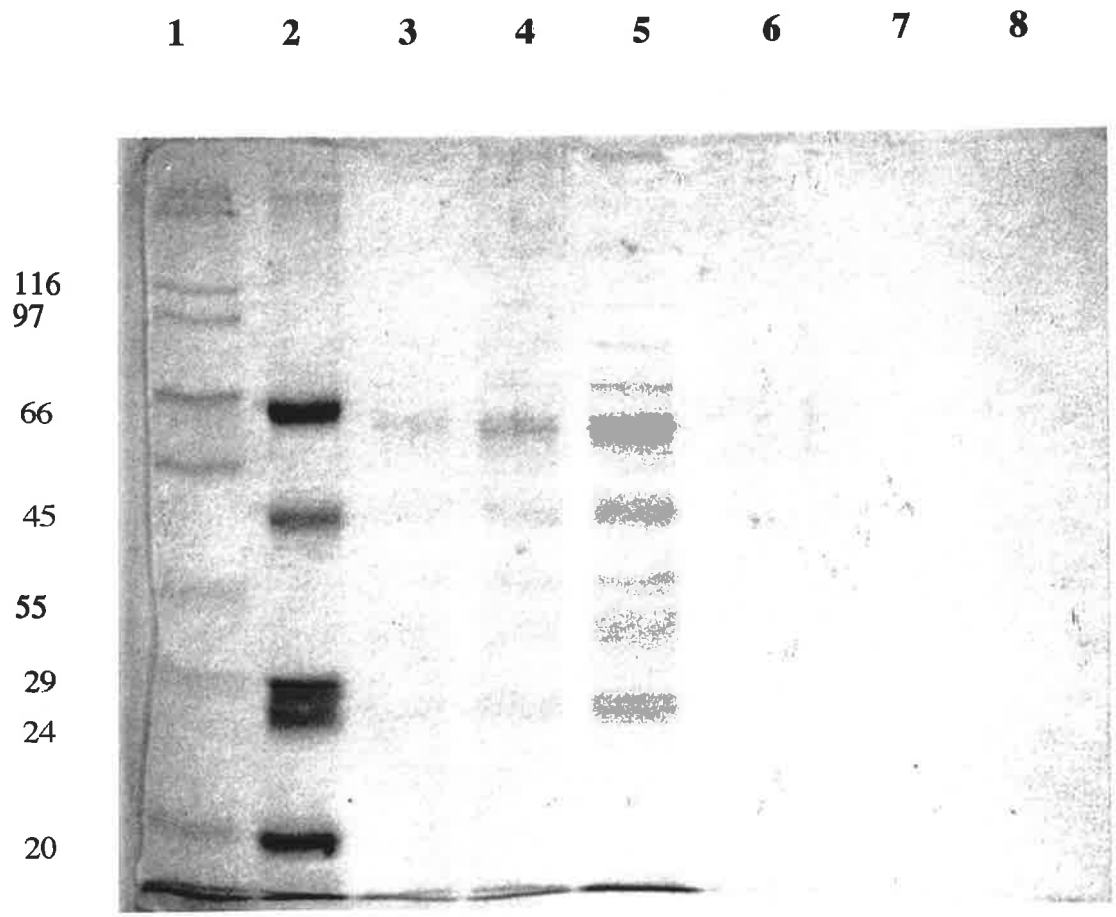


Figure 6.4 SDS-PAGE analysis of Triton X-100 extraction of proteins from *S. ruminantium* K2. (10% acrylamide gel)

Lanes 1 and 2, molecular weight markers (kDa); lanes 3, 4, 5, cells extracted with 1.0% v/v Triton X-100 in water; lanes 6, 7, 8, cells extracted with water-only.

Volumes of extracts loaded; lanes 3 and 6, 5µl; lanes 4 and 7, 10µl; lanes 5 and 8, 20µl. Cells were grown to A_{600} 0.8 in mBHI medium supplemented with 0.2% w/v gallic acid.

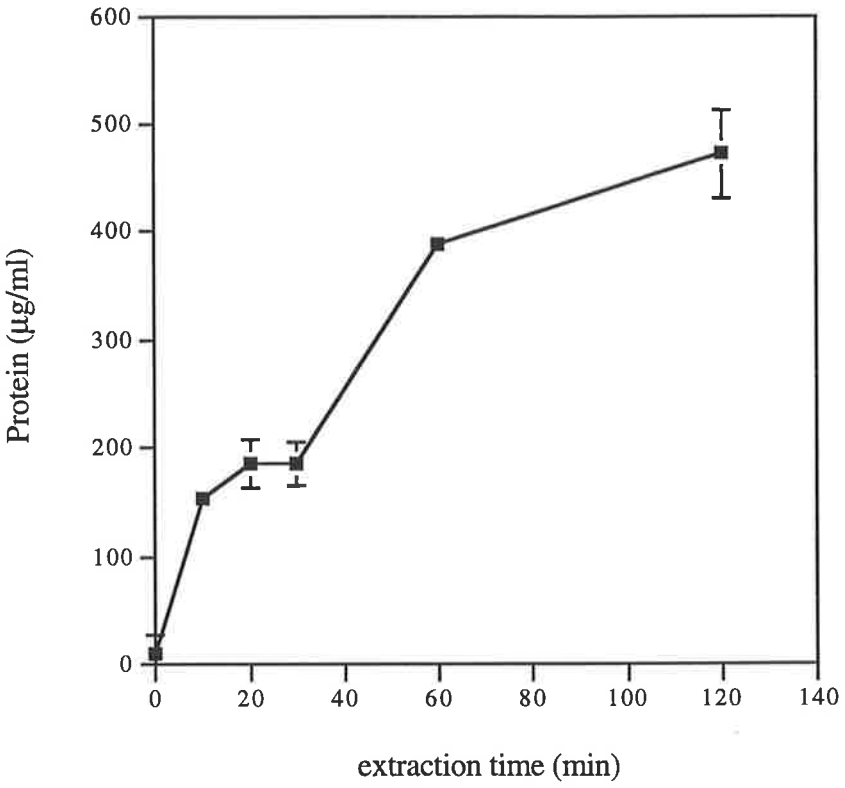


Figure 6.5 Triton X-100 extraction of *S. ruminantium* K2 proteins: Effect of extraction duration on protein yield. Values are means ± standard deviation of protein concentration of duplicate aliquots.

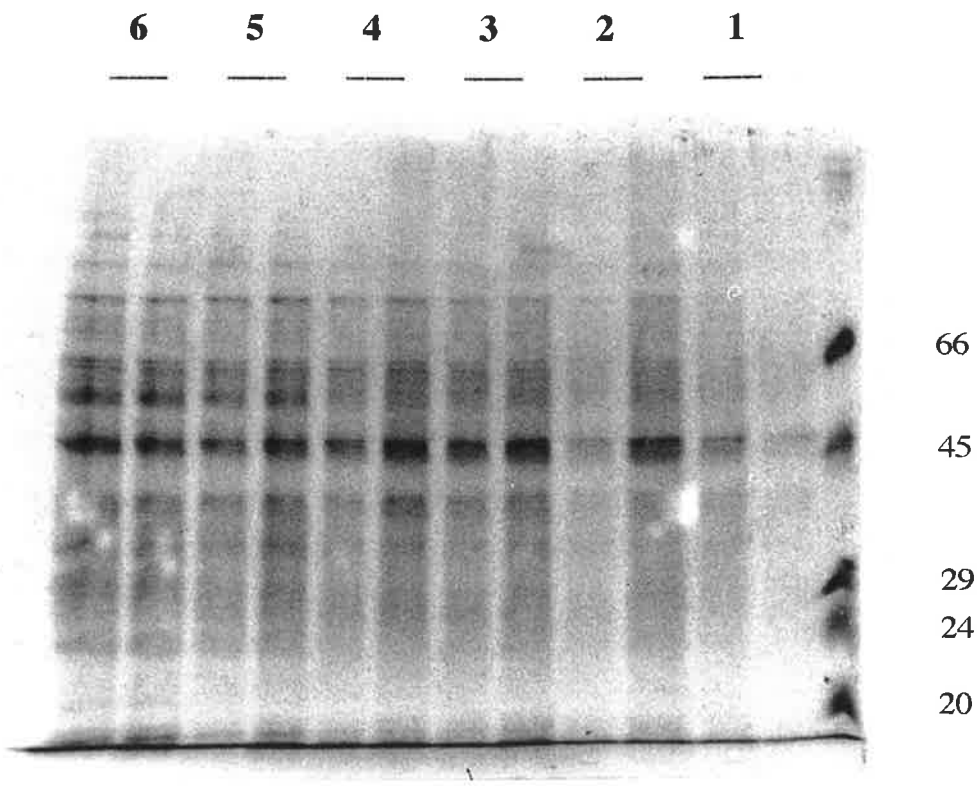


Figure 6.6 SDS-PAGE gel of Triton X-100-extracted proteins: Effect of extraction duration on protein profile. Cells were extracted for; lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 60 min; lane 6, 120 min. Molecular weight markers (kDa) are indicated on the right.

A preliminary study revealed that the acetone-precipitated proteins resulting from extraction of K2 cells with Triton X-100 retained tannin acylhydrolase activity. Consequently, the specific activity of these extracts was determined. The results (Table 6.3) show similar trends in enzyme activity as were obtained from both cell-free extracts produced by sonication and from whole-cell suspensions. Tannin acylhydrolase activity was increased 9-, 3-, and 10-fold in Triton X-100 extracts prepared from cells grown in the presence of gallic acid, GAME and tannic acid compared to cells grown in the absence of phenolics. Furthermore, the absolute values for these specific activities are similar to those obtained from cell-free extracts.

Table 6.3 Tannin acylhydrolase activity of Triton X-100 extracts of *S. ruminantium* K2 grown in the presence of phenolic compounds.

Medium*	Specific activity μmoles gallic acid produced/min/mg protein)
mBHI	0.58 ± 0.09
mBHI/TA	6.04 ± 0.14
mBHI/GAME	1.50 ± 0.16
mBHI/GA	5.20 ± 0.65

* see Table 6.1 for description of media. Values represent means ± standard deviations of triplicate assays.

6.2.4 Methylumbelliferyl acetate esterase activity

In Chapter 5 the substrate specificity of the K2 tannin acylhydrolase was examined and results showed that cell-free extracts displayed no activity against the common esterase substrates α-napthyl acetate and ρ-nitrophenyl acetate. Cell-free extracts did however display activity against methylumbelliferyl acetate. In order to determine if the tannin acylhydrolase was responsible for the observed methylumbelliferyl acetate esterase activity, the specific activities of cell extracts were determined using methylumbelliferyl acetate as substrate. In addition, a

zymogram technique was used to determine the size of the protein responsible for hydrolysis of methylumbelliferyl acetate.

The results presented in Tables 6.3 and 6.4 suggest that 2 different enzymes are responsible for the hydrolysis of GAME and methylumbelliferyl acetate. If the same enzyme was responsible for the hydrolysis of both substrates, one would expect the relative differences in specific activities for cells grown in each medium for one substrate to be mirrored in the specific activities for the other substrate; however this was not the case. The methylumbelliferyl acetate activities were consistently approximately 2-fold higher in the extracts prepared from cells grown in medium containing the 3 phenolic compounds compared to those grown without phenolics, whereas the tannin acylhydrolase activities for the same extracts were much more variable and ranged from 3- to 10-fold higher.

Table 6.4 Methylumbelliferyl acetate esterase activity of Triton X-100 extracts of *S. ruminantium* K2 grown in the presence of phenolic compounds.

Medium*	Specific activity (μmoles methylumbelliferone produced/min/mg protein)
mBHI	36.0 ± 0.4
mBHI/TA	87.1 ± 5.8
mBHI/GAME	87.3 ± 8.6
mBHI/GA	76.7 ± 8.3

* see Table 6.1 for description of media. Values represent means + standard deviations of triplicate assays

Cell extracts were separated on 10% SDS-PAGE gels and the methylumbelliferyl acetate esterase activity was detected by zymogram. From Figure 6.7 it can be seen that the enzyme

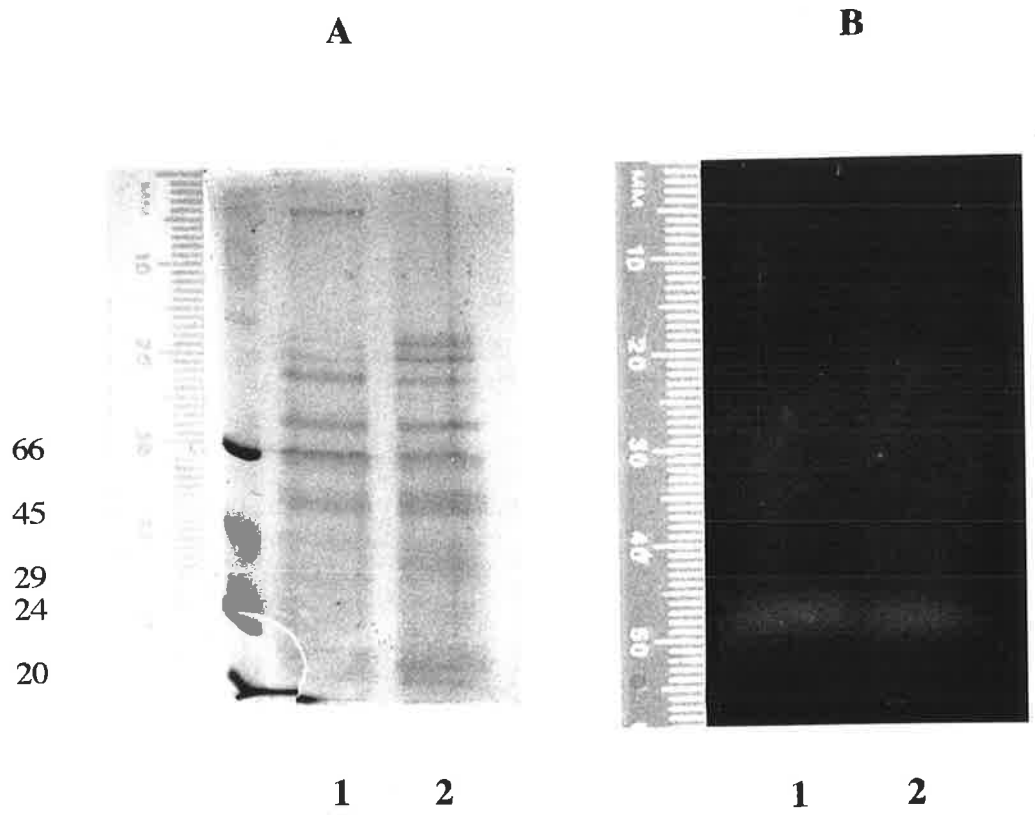


Figure 6.7 SDS-PAGE and zymogram analysis of methylumbelliferyl acetate esterase activity in Triton X-100 extracts of *S. ruminantium* K2. (10% acrylamide gel)

Protein was derived from cells grown in medium containing; lane 1, 0.2% w/v gallic acid; lane 2, no phenolics. MW, molecular weight markers (kDa) are indicated on the left. A, Coomassie Blue R250-stained gel; B, zymogram gel.

responsible for the hydrolysis of methylumbelliferyl acetate has a molecular weight of approximately 30 kDa.

6.2.5 Distribution of tannin acylhydrolase activity among isolates of *Selenomonas ruminantium*

Various strains of *S. ruminantium* and a *Streptococcal* species previously reported to be resistant to tannic acid were tested for the presence of tannin acylhydrolase. An attempt was also made to correlate the presence of tannin acylhydrolase activity with the ability of the strain to grow on tannic acid as a sole energy source. The results (Table 6.5) show that tannin acylhydrolase activity is not common to all strains of *S. ruminantium*. Strain K2 was the only bacterium to display high tannin acylhydrolase activity; and this activity could be detected after only 2 minutes incubation. In contrast, strains MA7, MA13, ML5 and S23 displayed weak activity which required 2 hours incubation to be detectable by the assay described in Chapter 2 section 2.4.5. The type strains of *S. ruminantium* , HD4 and MP72, displayed no activity.

Tannin acylhydrolase activity was not detected in the tannic acid-susceptible *Streptococcus bovis* H_{24} nor in the tannic acid-resistant *Streptococcus bovis* biotype I (Osawa, 1990) and *Streptococcus caprinus* 2.2 (Brooker *et al*, 1994).

S. ruminantium strain K2 was the only isolate able to grow on tannic acid as a sole energy source.

6.5

Table ~~7.1~~ Tannin acylhydrolase activity in rumen bacteria

Bacteria	Tannin acylhydrolase activity[1]	Growth on tannic acid[2]
Selenomonas ruminantium		
MA2	-	-
MA7	-/+	-
MA11	-	-
MA13	-/+	-
MA19	-	-
MA21	-	-
MA30	-	-
ME13	-	-
ME14	-	-
ME23	-	-
ML4	-	-
ML5	-/+	-
ML9	-	-
ML12	-	-
ML13	-	-
S23	-/+	-
K2	+	+
HD4 type strain	-	-
MP72 type strain	-	-
Streptococcus bovis		
biotype I (KO-1)	-	-
H_{24} type strain	-	-
Streptococcus caprinus 2.2	-	-

[1] Tannin acylhydrolase activity was determined by visual assessment of GAME hydrolysis by a dense cell suspension. + represents positive result ie hydrolysis of GAME, - represents no hydrolysis of GAME, -/+ represents weak positive result.

[2] Growth on tannic acid as sole energy source. + represents observed growth, - represents no observed growth.

6.3 Discussion

As a general conclusion, the data presented here suggest that tannin acylhydrolase is expressed constitutively in cells however its activity is increased in cells grown in medium containing gallic acid, tannic acid and GAME. The extent to which activity is increased varied depending on the method used to determine specific activity and also on the phenolic compound present in the medium. Consequently, it is not possible to conclude the absolute level to which tannin acylhydrolase is induced by gallic acid, tannic acid and GAME, nor is it possible to conclude if these three compounds produce the same degree of induction. For a variety of reasons, it is difficult to obtain accurate estimates of specific activity of this enzyme and each of the three methods described here has its disadvantages. In order to prepare cell-free extracts, a reliable and effective cell-disruption protocol needs to be established. A variety of methods were tested; sonication, French pressure-cell and grinding cells in the presence of liquid nitrogen and sand. It was concluded that sonication produced the best results. However, as stated previously, due to equipment breakage several different probes were used on the sonicator over the course of this work and some were more effective than others. This meant that on occasions the activity of tannin acylhydrolase was lower than expected, possibly due to overheating in the sample.

Of the three methods used to obtain specific activity data, the measurement of tannin acylhydrolase activity in whole-cell suspensions is probably the most reliable. This is because it does not rely on lysing cells and therefore is not subject to errors resulting from inconsistent lysis or heat-inactivation of the enzyme. This procedure does however require the accurate determination of cell number so that activity can be expressed on a per-cell basis. In this thesis, cell number was determined by direct-cell count using a haemocytometer as it was believed this would provide a more accurate estimate than by determining viable cell count by plating dilutions of bacterial cultures.

Although the extraction of tannin acylhydrolase by Triton X-100 is extremely simple and effective, further work needs to be done before it can be recommended as a routine protocol for the determination of specific activity. For example, a protocol for measuring protein

concentration of cell extracts containing Triton X-100 is required. The procedure developed by Peterson (1977) may prove helpful; this author found that the addition of excess SDS to a sample containing Triton X-100 results in the formation of mixed micelles of the detergents which do not interfere with the protein assay method of Lowry *et al* (1951). If this procedure does not work in the specific case of K2 cell-extracts, methods for removing the Triton need to be explored so that accurate measures of protein concentration in the extracts can be obtained. Although acetone-precipitation of proteins was used in the experiments detailed in this thesis, further work needs to be conducted to determine if this is the optimal procedure. The use of centrifuge-operated microconcentrators such as Amicon "Centricon-30's" may be more appropriate as they do not suffer from solubility problems inherent with protein precipitation methods. However, microconcentrators are not suitable for removing Triton X-100 from cell suspensions containing tannic acid because the tannic acid does not pass through the membrane and is concentrated along with the proteins.

The expression of the K2 tannin acylhydrolase activity differs from the fungal enzymes in a number of ways. Firstly, the bacterial enzyme is constitutively expressed whereas the fungal enzymes are not. Secondly, the fungal enzymes are induced by GAME and tannic acid but not by gallic acid. Since the product of the fungal tannin acylhydrolase is gallic acid which can be metabolised by the fungi; this mode of control of enzyme expression represents classical catabolite repression. In contrast, catabolite repression does not appear to control expression of tannin acylhydrolase activity in K2 since gallic acid increased enzyme activity and yet does not appear to be degraded by this bacterium. The fact that neither ferulic acid nor catechin were able to increase the activity of the tannin acylhydrolase provides further evidence that this enzyme may display specificity for esters containing gallic acid. Ferulic acid and catechin were chosen because they are phenolic compounds commonly encountered by ruminants, and in particular, catechin is often a component of condensed tannins. The mode of control of tannin acylhydrolase activity in relation to the role of this enzyme in this bacterium will be further discussed in Chapter 9.

Zymogram studies indicated that K2 tannin acylhydrolase is a single polypeptide of molecular mass 60 kDa with an isoelectric point of approximately 7. This is in contrast to the *Aspergillus niger* enzyme which is a glycoprotein of mass 186 kDa with an isoelectric point of 4.3 (Barthomeuf *et al*, 1994). This difference indicates that the bacterial protein is probably unrelated to the fungal protein and may represent a new enzyme. The presence of two zones of tannin acylhydrolase activity within each cell extract in the isoelectric focusing gels may be a misleading experimental artifact or it may indicate the presence of two proteins displaying tannin acylhydrolase activity with slightly different isoelectric points. Within any one cell extract, the activity of each of these proteins appears to differ since the intensity of the corresponding gallic acid-rhodanine complexes are different. Whether this difference in activity is a consequence of differences in the amount of protein or reflects a true difference in activity is unknown. When cell extract proteins were separated on SDS-PAGE gels, only one band of activity was detected in each sample, suggesting either that if two tannin acylhydrolases were present they each had identical molecular weights, or only one regained activity after removal of the SDS. Purification of the tannin acylhydrolase may help to resolve this question as this will reveal whether or not there are multiple proteins displaying tannin acylhydrolase activity.

No conclusions as to the location of the tannin acylhydrolase within K2 cells can be drawn from the Triton X-100 experiments. From the data presented it is not clear if the enzyme is located in the periplasm, the cytoplasm or in vesicles generated by blebbing of the outer membrane, only that it is Triton X-100-extractable. The fact that the magnitude of the specific activities measured in Triton extracts was very similar to those measured in extracts produced by sonication suggests that the Triton may have released the majority of cellular proteins, ie it may have lysed the cells. The cytoplasmic membrane of *E. coli* (Schnaitman, 1971) and *Bacillus stearothermophilus* (Gaillard *et al*, 1996) has been shown to be solubilised by Triton X-100 in preference to the outer membrane and this selectivity has been exploited as a method for isolating membrane fractions. The cytoplasmic membrane of *S. ruminantium* may also be Triton-soluble. Alternatively, the outer membrane may be Triton-soluble and the enzyme could be released from the periplasm. Periplasmic proteins have been defined operationally as those

proteins which are released by mild osmotic shock (Heppel, 1971), hence it would be interesting to determine if the tannin acylhydrolase could be released from cells by osmotic shock. Such a procedure has been used by Kawagishi *et al* (1994) to remove the periplasmic DNase from the marine bacterium *Vibrio alginolyticus* prior to electroporation.

Many Gram-negative bacteria are known to produce extra-cellular membrane vesicles. *Pseudomonas aeruginosa* and other organisms have been shown to release these vesicles filled with periplasmic components into the medium during growth (Kadurugamuwa and Beveridge, 1995). The vesicles of *P. aeruginosa* contain virulence factors such as peptidoglycan hydrolases (autolysins) while the vesicles of *Porphyromonas gingivalis* have been shown to contain deoxyribonuclease (Leduc *et al*, 1995) and cysteine proteinases (Shah *et al*, 1991). It may be productive to conduct transmission and scanning electron microscopy studies to examine K2 cells and culture supernatants for membrane vesicles or "blebs" analogous to those described in an oral Selenomonad by Shah (*pers. comm.*, 1995). If such vesicles were present, a method for their isolation would need to be developed so that the presence or absence of the tannin acylhydrolase within them could be established by enzyme assay.

Finally, since the enzyme responsible for the hydrolysis of methylumbelliferyl acetate had a molecular weight of approximately 30 kDa and the enzyme responsible for the hydrolysis of GAME had a molecular weight of approximately 60 kDa, and the fact that changes in specific activity with GAME were not mirrored with methylumbelliferyl acetate, it can be concluded that two different enzymes are responsible for the hydrolysis of each substrate. The results presented in Table 6.4 may imply that the enzyme which hydrolyses methylumbelliferyl acetate is also induced by compounds containing gallic acid, including tannins, but further work needs to be conducted before this can be verified.

The results of section 6.2.5 show that tannin acylhydrolase activity is uncommon amongst isolates of *S. ruminantium*. This result helps to explain why such activity has never been reported before. It should be noted however that the strains tested were not isolated from

ruminants consuming tannins. Therefore, no comment can be made on any possible correlation between a strain's tannin acylhydrolase activity and its host's diet. However, it is evident that there may be a positive correlation between tannin acylhydrolase activity and ability to grow on tannic acid as sole energy source. The weak positive results exhibited by strains ML5, MA13, MA7 and S23, together with their inability to grow on tannic acid as a sole energy source may be interpreted to mean that a minimum level of tannin acylhydrolase activity is required for growth on tannic acid. It would be interesting to quantitate the level of tannin acylhydrolase activity in these strains and compare this to that of strain K2. Given the problems previously encountered measuring activity in K2 by enzyme assay; it would be appropriate to use an independent method in this case, such as quantitation of the tannin acylhydrolase RNA transcript. This may provide an insight into the fundamental differences observed between these strains and K2 with respect to its ability to grow on tannic acid.

As stated in Chapter 5, tannin acylhydrolase activity has never previously been demonstrated in an anaerobic bacterium. Several authors have reported the isolation of *Streptococci* which are highly resistant to tannic acid and form clear zones around colonies grown on nutrient agar plates containing 0.5% w/v tannic acid. Some of these authors have attributed these clear zones to hydrolysis of the tannin-protein complex by a tannin acylhydrolase (Osawa and Walsh, 1993); however, as noted in Chapter 4, no experimental evidence of an enzymatic basis for this hydrolysis has been provided. Brooker *et al* (1994) suggest that the observed clear zones may be due to lactic acid-mediated hydrolysis of the tannin-protein complex. In the work presented here, the *Streptococcus* isolated by Osawa (biotype I or KO-1) and the *Streptococcus* isolated by Brooker *et al* (*S. caprinus* 2.2) were tested for tannin acylhydrolase activity as measured by hydrolysis of gallic acid methyl ester. No activity was detected. This confirms the hypothesis that clearing of tannin-protein complexes by these bacteria is due to a mechanism other than tannin acylhydrolase activity.

CHAPTER 7 Towards cloning the tannin acylhydrolase gene.

7.1 Introduction

In order to allow further characterisation of the tannin acylhydrolase enzyme in *S. ruminantium* K2, it was decided that every effort should be made to clone the gene encoding it. If successful, this would provide many opportunities for answering some of the questions raised by this work; such as the role of this enzyme in the tannin-resistance of K2 and the molecular basis of the regulation of tannin acylhydrolase expression by phenolic compounds.

As noted in Chapter 2, *S. ruminantium* is one of the most common bacterial species present in the rumen and it has been the subject of much research effort world-wide. However, despite the generally-accepted importance of this species, no chromosomal genes have been cloned and sequenced from any isolate. Knowledge of *Selenomonas* genetics is very scant and is derived from relatively few publications: Zhang *et al* (1991) showed that a high level of genetic diversity exists among members of this species; at least 3 cryptic plasmids have been reported in *S. ruminantium*, 2 of which have been sequenced (Martin and Dean, 1989; Attwood and Brooker, 1992; Zhang and Brooker, 1993); temperate bacteriophage have been reported in this species (Lockington *et al*, 1988; Cheong and Brooker, 1995); and finally, genetic transfer of lactate-utilising ability between strains of *S. ruminantium* has recently been reported, although the genetic basis for this transfer is unknown (Gilmour *et al*, 1996).

One of the major factors limiting the study of *S. ruminantium* genetics is the lack of a developed transformation system. Consequently, many of the commonly-used strategies to clone bacterial genes such as transposon mutagenesis or mutation complementation could not be adopted in this case. In addition, since no nucleic acid or protein sequence data exists for the fungal and yeast tannin acylhydrolases; strategies based on sequence homology were also not an option. Therefore, alternative strategies needed to be adopted to clone the tannin acylhydrolase gene and these are described in this chapter.

7.2 Results

7.2.1 Screening plasmid and bacteriophage libraries for expression of tannin acylhydrolase activity

In an attempt to clone the tannin acylhydrolase gene from *S. ruminantium* K2, several plasmid libraries of K2 genomic DNA were constructed in *Escherichia coli* vectors. A substrate overlay procedure was developed to allow screening of these libraries for tannin acylhydrolase expression. This procedure relied on the detection of gallic acid liberated from GAME by the action of tannin acylhydrolase. The procedure was developed using *S. ruminantium* K2 as the positive control. When colonies of K2 were overlaid with agarose containing GAME and incubated for 30 minutes, purple haloes could be observed surrounding the colonies when the plates were flooded with rhodanine and alkali solutions. These purple haloes were indicative of gallic acid released from the substrate GAME.

A number of different combinations of restriction endonuclease-digested DNA, vector and *E. coli* genotype were tested, however most libraries were unsatisfactory because they were too small to represent the entire K2 genome. The best library was constructed by ligating a *Sau* 3AI-partial digest of K2 DNA into the *Bam* HI site of pGEM-3Z (Promega) and electroporating this ligation mix into *E. coli* ER2267 electrocompetent cells. This library consisted of approximately 7 x 10^3 insert-containing transformants. When this library was screened for expression of tannin acylhydrolase activity using the substrate-overlay procedure outlined above; no positive colonies were detected.

Due to the difficulties encountered in constructing a representative library of the K2 genome in a plasmid vector, the possibility of constructing a genomic library in a bacteriophage vector was explored. Two different *E. coli* phage vectors were used; the replacement vector λDASH II (Stratagene) and the insertion vector λgt11. A successful library of K2 genomic DNA was constructed in each vector. The λDASH II library consisted of 4500 plaques before amplification. This vector is only viable if it contains an insert of 9-23 kb in size. Hence the library contained 40.5-103.5 Mb of K2 DNA which represents approximately 10-26 genomes,

assuming that the K2 genome is similar in size to that of *Escherichia coli*. The λgt11 library contained 1.5 x 10^6 insert-containing plaques before amplification. This vector accepts DNA inserts up to 7 kb, hence this library also represented the entire K2 genome.

Approximately 4000 plaques from the λDASH II and 90,000 plaques from the λgt11 amplified libraries were screened for expression of tannin acylhydrolase activity using the substrate overlay method. No positive clones were detected.

7.2.2 Cloning a tannic acid-inducible promoter

On the basis of the results presented in Chapter 6 which showed an increased expression of tannin acylhydrolase activity in K2 cells grown in the presence of certain phenolic compounds; I attempted to clone a tannin-inducible promoter from K2. If successful, this promoter element could be used as a probe to clone the full-length tannin acylhydrolase gene.

The prokaryotic promoter selection vector pKK232-8 (Brosius and Lupski, 1987), was used to isolate promoter-active DNA fragments from the chromosome of *S. ruminantium* K2. This plasmid vector contains a promoterless chloramphenicol acetyltransferase (*cat*) gene, flanked by transcription terminators of the *E. coli rrn*B ribosomal RNA operon. In addition, the region between the multiple cloning site (MCS) and the start codon of the *cat* gene contains translational stop codons in all three reading frames. In order to maximise the chance of cloning a tannin-inducible promoter, 2 libraries were constructed; one based on a *Hae* III digest of K2 DNA and the other based on an *Alu* I digest.

A total of 138 chloramphenicol-resistant clones were produced from the *Alu* I digest and 130 chloramphenicol-resistant clones from the *Hae* III digest. Each clone was patched onto plates containing 4 different concentrations of chloramphenicol in the absence and presence of tannic acid (0.2% w/v) as described in Chapter 2 section 2.5.4. The plates were screened for clones whose resistance to chloramphenicol increased in the presence of tannic acid; thereby indicating the presence of a tannin-responsive promoter element. In general, the clones displayed a variety

of levels of resistance to chloramphenicol, ie some clones were resistant to the highest level tested (400 μg/ml) while others were only resistant to the lowest level tested (25 μg/ml). This result suggests that the cloned K2 DNA fragments which acted as promoters did not provide the same level of transcription of the CAT gene in every case. However, no clone was identified that displayed an altered level of chloramphenicol resistance, either increased or decreased, in the presence of tannic acid.

7.2.3 Preparation of tannin acylhydrolase-specific polyclonal antibodies

Following the unsuccessful cloning strategies described above, the conclusion was reached that any procedure relying on the detection of tannin acylhydrolase activity in a heterologous host would be likely to fail. Consequently an alternative procedure for detecting the cloned tannin acylhydrolase was required. I therefore attempted to raise rabbit polyclonal antibodies to the tannin acylhydrolase. These antibodies could then be used to screen the λgt11 library constructed earlier. This λ vector contains a single *Eco* RI cloning site 53bp upstream from the end of the *lac* Z β-galactosidase gene. If a fragment of DNA coding for a gene is cloned into this site in the same reading-frame as the *lac* Z gene, a β-galactosidase fusion protein will be expressed which could be detected with specific antibodies. Hence, many plaques can be screened at one time by transferring the expressed fusion proteins to nylon or nitrocellulose filters and incubating with primary antibodies and a secondary antibody-enzyme conjugate in a manner analogous to a Western blot.

Initially, tannin acylhydrolase was purified for immunisation by combining analytical isoelectric focusing in polyacrylamide gels (pH 5-8) with SDS-PAGE, as described in Chapter 2 (section 2.5.5). Test bleeds taken from 4 New Zealand White rabbits after primary and boost immunisations were analysed for the presence of tannin acylhydrolase-specific antibodies by immunoblot (Figure 7.1). Despite many bands being apparent, the same bands were produced by probing with the preimmune sera as the immune sera; indicating the absence of any tannin

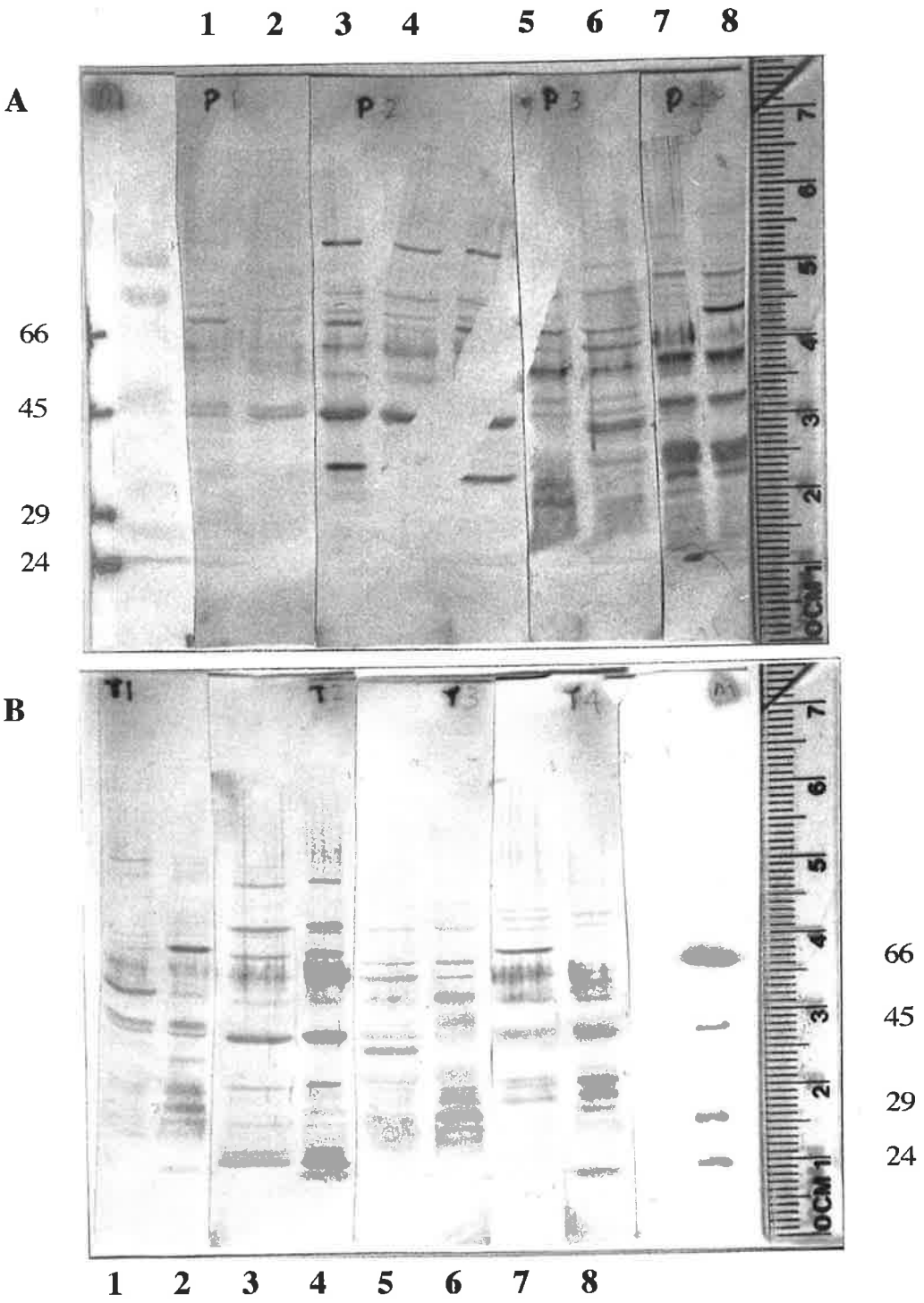


Figure 7.1 Immunoblot of SDS-PAGE fractionated cell-free extract proteins from *S. ruminantium* K2 grown in mBHI/GAME.

Membrane strips were probed with 100-fold dilutions of sera from: lanes 1 and 2, rabbit 1; lanes 3 and 4 , rabbit 2; lanes 5 and 6, rabbit 3, lanes 7 and 8, rabbit 4. A. membrane strips probed with preimmune sera, B. membrane strips probed with immune sera after 1st boost. Molecular weight markers (kDa) are indicated on the left (A) and right (B).

acylhydrolase-specific antibodies. Since the antisera was not preabsorbed against total *Selenomonas* extract before being used in the immunoblot, the large number of bands produced reflects the background level of non-specific antibody-protein interactions at the concentration of antisera tested.

The failure to produce specific antibodies after the primary and secondary immunisations was possibly due to insufficient quantity of immunogen. Consequently, the rabbits were immunised with approximately 4-fold more protein than was used initially. Only two rabbits were given this third immunisation due to the limited amount of material available. Protein for these immunisations was purified by combining preparative isoelectric focusing in granulated gel with preparative SDS-PAGE. The preparative isoelectric focusing in the granulated gel bed did not work as well as the small-scale experiments in a polyacrylamide gel because the proteins in the cell-lysate did not focus completely into discreet bands. However, proteins eluted from a region of IEF gel corresponding to the neutral pH zone displayed high tannin acylhydrolase activity. When an aliquot of this sample (fraction #24) was run on an SDS-PAGE gel, an intense band corresponding to the size of the tannin acylhydrolase was observed (Figure 7.2). Subsequent zymogram analyses confirmed that this band was in fact the tannin acylhydrolase (data not shown).

In order to avoid acid-fixation of the proteins in the polyacrylamide, gels were stained with copper chloride to visualise the protein to be excised. However, copper chloride staining is less sensitive than Coomassie Blue R250 and consequently it was not possible to excise a single band of protein corresponding to the tannin acylhydrolase. Therefore, the 3 proteins of approximately 60 kDa molecular weight shown in Figure 7.2 were excised together and used as the immunogen in subsequent boosts. Antisera from these immunisations were analysed for tannin acylhydrolase-specific antibodies by immunoblot. The results appear in Figures 7.3 and 7.4 and are very similar to those shown in Figure 7.1. All of these immunoblots contained the same cell-free extract proteins bound to the PVDF membranes. Two cell extracts were included in each case, each derived from a separate culture of K2 grown in mBHI/GAME, in order to

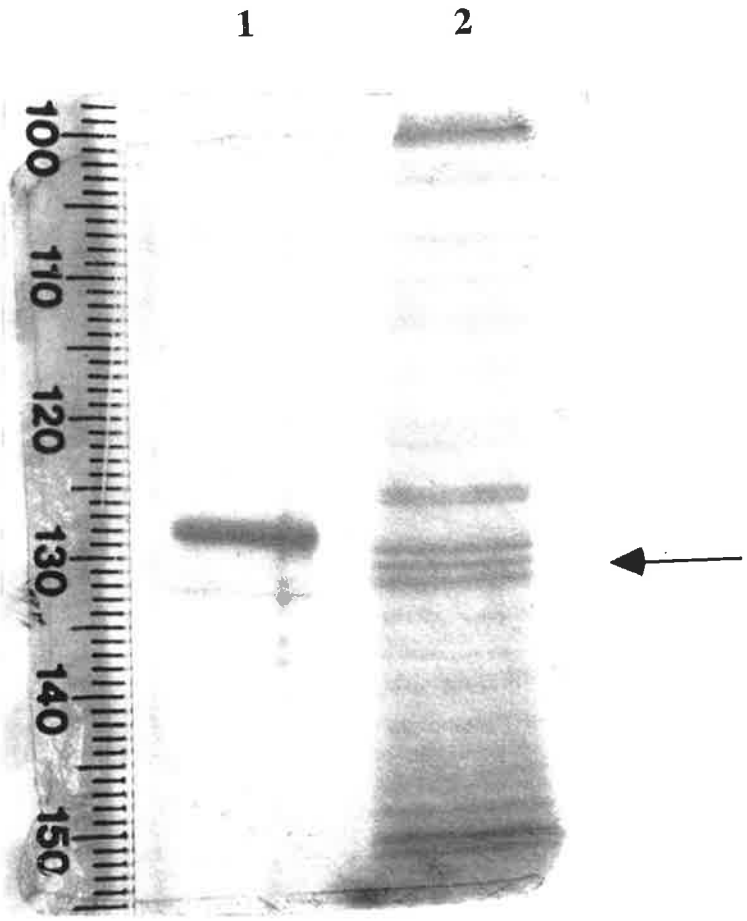


Figure 7.2 SDS-PAGE gel of fraction #24 from preparative isoelectric focusing of *S. ruminantium* K2 cell-free extract proteins.

Lane 1, molecular weight marker (Bovine Serum Albumin, 66 kDa); lane 2, fraction #24. Arrow indicates tannin acylhydrolase.

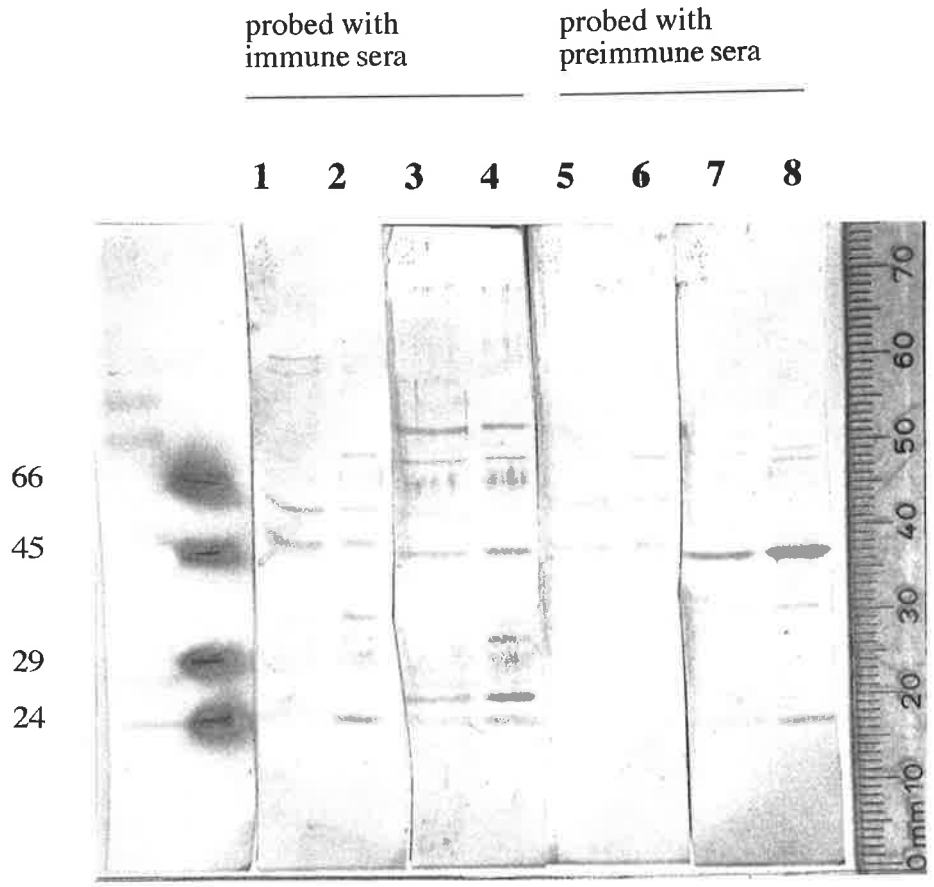


Figure 7.3 Immunoblot of SDS-PAGE fractionated cell-free extract proteins from *S. ruminantium* K2 grown in mBHI/GAME.

Membrane strips were probed with 100-fold dilutions of: lanes 1 and 2, immune sera from rabbit 1 after 2nd boost; lanes 3 and 4, immune sera from rabbit 2 after 2nd boost; lanes 5 and 6, preimmune sera from rabbit 1; lanes 7 and 8, preimmune sera from rabbit 2. Molecular weight markers (kDa) are indicated on the left.

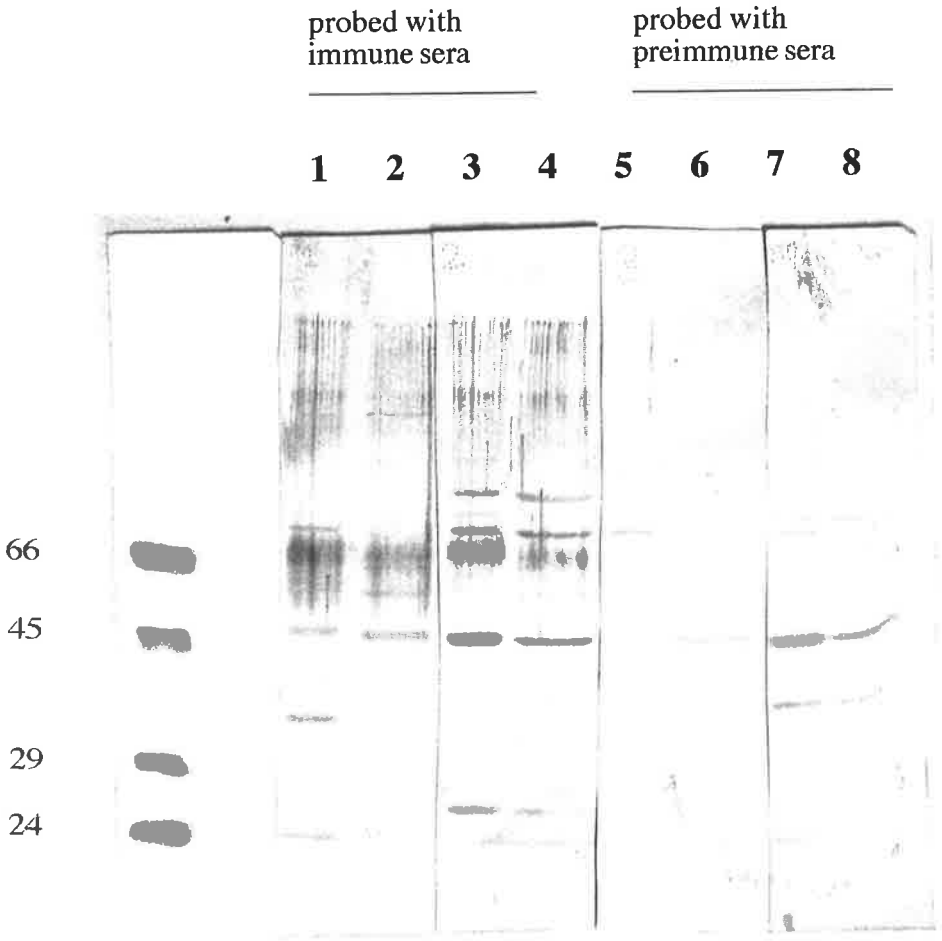


Figure 7.4 Immunoblot of SDS-PAGE fractionated cell-free extract proteins from *S. ruminantium* K2 grown in mBHI/GAME.

Membrane strips were probed with 50-fold dilutions of: lanes 1 and 2, immune sera from rabbit 1 after 3rd boost; lanes 3 and 4, immune sera from rabbit 2 after 3rd boost; lanes 5 and 6, preimmune sera from rabbit 1; lanes 7 and 8, preimmune sera from rabbit 2. Molecular weight markers (kDa) are indicated on the left.

ensure consistency of results. From Figures 7.1, 7.3 and 7.4 it is evident that this consistency was achieved since within any one antisera tested, the same proteins in each cell extract reacted with the antisera.

Despite having immunised the rabbits with 3 discreet proteins, many bands are evident in the immunoblot conducted after the final boost (Figure 7.4) and it was suspected that this still represented the background level of non-specific protein binding. Consequently an immunoblot was conducted against proteins in fraction #24, the source of the immunogen, and cell lysate proteins derived from *S. ruminantium* HD4, a bacterium previously shown to have no tannin acylhydrolase activity. The results (Figure 7.5) indicate that both rabbit's antisera reacted with the majority of HD4 cell lysate proteins and did not appear to react strongly with the desired proteins in fraction #24.

From these blots it was concluded that after a total of 4 immunisations with differing amounts of protein, no tannin acylhydrolase-specific antibodies were detected.

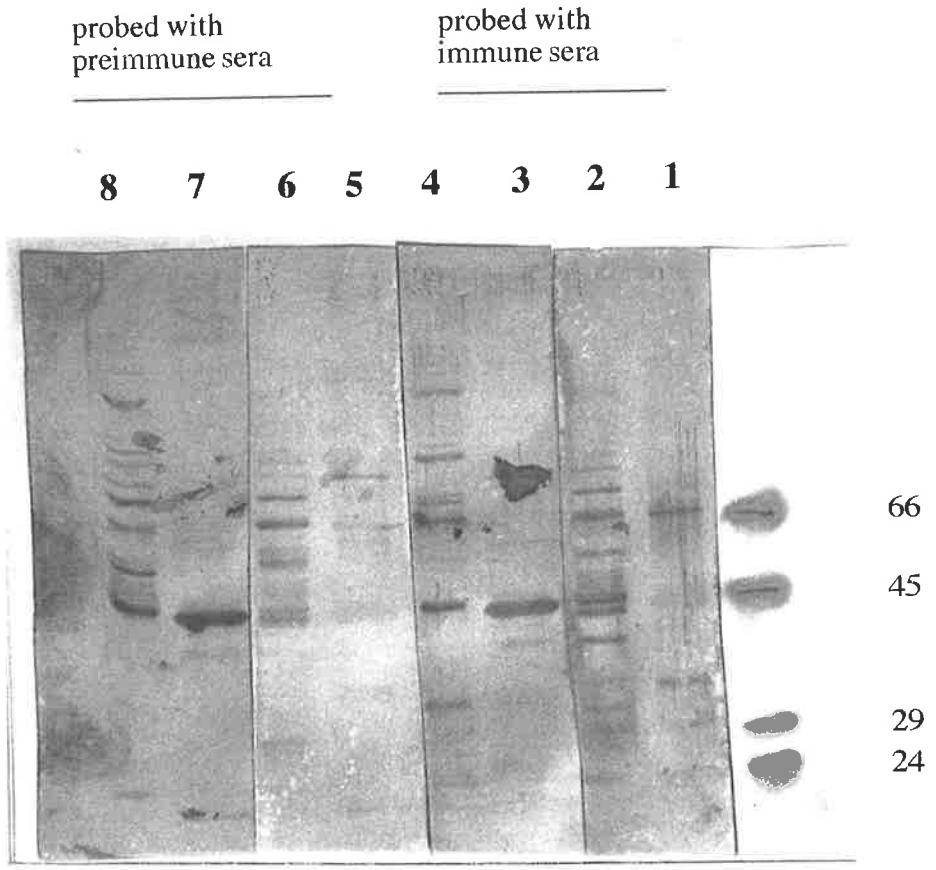


Figure 7.5 Immunoblot of SDS-PAGE fractionated proteins.

Proteins were derived from: lanes 1, 3, 5 and 7, fraction #24; lanes 2, 4, 6 and 8, *S. ruminantium* HD4. Membrane strips were probed with 50-fold dilutions of: lanes 1 and 2, antisera from rabbit 1 after 3rd boost; lanes 3 and 4, antisera from rabbit 2 after 3rd boost; lanes 5 and 6, preimmune sera from rabbit 1; lanes 7 and 8, preimmune sera from rabbit 2. Molecular weight markers (kDa) are indicated on the right.

7.2.4 Screening phage libraries with a synthetic degenerate oligonucleotide

After the failure to generate tannin acylhydrolase-specific antibodies, an alternative approach to cloning the tannin acylhydrolase gene was required. Since I had already prepared genomic libraries of K2 in phage vectors and I had developed a procedure for purifying small quantities of the tannin acylhydrolase, I decided to attempt to obtain partial protein sequence from which I could design a synthetic degenerate oligonucleotide probe.

The tannin acylhydrolase was purified from fraction #24 by SDS-PAGE as described in Chapter 2 (section 2.5.8). This sample was then subjected to N-terminal sequencing using the Edman degradation technique which was carried out by the Department of Biochemistry at the University of Adelaide. The sample yield was low (approximately 1-2 pmoles) and the background was high at the start of sequencing. Consequently this caused detection problems for the first few residues. Nevertheless, the following amino acid sequence was obtained:

Table 7.1 N-terminal sequence of K2 tannin acylhydrolase

Residue No.	Amino acid[1]	IUB code
1.	?	
2.	?	
3.	Proline	P
4.	?	
5.	Alanine	A
6.	? (Alanine)	
7.	Asparagine	N
8.	Glutamine	E
9.	?	
10.	Arginine	R
11.	Glycine	G
12.	Isoleucine	I
13.	Tyrosine	Y
14.	Methionine	M
15.	Phenylalanine	F
16.	?	
17.	?	
18.	Asparagine	N
19.	? (Lysine)	
20.	Tyrosine	Y

[1] ?, residue identity unknown; possible identity listed in brackets.

This protein sequence is encoded by the following nucleotide sequence:

?	?	P	?	A	?	N	E	?	**R**	**G**	**I**	**Y**	**M**	**F**	?	?	N	?	Y
---	---	CCG	---	GCT	---	AAC	GAA	---	CGT	GGT	ATT	TAT	ATG	TTT	---	---	AAC	---	TAT
		CCA		GCC		AAT	GAG		CGC	GGC	ATC	TAC		TTC			AAT		TAC
		CCC		GCA					CGA	GGA	ATA								
		CCT		GCG					CGG	GGG									
									AGA										
									AGG										

From this possible nucleotide sequence, a degenerate oligonucleotide was designed and synthesised based on residues 10-15 (highlighted in bold type). This oligonucleotide incorporated the neutral base inosine at two positions in order to reduce number of synthetic oligonucleotides required. For purposes of identification, it will be referred to as DO1:

5'	CGI	GGI	ATT	TAT	ATG	TTT	3'
	A		C	C		C	
			A				

DO1: 18-mer, 24-fold degeneracy, based on the published convention that inosine =1

DO1 had an estimated minimum melting temperature (T_m) of 44°C. Preliminary hybridisation experiments were conducted as described in Chapter 2 (section 2.5.9) in an attempt to determine the optimum hybridisation temperature. DO1 was end-labelled with ^{32}P and used to probe a series of restriction endonuclease-digested samples of K2 genomic DNA at several different hybridisation and wash temperatures. During these experiments it was observed that the temperature and duration of washing was extremely critical and needed to be closely monitored. Consequently, following hybridisation, the membranes were washed initially at room

temperature and then at gradually higher temperatures until the counts from the membrane, as measured by a hand-held Geiger counter, began to fall. The results (Figures 7.6-7.9) indicate that a hybridisation temperature of >34°C produced the least amount of non-specific hybridisation.

Approximately 24,000 plaques from the λgt11 library were screened with the end-labelled DO1 at a temperature of 40°C as described in Chapter 2 (section 2.5.10). Eighteen positively-hybridising plaques were observed and these were then picked and subjected to a second round of hybridisation with DO1, in order to purify them. Unfortunately, for reasons unknown, this second screening was unsuccessful and time restrictions meant that the experiment had to be ended before the positive plaques could be purified and characterised.

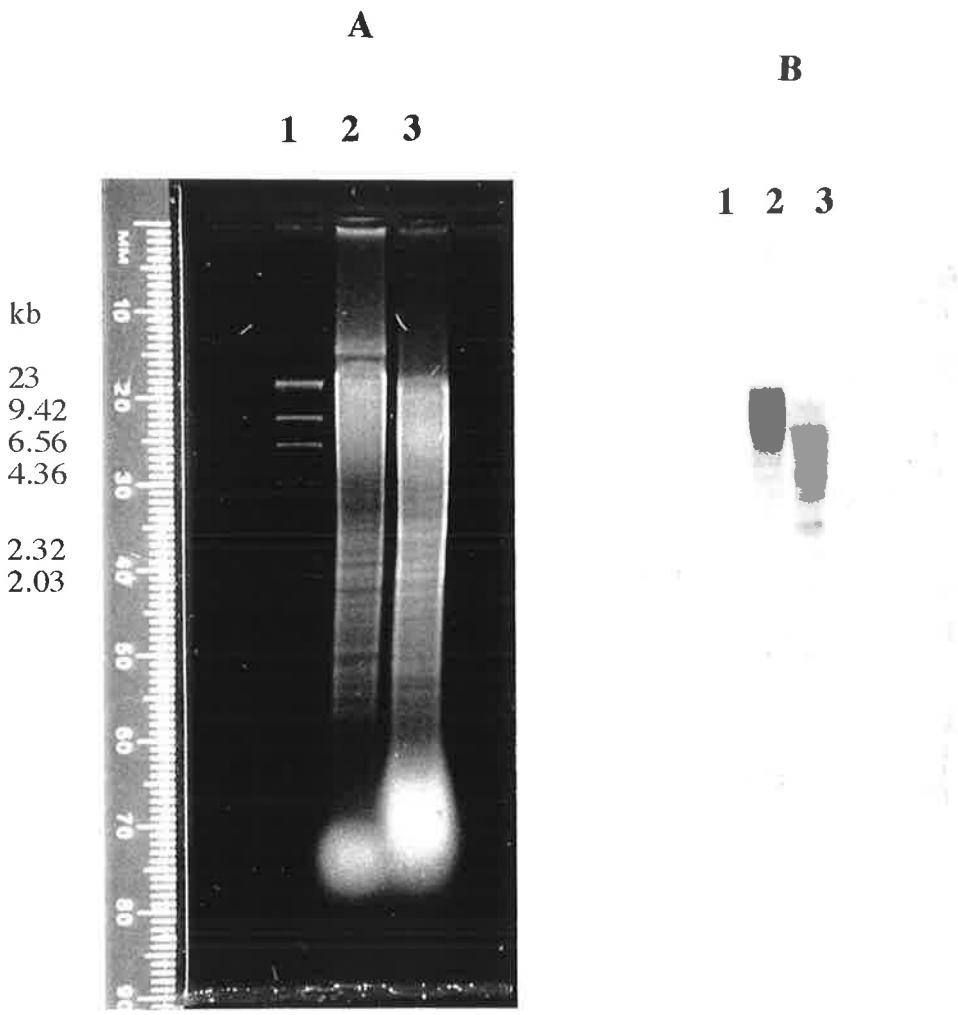


Figure 7.6 Hybridisation of DO1 to restriction endonuclease-digested *S. ruminantium* K2 DNA.

Lane 1, molecular weight marker (λ DNA digested with *Hind* III); lanes 2-3, K2 DNA was digested with: lane 2, *Eco* RI; lane 3,*Acc* I. Membrane was hybridised at 27°C and washed at room temperature (22°C) in 6X SSC/0.1% SDS. A. Ethidium bromide-stained gel; B. autoradiograph of gel after transfer and hybridisation.

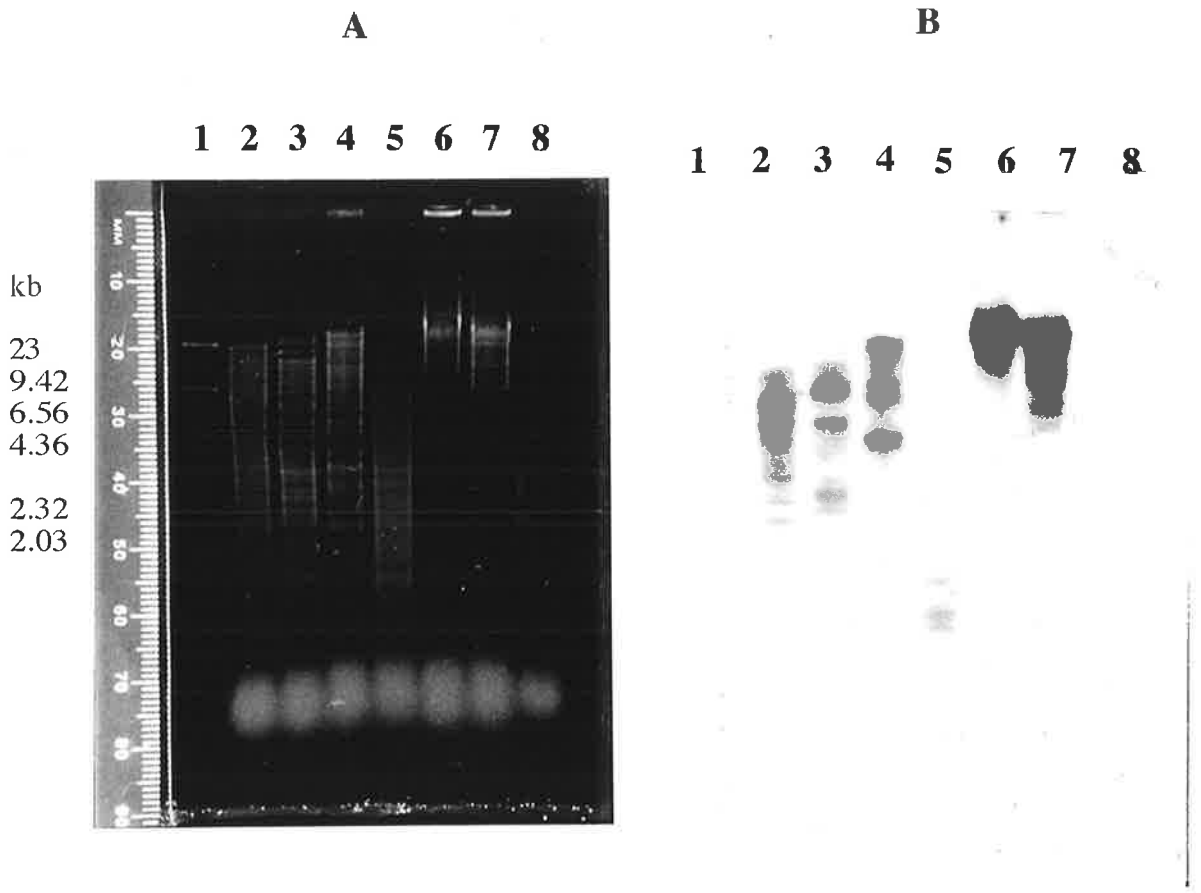


Figure 7.7 Hybridisation of DO1 to restriction endonuclease-digested *S. ruminantium* K2 DNA.

Lane 1, molecular weight marker (λ DNA digested with *Hind* III); lanes 2-7, K2 DNA was digested with: lane 2, *Eco* RI; lane 3, *Hind* III; lane 4, *Sph* I; lane 5, *Sau* 3AI; lane 6, *Sal* I; lane 7, *Sac* I; lane 8, *S. bovis* H_{24} DNA digested with *Hind* III. Membrane was hybridised at 24°C and washed at 30°C in 6X SSC/0.1% SDS. A. Ethidium bromide-stained gel; B. autoradiograph of gel after transfer and hybridisation.

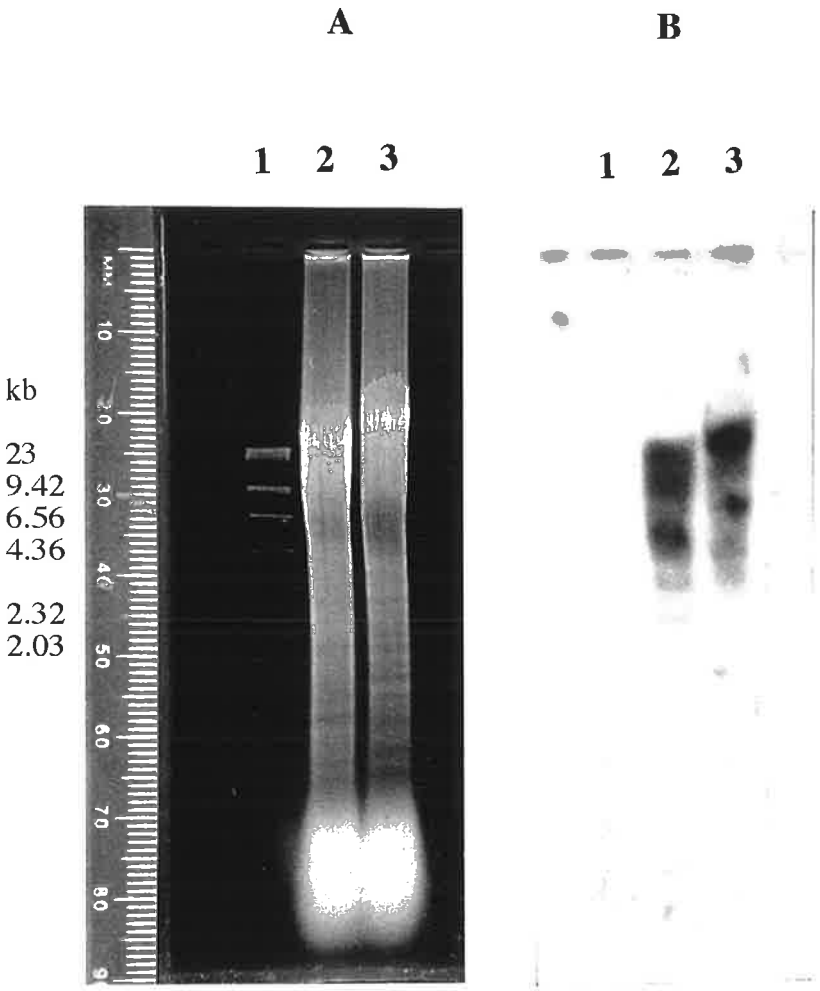


Figure 7.8 Hybridisation of DO1 to restriction endonuclease-digested *S. ruminantium* K2 DNA.

Lane 1, molecular weight marker (λ DNA digested with *Hind* III); lanes 2-3, K2 DNA was digested with: lane 2, *Eco* RI; lane 3, *Hind* III. Membrane was hybridised at 34°C and washed at 41°C in 6X SSC/0.1% SDS. A. Ethidium bromide-stained gel; B. autoradiograph of gel after transfer and hybridisation.

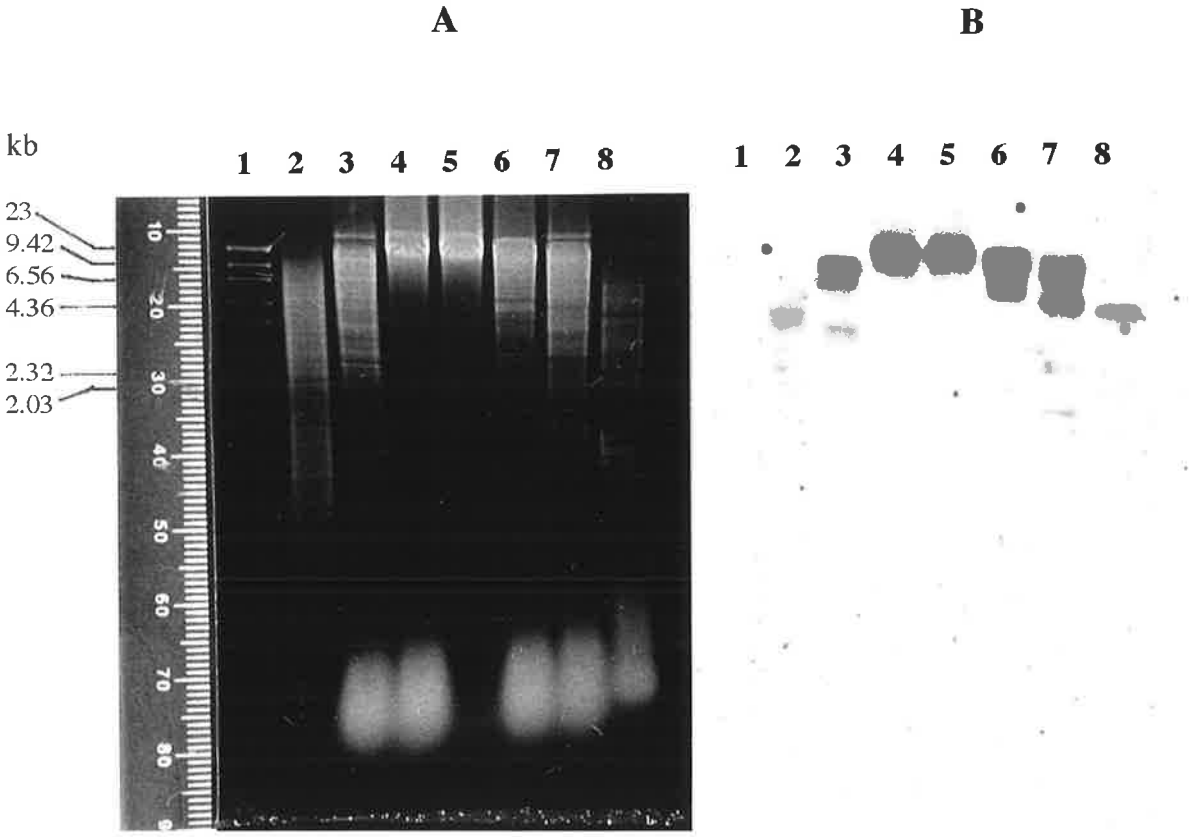


Figure 7.9 Hybridisation of DO1 to restriction endonuclease-digested *S. ruminantium* K2 DNA.

Lane 1, molecular weight marker (λ DNA digested with *Hind* III); lanes 2-7, K2 DNA was digested with: lane 2, *Eco* RI; lane 3, *Hind* III; lane 4, *Bam* HI; lane 5, *Pst* I; lane 6, *Xma* I; lane 7, *Sph* I; lane 8, *S. bovis* H_{24} DNA digested with *Hind* III. Membrane was hybridised at 34°C and washed at 40°C in 6X SSC/0.1% SDS. A. Ethidium bromide-stained gel; B. autoradiograph of gel after transfer and hybridisation.

7.2.5 PCR with DO1 and M13 forward/reverse primers

A PCR strategy was developed as an alternative to screening λgt11 plaques by hybridisation to DO1. This strategy (Figure 7.10) involved the use of the tannin acylhydrolase-specific primer DO1 and a plasmid vector-specific primer (Chapter 2 section 2.5.11). A range of products was observed in all reactions except those lacking Mg^{2+} ions (Figure 7.11A). The positive controls which contained both vector-specific M13 forward and reverse primers produced the expected 223bp product (Figure 7.11B). One product of approximately 400 base pairs in size (Figure 7.11A), arising from the DO1/M13 reverse primer combination, was purified from the gel and reamplified with DO1 and M13 (reverse), in order to produce a single product (Figure 7.12). This 400bp product, designated "pcr1", was probed with end-labelled DO1 and strong hybridisation occurred (Figure 7.13A and B), indicating that it contained sequence with high homology to DO1. Pcr1 was then labelled by random priming and used to probe restriction endonuclease-digested K2 DNA (Figure 7.14A and B). Strong signals of hybridisation were evident in each track, suggesting that pcr1 was derived from K2 genomic DNA as expected. The PCR product was then cloned into pBluescript SK to give plasmid pBS.pcr1 (Figure 7.15) and sequenced in one direction only using dye primer cycle sequencing with the M13 forward sequencing primer (Figure 7.16). The reverse primer could not be used in the sequencing reaction because the PCR product contained part of pBluescript incorporating this sequence. This would have led to priming at multiple sites and consequently incorrect sequence data.

There are several points to note in the sequence presented in Figure 7.16. Firstly, pcr1 represents the sequence between the two *Hinc* II sites. Secondly, nucleotides 49 through to 157 represent pBluescript sequence that made up part of the PCR product as a result of amplifying with the M13 reverse primer. The *Bam* HI site of pBluescript into which K2 DNA was originally ligated is at nucleotide 158 and hence sequence derived from K2 DNA lies between nucleotides 158 and 446. These features are illustrated diagramatically below the nucleotide sequence in Figure 7.16.

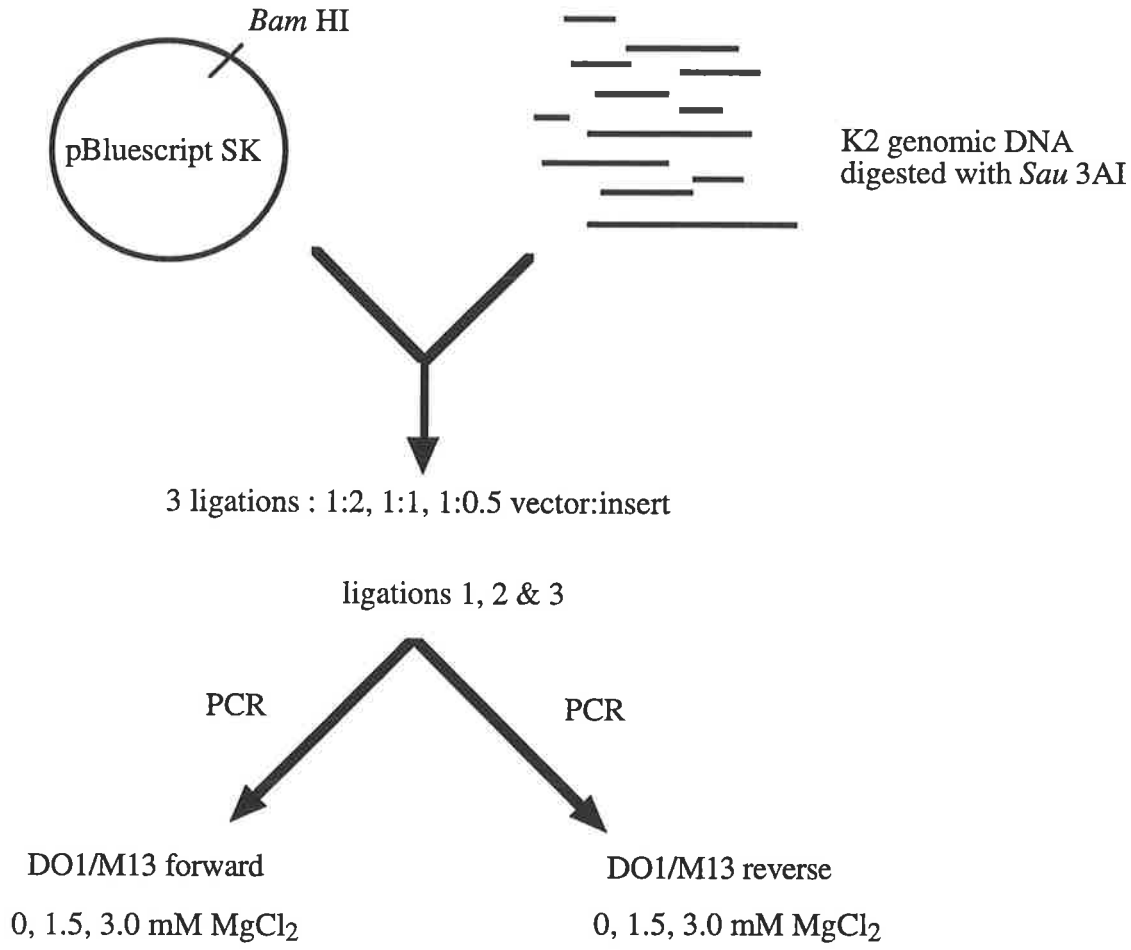


Figure 7.10 PCR strategy using DO1 and M13 vector-specific primers.

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Figure 7.11 PCR with DO1 and vector-specific primers.

A. Products from reactions using ligations as templates and DO1 and vector-specific primers.

lanes 1 and 20, pGEM molecular weight markers.

lanes 2-4, ligation 1 as template, DO1 and M13 reverse primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 5-7, ligation 1 as template, DO1 and M13 forward primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 8-10, ligation 2 as template, DO1 and M13 reverse primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 11-13, ligation 2 as template, DO1 and M13 forward primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 14-16, ligation 3 as template, DO1 and M13 reverse primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 17-19, ligation 3 as template, DO1 and M13 forward primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

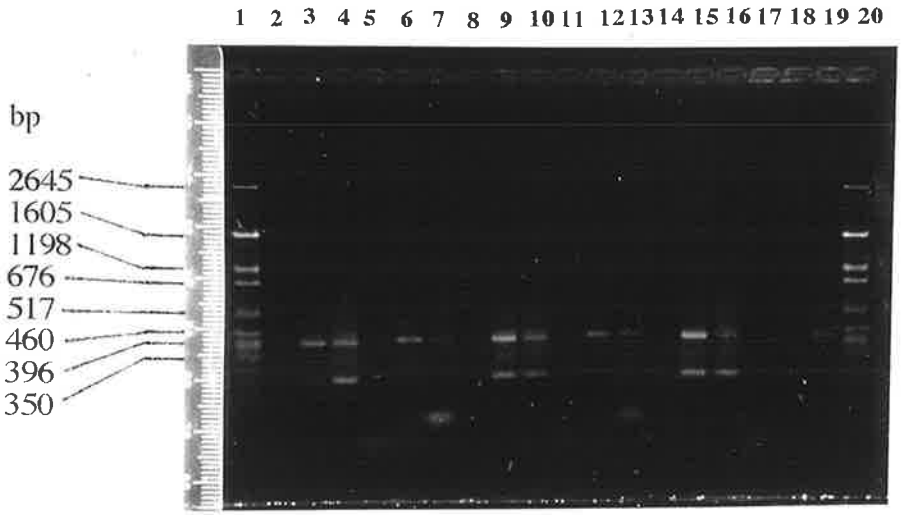
B. Positive control PCRs

lane 1, pGEM molecular weight markers.

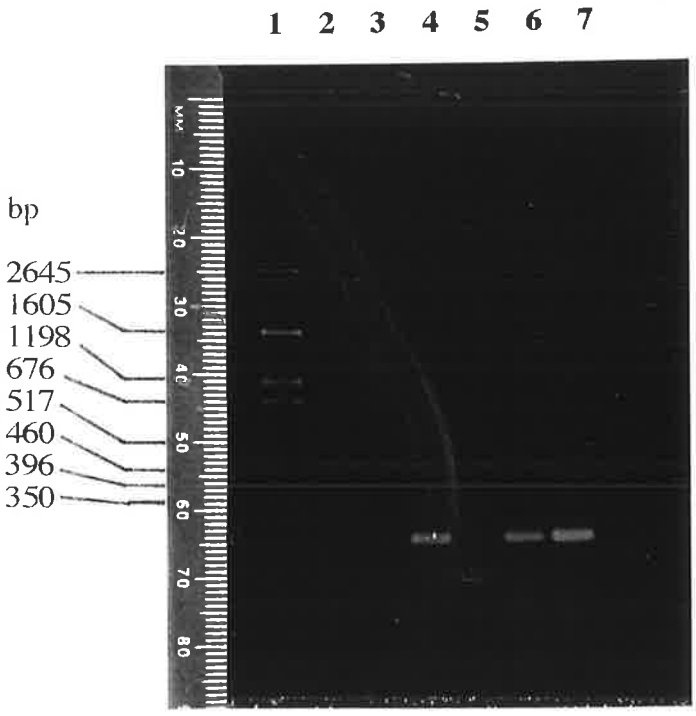
lanes 2-4, cut and religated pBluescript as template, M13 forward and reverse primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

lanes 5-7, uncut pBluescript as template, M13 forward and reverse primers, 1.5, 3.0 and 4.5 mM $MgCl_2$ respectively.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
bp
2645
1605
1198
676
517
460
396
350

B


1 2 3 4 5 6 7
bp
2645
1605
1198
676
517
460
396
350

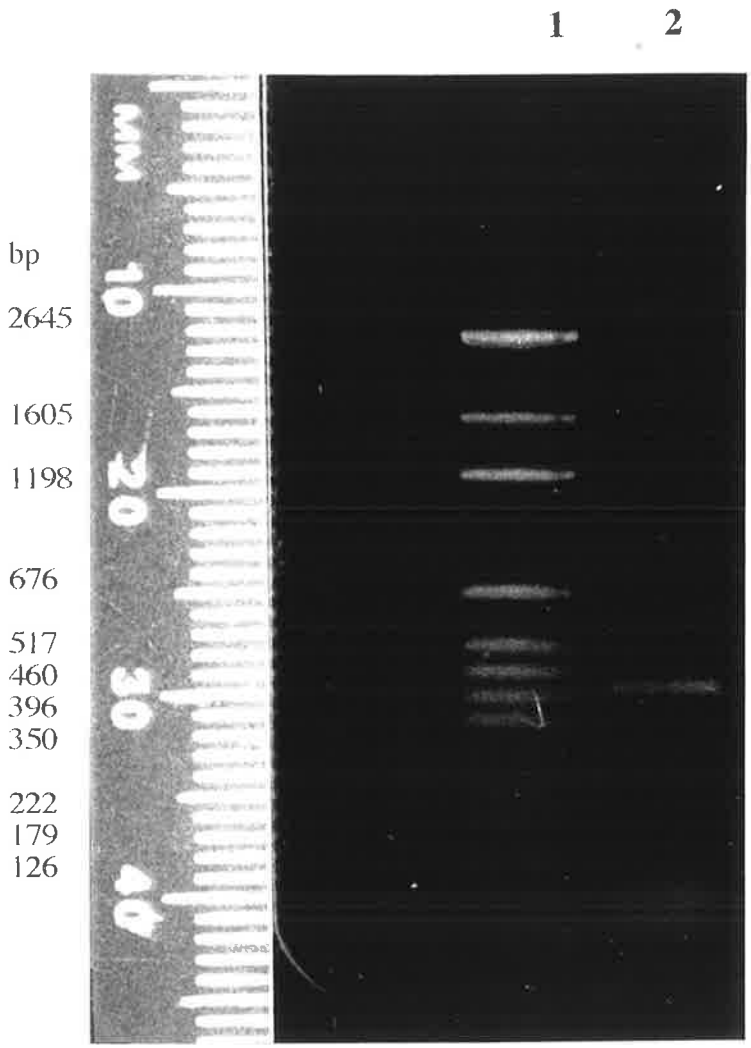


Figure 7.12 Reamplification of 400bp product by stab-PCR.

Lane 1, pGEM molecular weight markers; lane 2, 400bp product from DO1 and M13 reverse primers.

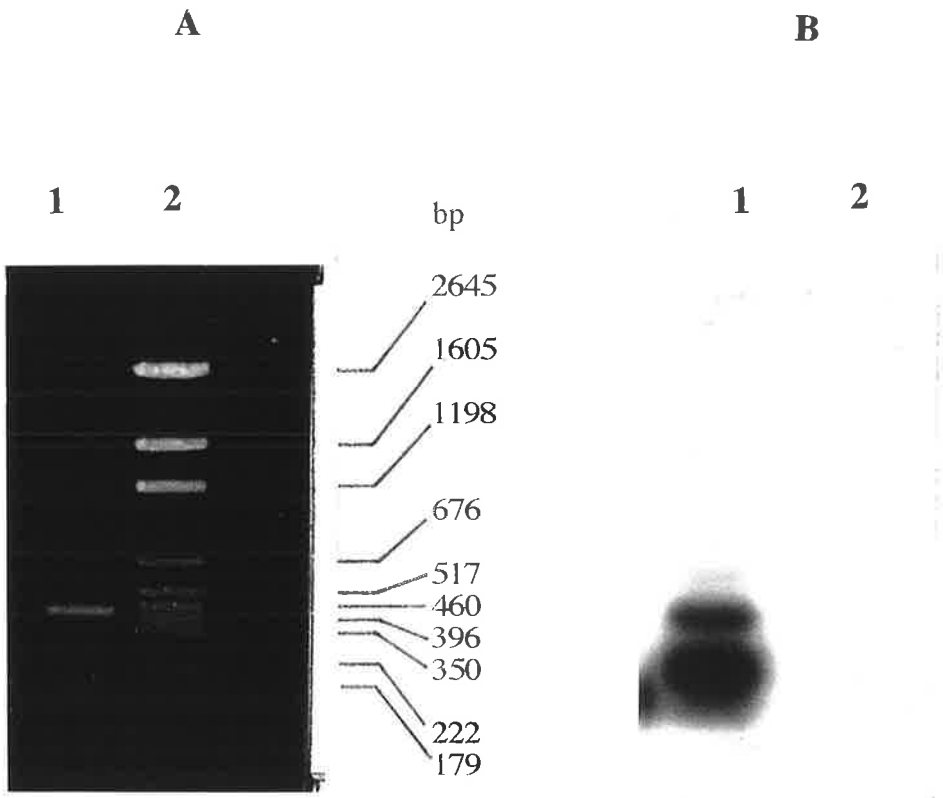


Figure 7.13 Hybridisation of DO1 to PCR product pcr1.

Lane 1, 400bp product (pcr1) from DO1 and M13 reverse primers; lane 2, pGEM molecular weight markers. Membrane was hybridised at 34°C and washed at 40°C in 6X SSC/0.1% SDS. A. Ethidium bromide-stained 0.8% agarose gel; B. autoradiograph of gel after transfer and hybridisation.

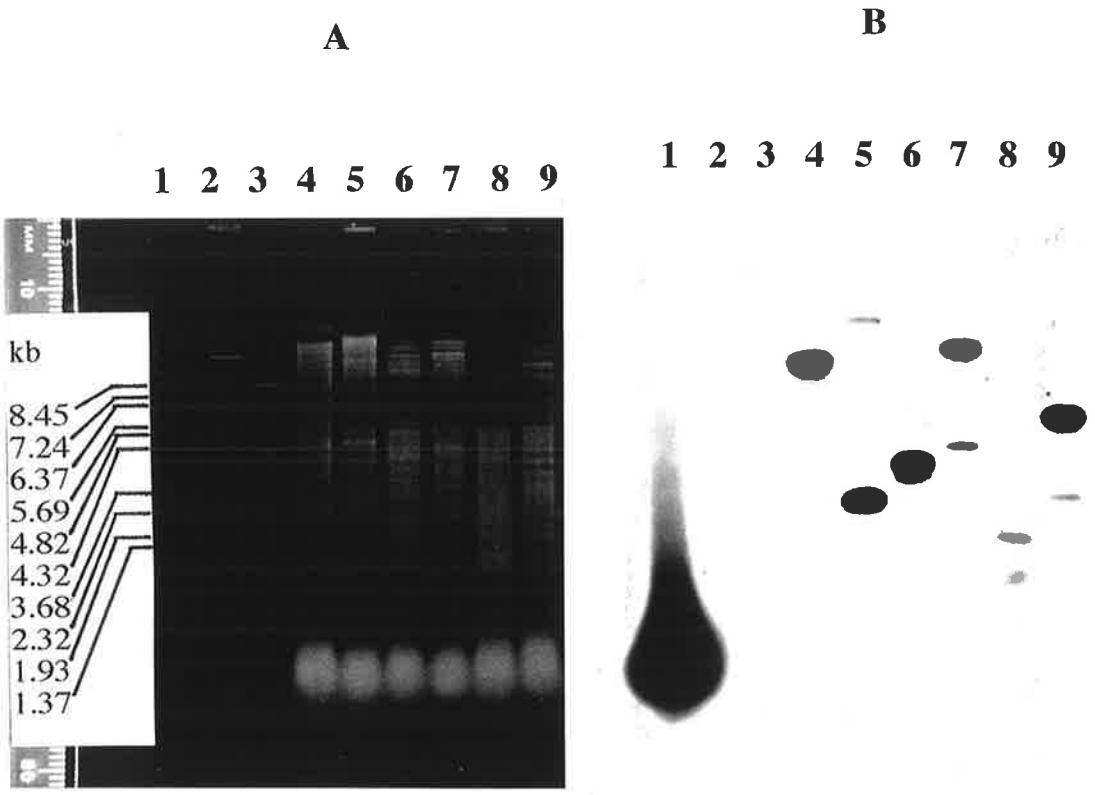


Figure 7.14 Hybridisation of pcr1 to *S. ruminantium* K2 restriction endonuclease-digested DNA.

Lane 1, pcr1; lanes 2-3, molecular weight markers; lanes 4-9, K2 DNA was digested with: lane 4, *Eco* RI; lane 5, *Sph* I; lane 6, *Eco* RV; lane 7, *Hind* III; lane 8, *Sau* 3AI; lane 9, *Hind* II. Membrane was hybridised at 60°C and washed stringently in 0.5X SSC/0.1% SDS at 42°C. A. Ethidium bromide-stained 0.8% agarose gel; B. autoradiograph of gel after transfer and hybridisation.

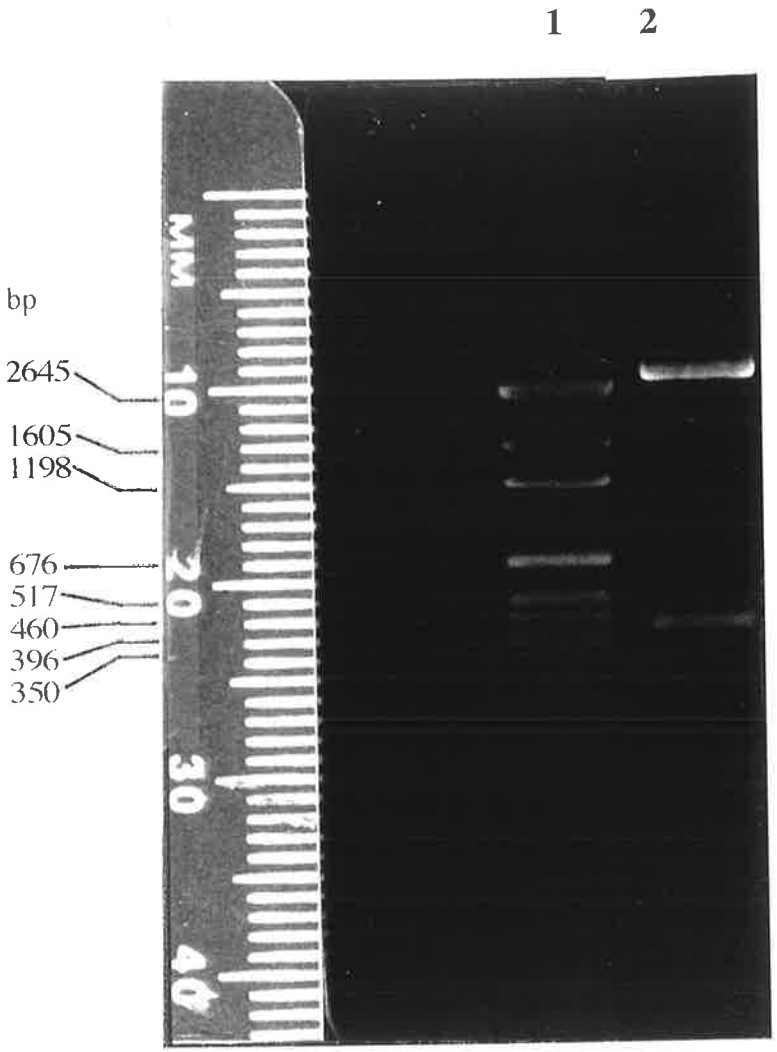


Figure 7.15 Cloning of pcr1 into pBluescript S/K.

Lane 1, pGEM molecular weight markers; lane 2, plasmid clone pBS.pcr1 double-digested with *Cla* I and *Xho* I to release 400bp pcr1 insert.

Hinc II
ACGACTCACTCATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTC|CA 50

CACAGGAAACAGCTATGACCATGATTACGCCAAGCCCGAAATTAACCCTC 100

ACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGA 150

Bam HI
ACTAGTG|GATCCGGCTATGAAGCATATTGCTCCCCTAAATCCCGTAGTGG 200

CTCTTGGTATTATGATGAATACGCTTGGAGGTGCTGCACCAAGCGTGGCG 250

GACAGCTTCCGGCCAATGGC**AGCTGTACTGATTATGTTTG**CAAAGTTTAA 300

TACATTGGGGGGCGACAGAGTATCAAAATAGTCTATCTATGGAGTATTTT 350

TCCTGCCAGAAATTTTTGCCGGTAAATAATACTTCCGGCTGGCAATATTT 400

Hinc II
TAGTAACGATGGAAATCCCGCTGCATGGTCATAGCTGTTTCCTGTGT|GACG 450

GTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGATCCACTAGT 500

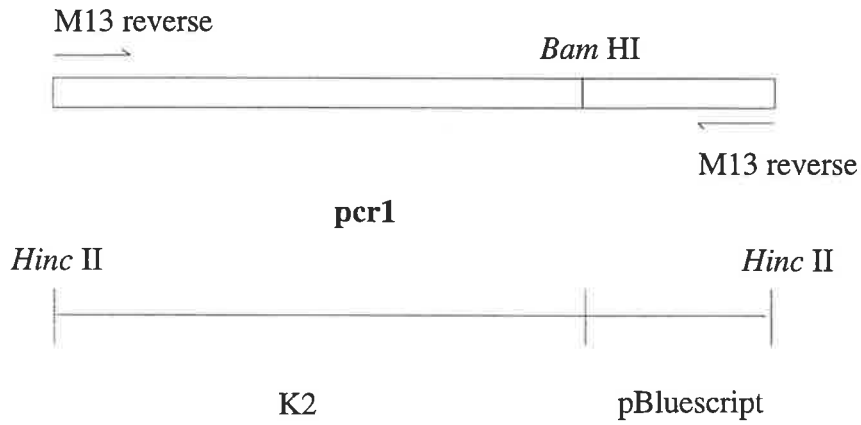


Figure 7.16 Nucleotide sequence derived from pBS.pcr1 using M13 forward primer. Nucleotides are numbered from the 5' end. Underlined sequences are M13 reverse primer-binding sites. Boxed sequence is putative DO1-hybridisation site; nucleotides in bold type represent those in common with DO1.

Unfortunately, it is evident that pcr1 displays the sequence of the M13 reverse primer on both ends; hence it appears that it was derived from a mispriming by the M13 reverse primer alone and not by a combination of DO1 and the M13 reverse primer as expected.

7.2.6 Internal amino acid sequencing and degenerate oligonucleotide primed-PCR

A sample of the tannin acylhydrolase was purified as described in Chapter 2 (section 2.5.12) and subjected to proteolysis with Endoproteinase Lys-C. This endoproteinase was chosen because it specifically cleaves peptide bonds C-terminally at lysine and consequently produces fewer peptides than trypsin which cleaves C-terminally at either lysine or arginine. The resulting peptides were eluted from the gel and separated on reverse-phase high performance liquid chromatography (HPLC) according to the protocol described in Chapter 2 section 2.5.12. The chromatograms of the non-protein containing blank and the protein sample are presented in Figure 7.17. Due to the low amount of starting material, the peptide peaks were very small and consequently the choice of peptides to sequence was very limited. The most abundant peptide, as judged from the peak height on the chromatogram in Figure 7.17, was then subjected to N-terminal protein sequencing and yielded the sequence listed in Table 7.2. This sample corresponded to 15 pmol of peptide and generated a sequence that was clear and unambiguous until the signal was no longer detectable. As the sequencing process nears the C terminus of a peptide, short hydrophilic di- and tri- peptides can be stripped from the sequencing column and lost which probably explains why a C terminal lysine was not found as expected.

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Figure 7.17 Separation of Endo-Lys-C-derived peptides by HPLC. The signals from the 214nm detector are shown, representing the signal from peptide bonds. Top chromatogram (solid line) illustrates separation profile from tannin acylhydrolase digested with Endo-Lys-C; Bottom chromatogram (dashed line) illustrates separation profile from non-protein blank digested with Endo-Lys-C. Arrow indicates peptide peak chosen for sequencing.

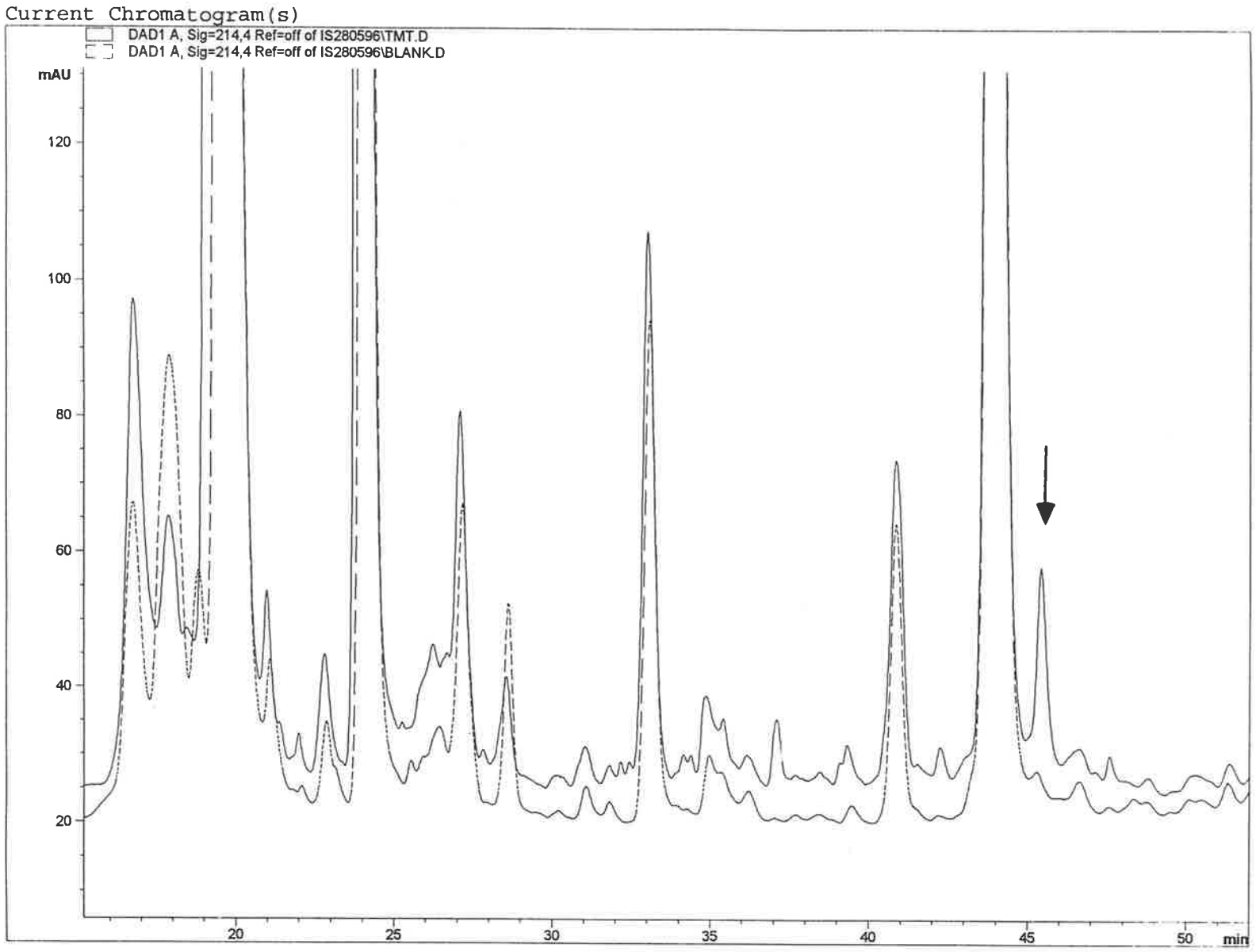
Current Chromatogram(s)
DAD1 A, Sig=214,4 Ref=off of IS280596\TMT.D
DAD1 A, Sig=214,4 Ref=off of IS280596\BLANK.D
mAU
120
100
80
60
40
20
20
25
30
35
40
45
50
min

Table 7.2 Amino acid sequence of Endo-Lys-C-derived peptide

Residue No.	Amino acid	IUB code
1.	Leucine	L
2.	Methionine	M
3.	Asparagine	N
4.	Proline	P
5.	Leucine	L
6.	Asparagine	N
7.	Phenylalanine	F
8.	Isoleucine	I
9.	Glycine	G
10.	Threonine	T

This peptide is encoded by the following possible nucleotide sequence:

L	**M**	**N**	**P**	**L**	**N**	**F**	**I**	**G**	T
TTA	ATG	AAC	CCG	TTA	AAC	TTT	ATA	GGG	ACG
TTG		AAT	CCA	TTG	AAT	TTC	ATC	GGA	ACA
CTG			CCC	CTG			ATT	GGC	ACC
CTA			CCT	CTA				GGT	ACT
CTC				CTC					
CTT				CTT					

A degenerate oligonucleotide was synthesised as the reverse complement of the residues highlighted in bold type. This oligonucleotide contained 3 inosine residues to reduce the number of synthetic oligonucleotides required and was designated DO2:

5'	CC	TAT	AAA	GTT	IAA	IGG	GTT	CAT	3'
		G	G	A	IAG		A		
		A							

DO2: 23-mer, 48-fold degeneracy based on the published convention that inosine =1

Degenerate oligonucleotides DO1 and DO2 were used as primers in degenerate oligonucleotide-primed polymerase chain reactions (DOP-PCR) with K2 genomic DNA template (Chapter 2 section 2.5.13). Reaction conditions such as Mg^{2+} concentration, template DNA concentration and primer annealing temperature were optimised such that a single product was obtained. The results illustrated in Figure 7.18 show a product of approximately 170bp in size was generated when 5µg DNA was used as template. This product was only produced in reactions containing both primers and was absent in control reactions containing a single primer. This same product was also generated in reactions containing both primers and 0.5µg and 0.05µg template DNA respectively; however additional products were also evident. These products possibly arose from non-specific annealing of primers to template as a consequence of the high ratio of primer to template concentration.

Initial attempts to clone the 170bp product were unsuccessful and consequently it was necessary to repeat the DOP-PCR in order to provide more product for cloning. Identical reaction conditions were used in this repeat experiment. Unfortunately, several products in addition to the expected 170bp product arose from the repeat experiment (Figure 7.19) As a consequence, the 170bp product was reamplified by stab-PCR in order to purify it and the products of this PCR were then cloned into the *Hinc* II site of pBluescript SK. Plasmid mini-preps were performed on 14 randomly chosen clones and analysed by gel electrophoresis (Figure 7.20). Eight of the fourteen clones contained inserts considerably smaller than expected, possibly arising from spurious products of the PCR. These clones were not analysed any further. Of the remaining six clones, three were of the expected size, one was slightly smaller and two were slightly larger than expected. This result indicates that the original 170bp

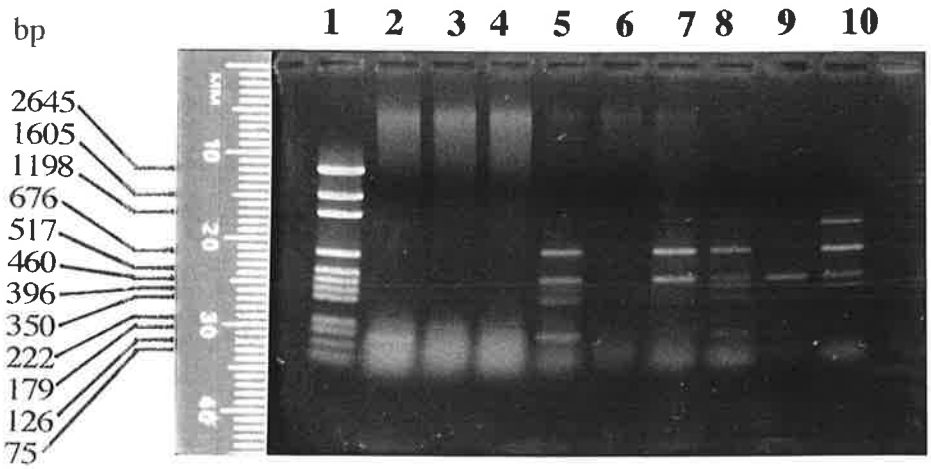

Figure 7.18 PCR using degenerate primers derived from protein sequence of K2 tannin acylhydrolase.

Lane 1, pGEM molecular weight markers; lanes 2, 5 and 8 contain 5.0, 0.5 and 0.05µg K2 template DNA respectively and were primed with both DO1 and DO2; lanes 3, 6 and 9 contain 5.0, 0.5 and 0.05µg K2 template DNA respectively and were primed with DO1 only; lanes 4, 7 and 10 contain 5.0, 0.5 and 0.05µg K2 template DNA respectively and were primed with DO2 only.

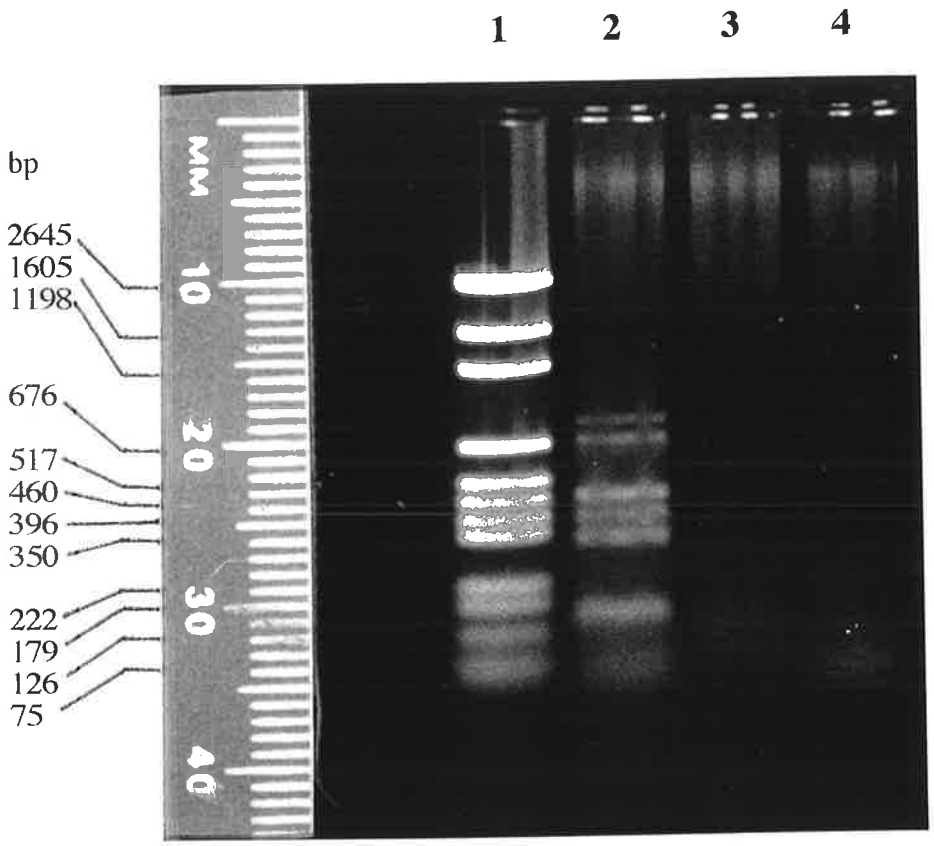

Figure 7.19 Repeat PCR using degenerate primers derived from protein sequence of K2 tannin acylhydrolase.

Lane 1, pGEM molecular weight markers; lane 2, products from DOP-PCR with both DO1 and DO2; lane 3, DOP-PCR with DO1 primer only; lane 4, DOP-PCR with DO2 primer only.

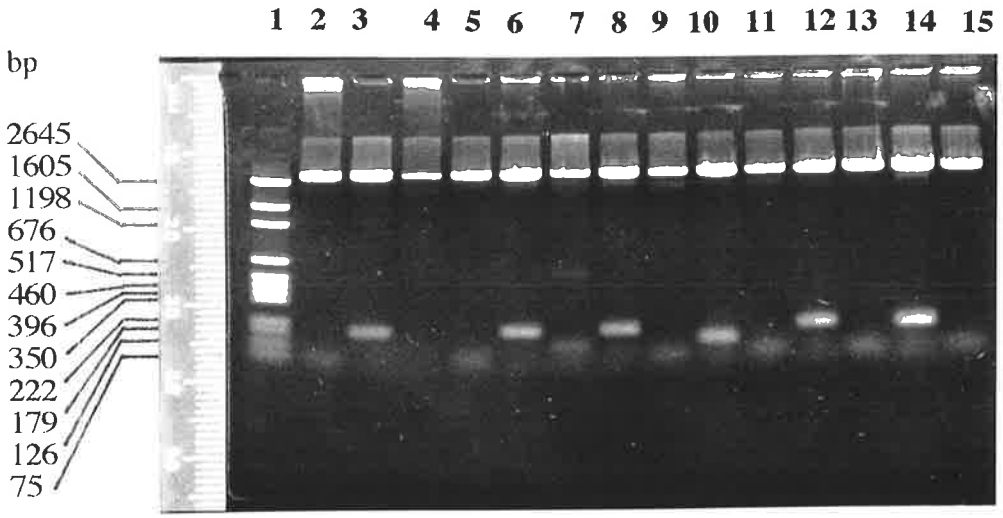

Figure 7.20 Cloning of products from DOP-PCR into pBluescript S/K.

Lane 1, pGEM molecular weight markers; lanes 2-15, mini-preps of pBluescript containing inserts double-digested with *Cla* I and *Xho* I. Lanes 3, 10 and 12 contain plasmids pBS.pcr2, pBS.pcr9 and pBS.pcr11 respectively.

product was in fact a mixture of similar-sized products and not a unique sequence. A representative of each of these (pBS.pcr2, pBS.pcr9 and pBS.pcr11) was then sequenced by automated cycle sequencing using dye-labelled M13 forward and reverse primers. The sequence of each PCR product appears in Figure 7.21. While displaying the sequence of DO1 and DO2 on each end, none of these products show the internal nucleotide sequence required to give the amino acid sequence determined previously. In addition, the internal nucleotide sequences of pcr2, pcr9 and pcr11 show no homology. These results therefore suggest that pcr2, 9 and 11 represent 3 unrelated sequences resulting from mispriming of both DO1 and DO2 in each case and do not represent a portion of the tannin acylhydrolase gene.

pcr2 CGGGGGATTT ACATGTTCCC GATATGGGAT GTATCCGCCA GCCAAAAGGA 50

pcr9 CGGGGGATTT ACATGTTCCT AAAGTTTGAA TTTGATTATA ATATCTTGTT 50

pcr11 CGGGGGATTT ATATGTTTGG ACGGCGGCTG TGGTGGCATG AACAGGTTAA 50

pcr2 GACGTTACCG GCAGATCGGC AGCATGGACT CCGCCGCTAT ATAAGGATAG 100

pcr9 AAATCTGTAT CACCTAAGCT CAAAGAATAC CCGAAAACTC CATGAACCCC 100

pcr11 GGACGTTCTG GGCTGGAATA ATGATTGCAA GGCAAAGTTT ATGGTTTGCA 100

pcr2 GCAAAAGCCT ATGATAGCTC CTGCCTTTCC TATGGTTCGC ATGAACCCCC 150

pcr9 CTCAACTTCA TCGG 114

pcr11 AGCACCATAT TTCCCACGCA GCTTCGGCTT TTTTTCCATC ACCCTATGAA 150

pcr2 TCAACTTCAT CGG 162

pcr11 CCCCCTCAAC TTCATCGG 168

Figure 7.21 Nucleotide sequence of cloned PCR products generated by DOP-PCR with DO1 and DO2. Nucleotides are numbered from the 5' end. Underlined nucleotides represent sequence of DO1 and double-underlined nucleotides represent reverse complement sequence of DO2.

7.3 Discussion

7.3.1 Expression of K2 tannin acylhydrolase in *Escherichia coli*

Many genes have been cloned from a number of rumen bacteria, and in all but two cases (the glutamine synthetase from *Butyrivibrio fibrisolvens* (Goodman and Woods, 1993) and the malic enzyme from *Streptococcus bovis* (Kawai *et al*, 1996)), these have coded for glycosidases or polysaccharidases, in particular endoglucanases and xylanases (see Wallace, 1994). There are two reasons for this bias. Firstly it represents the importance which has been placed on fibre digestion in the rumen; but more significantly it can be attributed to the fact that plate assays, often using chromogenic substrates, have been developed for such enzymes which allow the rapid screening of a large number of recombinant bacteria/phage for cloned enzyme activity. It was therefore a logical first-step to develop the rhodanine-gallate assay into such a plate assay and then use it to screen recombinant libraries of *S. ruminantium* K2 DNA for the expression of tannin acylhydrolase activity.

The success of such an approach relies on the satisfaction of two criteria. Firstly, it must be possible to construct a complete library of the desired organism in the chosen cloning system. This means that every possible sequence of the organism of interest must be represented in the library at least once. Secondly, the cloned genes must be expressed in the chosen host, either from their own promoters or from host encoded promoters, and the expressed products must retain function in order to be detected.

For *S. ruminantium* K2, in order to satisfy the first criterion it was necessary to construct the recombinant libraries in *E. coli* bacteriophage vectors, because great difficulty was experienced when trying to generate complete genomic libraries of K2 in *E. coli* plasmid vectors. Although not officially reported, this problem has been experienced by other workers attempting to generate recombinant libraries of *Selenomonas* in *E. coli* using plasmid vectors (Flint and Brooker, *pers. comm.*). Possible explanations for this problem are not readily apparent. Restriction/modification of K2 DNA by *E. coli* ~~can be discounted~~ is unlikely because strains were chosen although there are illegitimate recombination systems. which were deficient in all restriction and modification genes. The *E. coli* strain ER2267 has

been developed by New England Biolabs specifically for the purpose of cloning problematic DNA and has the phenotype McrA$^-$, McrBC$^-$, *Eco* K(R and M)$^-$, Mrr$^-$. Analysis of recombinant plasmids revealed relatively small insert sizes (<2.0 kb) and so it was initially suggested that large fragments of *Selenomonas* DNA were unclonable because they somehow caused plasmid instability and subsequent non-replication. However, large pieces of *Selenomonas* K2 DNA were successfully cloned into two different phage vectors, which would suggest that if such a "toxic" mechanism exists, it is specific to plasmid replication and not phage replication. Plasmid instability may have been enhanced in high copy-number plasmids such as pUC-based vectors, however when the ~~lower copy number~~ vector pBR322 was used this also failed to produce complete libraries. Until many *Selenomonas* genes have been successfully cloned and analysed, the molecular basis for this apparent incompatibility between *Selenomonas* DNA and *E. coli* plasmids will remain unanswered.

As indicated above, the cloning of genes encoding enzymes by detecting activity of these enzymes in a heterologous host relies on gene expression. In order for the cloned gene to be expressed, the gene's promoter must usually also be cloned and must function in the host. In very rare circumstances using vectors such as pUC or λgt11 which contain the *lac* promoter, transcription can be driven by the host promoter. If the inserted DNA is cloned in the correct orientation and reading frame, transcription from the *lac* promoter may result in the correct mRNA being transcribed and subsequently translated, resulting in production and function of the desired enzyme. Assuming the average weight of an amino acid to be 136.75 and the size of the K2 tannin acylhydrolase to be 60 kDa, it can be calculated that the tannin acylhydrolase contains approximately 439 amino acids and is therefore encoded by a fragment of DNA approximately 1.3 kb in size. Furthermore, if it is assumed that the size of the K2 genome is similar to that of *E. coli* (approximately 4.5 Mb) it can be calculated that fusion of any portion of a 1.3 kb gene will occur once in every 3462 nucleotides and that one out of every six fusions will be in the proper orientation and reading frame. Therefore it is calculated that an in-frame fusion resulting in expression of the tannin acylhydrolase from the *lac* promoter would occur on average once in 21,000 recombinants. From this probability it is obvious that such an event

would have been impossible to detect in the plasmid libraries constructed in vectors containing the *lac* promoter because the best such library contained only 7,000 recombinants. Such an event may have occurred in the λgt11 library but given the size of this library after amplification ($1x10^{13}$ pfu/ml) and the rarity of this event, it is unlikely that enough clones were screened to detect it.

The probability of detecting expression of a cloned gene from its own promoter is much higher because it is independent of fragment orientation and reading frame and is therefore a mathematical function of gene, insert and library size. Therefore it is likely that the failure to detect a tannin acylhydrolase-expressing recombinant in any of the libraries was due to the inability of the *Selenomonas* promoter to function in *E. coli*. There is some previous experimental evidence to support this claim. Attwood (1991) cloned and sequenced a cryptic plasmid from *S. ruminantium* and conducted some transcript-mapping experiments. The results of these experiments showed that the transcription initiation site (TIS) of a plasmid encoded gene was different when the plasmid was replicating in *Selenomonas* compared to when it was cloned in *E. coli*, suggesting that a different sequence acted as the promoter in each case. Similar results have been observed for other rumen bacterial species. For example it has been shown that transcription is initiated at different sites in the native host compared to *E. coli* for 3 endoglucanase genes; *celB* (*Ruminococcus flavefaciens* FD-1, Vercoe *et al*, 1995b), *celA* (*Ruminococcus albus* AR67, Vercoe and Gregg, 1995) and *celD* (*R. flavefaciens* FD-1, Vercoe *et al*, 1995a). It is therefore suggested that *S. ruminantium* may be sufficiently unrelated to *Escherichia coli* that its promoter consensus sequence is unrecognised by the *E. coli* RNA polymerase σ-factors. This theory cannot be proven until a number of *Selenomonas* genes have been sequenced and the 5'-untranslated regions (UTR) analysed for a *Selenomonas* consensus motif.

However, the promoter-selection experiments described in this chapter could provide a basis for future experiments designed to solve this issue. The K2 sequences isolated in this experiment were inherently those which were recognised by *E. coli* RNA polymerase but they were not

necessarily true *Selenomonas* promoters. By sequencing these fragments and then conducting primer extension analyses using RNA isolated from both *Selenomonas* and from *E. coli* transformed with the cloned fragments, it would be possible to determine the TIS in both genotypes. It would then be possible to confirm whether or not the cloned fragments which drove transcription of the *cat* gene in *E. coli* were indeed *Selenomonas* promoters or not.

Under ideal circumstances, screening for *Selenomonas* promoters would have been conducted in *Selenomonas* using a promoter selection vector, instead of in *E. coli*. In this way the distinction between true *Selenomonas* promoter sequences and those that act as promoters in *E. coli* ie "false" promoter sequences, would not need to be made. However, as previously noted, *S. ruminantium* has not been successfully transformed *in vitro* and so this experiment was not possible. Until knowledge of *Selenomonas* genetics becomes advanced enough to overcome this obstacle, it may be more productive to attempt to clone *Selenomonas* genes using bacterial systems more closely related to this genus than *E. coli*, such as *Clostridia*. Such an approach has been used by Whitehead and Hespell (1990) and Whitehead *et al* (1991) to clone xylanase genes from *Prevotella ruminicola* (formerly *Bacteroides ruminicola*) using *Bacteroides fragilis*, *B. uniformis* and *B. thetaiotaomicron* expression systems.

7.3.2 Tannin acylhydrolase-specific polyclonal antibodies

The failure to raise tannin acylhydrolase-specific polyclonal antibodies in the rabbits is probably due to one of two reasons. Firstly it could be because not enough protein was used as the immunogen, or secondly, it could be because the protein used was not particularly immunogenic. Potentially, rabbits may already be immunised against Selenomonads since members of this genus are commonly found in the oral cavity and in the gastrointestinal tract. However, given that a mixture of three different proteins (fraction #24) was injected in the last two boosts and that none of these produced a marked immune response, the first possibility provides the most likely explanation. When material is limited as in this case, the only option is to inject as much as possible. In future, if this experiment was to be repeated, it would be better to increase the amount of material available for immunisations by first purifying the tannin

acylhydrolase. Such a procedure would then provide a source of immunogen that could be mixed with Freund's adjuvant and injected subcutaneously in the more common manner, thereby avoiding problems associated with proteins contained in polyacrylamide gel. The immune sera should also be preabsorbed against control *Selenomonas* extract before it is used in immunoblotting experiments in order to reduce the background of non-specific binding.

7.3.3 Use of a single degenerate oligonucleotide

Cloning genes by probing libraries with pools of oligonucleotides based on short stretches of amino acid sequence is an established technique which has been used successfully many times. Recent examples can be found in Kawai *et al* (1996), Williams *et al* (1995), Potuckova *et al* (1995), Smid *et al* (1995), Chui *et al* (1995), Kawahara *et al* (1995), Falcone *et al* (1994), Lonetto *et al* (1994), Berkelman *et al* (1994), Guicherit *et al* (1994) and Heinrichs *et al* (1993). The length and level of degeneracy of the probes used in these experiments has been remarkably variable, ranging from a 16-fold degenerate 14-mer (Potuckova *et al*, 1995) to a 1,152-fold degenerate 29-mer (Heinrichs *et al*, 1993).

One of the problems associated with this type of hybridisation experiment is the relatively low signal:noise (background) ratio. Because the probes are so short, even a 100% match between the target sequence and the probe sequence will not produce a particularly stable DNA duplex. Consequently, the hybridisation and washing conditions must be very carefully controlled such that the probe is not stripped from the target. Therefore in general, conditions are employed which inevitably generate both true matches and also mismatches; the true matches are then confirmed by alternative techniques such as sequencing or by hybridisation to a second, more specific probe. Determining the most appropriate temperature at which to conduct the hybridisations is another of the difficulties associated with these experiments. One can use estimates of probe melting temperature (T_m) as a guide and then hybridise at 15-20°C below the oligonucleotide with the lowest T_m, as recommended by Sambrook *et al* (1989). Alternatively, hybridisations can be conducted in solutions of 3M tetramethyl ammonium chloride (TMAC) instead of standard saline citrate (SSC). In 3M TMAC, the effect of G/C content on melting

temperature is negated and melting temperature therefore becomes a linear function of probe length. This therefore means that the pool of oligonucleotides can be regarded as having a single melting temperature and hence hybridisations can be conducted at temperatures much closer to the estimated T_m. The optimum hybridisation temperature can also be determined empirically. With bacteria which contain no introns, one way of doing this is to probe a Southern-transfer of restriction endonuclease-digested DNA from the target organism with the radiolabelled, degenerate oligonucleotides. In theory, if the oligonucleotides are derived from a unique sequence, it should be possible to determine hybridisation and washing conditions such that a single hybridising band can be detected in such an experiment. In practice however, the results are not so clear-cut, as illustrated by the results presented in this chapter (section 7.2.4). Difficulties in interpreting the results of such experiments arise because the signal from the true target match may be weak due to the low number of copies of target sequence bound to the membrane. This is compounded by the fact that the background of non-specific hybridisation can be high because short probes inevitably become trapped in the high molecular weight sequences near the top of the membranes and these are often impossible to remove without stripping the probe off the true target site.

It can be seen that the verification of recombinant clones isolated by hybridisation with degenerate oligonucleotides must be a priority before further work can proceed. Unfortunately, in the case of the work presented in this thesis, the only possible way of confirming the isolation of the tannin acylhydrolase gene was to sequence each DO1-positive clone and match this sequence to the amino acid sequence derived from the amino terminus of the tannin acylhydrolase protein. However, such a characterisation of λgt11 clones is a very time consuming task and since a number of DO1-positive clones were initially detected, time restrictions meant that this work had to be cut short before the correct clone had been identified. Given more time, it is believed that this experimental approach would be successful and result in the isolation of the K2 tannin acylhydrolase gene.

As a means of using the DO1 sequence to produce a longer and more tannin acylhydrolase-specific probe, a PCR strategy was tested which employed DO1 and a vector-specific primer. This strategy was similar to previously published methods except that a ligation reaction was used as the source of template DNA instead of subcloned DNA. Previous authors have used a degenerate oligonucleotide derived from the gene of interest and a vector-specific primer to amplify the 5' or 3' ends of a gene from a recombinant library (see Williams *et al*, 1995 for a recent example). In the work presented here, a ligation reaction was used in order to avoid the problems of generating plasmid libraries of *Selenomonas* DNA; ie it was presumed that the desired sequence may ligate to the plasmid vector but it may not necessarily have been represented in a library if *E. coli* was transformed with this ligation. The isolation of an incorrect sequence by this approach highlights the necessity of including single-primer control reactions in this type of experiment; only PCR products derived from a combination of both primers should be investigated further. The result also highlights the problems associated with hybridising with a degenerate oligonucleotide mix, since what appeared to be a DO1-positive sequence turned out to be a similar but mismatched sequence. From the sequence illustrated in Figure 7.16 it appears that DO1 was most probably hybridising to the boxed sequence comprising nucleotides 221 to 240, within which 15 nucleotides match the sequence of DO1 exactly.

7.3.4 PCR with two sets of degenerate oligonucleotide primers

Since the advent of PCR technology, the cloning of genes by degenerate oligonucleotide primed-PCR (DOP-PCR) has overtaken hybridisation with a single degenerate oligonucleotide as the method of choice. A search of the current literature reveals literally hundreds of examples of genes cloned using this method from a vast array of different organisms. A prerequisite is two separate amino acid sequences from the protein of interest so that two opposing degenerate primers can be designed which will generate a product by amplification. Many authors have had the advantage of cloning genes for which sequence (either nucleotide or protein) is available and they have often been able to design primers around regions of known sequence conservation. The advantages of this are two-fold. Firstly, by this approach genes for related

proteins can be cloned from completely unrelated organisms with little knowledge of the nucleotide sequence of the target gene. Secondly, by designing primers to areas of sequence conservation, a rough estimate of the size of the desired PCR product from the organism of interest can be made, thereby facilitating the isolation of the correct sequence.

Without any knowledge of sequence conservation upon which to design primers, amino acid sequence data must be gained by purifying and sequencing the protein of interest. As was the case in the work presented here, this inherently means that when an internal peptide is sequenced it is impossible to predict where this sequence lies in the protein relative to the amino terminus. Consequently it is impossible to predict the size of the desired PCR product. In the experiments presented in this chapter, the PCR conditions were optimised such that a single product was formed and only when both degenerate primers were included in the reaction. However it appears that these conditions selected for mispriming events which resulted in the incorrect sequence being cloned. The correct PCR product may in fact be one of the other products shown in Figure 7.19. If this work were to continue, these products should be cloned and sequenced in order to unequivocally determine whether or not any of them represent a part of the K2 tannin acylhydrolase gene. This could easily be achieved by comparing the translated sequences to the amino acid sequence previously determined from isolated protein. Alternatively, the PCR products could be used to probe RNA isolated from tannin-induced and non-induced cells of K2. If the PCR products are derived from the tannin acylhydrolase gene, one would expect a greater signal of hybridisation to the RNA from the induced cells than from the uninduced cells, assuming that the control of tannin acylhydrolase gene expression is at the level of messenger RNA.

Conclusion

The experiments detailed in this chapter describe a variety of different approaches to clone the gene encoding the tannin acylhydrolase in *S. ruminantium* K2. Although unsuccessful, a foundation for future work has been provided by determining the partial sequence of the amino terminus and of an internal peptide. Degenerate oligonucleotides based on this amino acid

sequence have been designed and synthesised and may prove to be successful in isolating the tannin acylhydrolase gene by DOP-PCR. In addition, *Selenomonas* sequences have been cloned which act as promoters in *Escherichia coli*. These sequences may form the basis for future projects investigating the structure of *Selenomonas* promoters. Finally, genomic libraries of *S. ruminantium* K2 have been constructed in two separate *E. coli* bacteriophage vectors which may prove useful for isolating other *Selenomonas* genes.

CHAPTER 8 General Discussion.

8.1 Introduction

The inhibition of ruminant digestion by dietary tannins is a significant factor limiting livestock production in many areas of the world. Tannins inhibit ruminant digestion by complexing with proteins, carbohydrates and minerals forming insoluble complexes which are poorly digestible, and by inhibiting microbial growth and metabolism. Since the productivity of a ruminant is intimately dependent on the well-being of its rumen microbial population, inhibition of microbial activity by dietary tannins is regarded as an important contributor to the overall anti-nutritional effect of ingested tannins on ruminant nutrition.

Some ruminants, for example feral goats and camels in Australia, appear to efficiently utilise tannin-rich plants. This has led to the hypothesis that such animals may contain novel rumen microorganisms which by some mechanism contribute to these animals' ability to thrive on high levels of ingested tannins. There are two possible mechanisms by which this could be achieved. Firstly the rumen of such animals may contain a population of tannin-resistant organisms which are able to carry out their normal roles in the rumen, uninhibited by tannins. Alternatively or in addition, the rumen may contain novel organisms which can alter the chemical structure of tannins thereby reducing their toxic effects. The identification of microorganisms which play a role in the detoxification of tannins is now receiving world-wide attention. A tannin-resistant *Streptococcus* has been isolated by three separate research groups 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, analogous to the degradation of fibre.

Therefore, this research project aimed to identify other rumen microorganisms which may contribute to feral goats' ability to digest tannin-rich forage and to determine the mechanisms by which they achieve this.

8.2 General Discussion

The initial aims of this research project have been partially fulfilled, however many fundamental questions have been raised by this work and remain unanswered. The key questions which need to be addressed are: 1) What is the role of the tannin acylhydrolase in *S. ruminantium* K2; and, 2) does this role have any relevance to the ability of feral goats to efficiently metabolise plant material rich in tannins? The results and outcomes of this project will now be discussed, in particular in relation to answering these two questions.

Chapter 3 of this thesis described the isolation of tannin-resistant organisms from feral goat rumen fluid by enrichment with *Acacia*-derived tannins. Four morphologically-distinct bacteria were isolated as a result of this enrichment, confirming that resistance to tannins is a property shared by more than one organism. One of the isolates from the enrichment was chosen for further characterisation and was identified as a strain of *Selenomonas ruminantium* subspecies *ruminantium*, a common ruminal bacterial species.

This isolate, designated strain K2, was shown to be not only tannin-resistant but also able to grow on a hydrolysable tannin (tannic acid) as a sole energy source. This property has never previously been reported in an anaerobic bacterium and raises the possibility of a link between this activity and the ability of feral goats to consume a diet high in tannins.

K2 was unable to grow on pure, *Acacia*-derived condensed tannin as a sole energy source, suggesting that a mechanism for growth on hydrolysable tannins could be found in the fundamental differences in chemical structure between the two classes of tannins. Hydrolysable tannins consist of a central carbohydrate core such as glucose, to which a number of phenolic acid residues are esterified. Condensed tannins contain no carbohydrate core nor ester bonds and consist of polymers of flavanols linked by carbon-carbon bonds. In Chapter 4 it was shown that during growth of K2 on the hydrolysable tannin, tannic acid, gallic acid was released into the medium. Subsequent experiments demonstrated enzymatic hydrolysis of

tannic acid and the simple ester, gallic acid methyl ester. This enzymatic activity has never previously been demonstrated in any bacterium and provided an explanation for the growth of K2 on a hydrolysable tannin and the lack of growth on a purified condensed tannin. It is proposed that this bacterium obtained energy for growth from tannic acid by fermenting the glucose released by enzymatic hydrolysis of the tannin.

The enzyme responsible for this hydrolysis was characterised in Chapters 5 and 6 and it was shown to have properties which classified it as a tannin acylhydrolase, an enzyme previously described in aerobic fungi. The K2 enzyme hydrolysed esters containing gallic acid, including tannic acid, GAME and gallic acid lauryl ester but it did not hydrolyse common general esterase substrates. These properties suggested a specificity for gallic acid, a characteristic displayed by fungal tannin acylhydrolases. Further studies are needed to determine the substrate specificity of the K2 enzyme, as the results of these may aid our understanding of the role this enzyme plays in this organism. In particular, it is necessary to determine if other phenolic acid-alcohol esters can be hydrolysed by this enzyme. For example, it would be very interesting to know if this enzyme is capable of hydrolysing esters of ferulic acid and arabinose, such as are commonly found cross-linking hemicellulose in the cell walls of grasses. Ferulic acid esterase activity has been demonstrated in the rumen bacteria *Fibrobacter succinogenes* S85 (McDermid *et al*, 1990) and *Butyrivibrio fibrisolvens* 49 (Akin *et al*, 1993), and separate ferulic acid esterases have been characterised from the fungus *Aspergillus niger* (Faulds and Williamson, 1994 and 1995) and the actinomycete *Streptomyces olivochromogenes* (Faulds and Williamson, 1993). These enzymes are believed to play an important role in the complex process of fibre degradation because it is thought they act as "debranching enzymes" by breaking the cross-links between cell wall polymers.

If it is shown that the K2 tannin acylhydrolase can hydrolyse a range of phenolic acid esters then perhaps this points to a more generalised role for the enzyme in this bacterium. Alternatively, if it is shown that the enzyme does indeed show specificity for gallic acid esters then a more specific role, perhaps associated with the biodegradation of hydrolysable tannins,

particularly gallotannins, may be attributed to it. In either case, it may be ultimately proven that this enzyme has evolved as a means by which this bacterium can gain energy from phenolic acid-carbohydrate esters, thereby exploiting a widely-available but microbially under-utilised resource.

In order to further examine substrate specificity the K2 tannin acylhydrolase must be purified so that assays can be conducted using a single protein instead of a mixture of proteins. Additionally, a range of phenolic acid esters, including a number of different-sized galloyl glucose esters, would need to be obtained. Unfortunately, many phenolic acid-alcohol esters would need to be chemically synthesized as they are not commercially available. Obtaining pure samples of galloyl glucose esters of a specific size for testing as substrates would also be difficult. Tannic acid has been fractionated using HPLC by Beasley *et al* (1977), Versele and Delahaye (1983) and Murdiati *et al* (1992) but discreet peaks were difficult to obtain and it is doubtful if enough material could be produced. Chemically-synthesised galloyl glucose esters have been used in a study by Simionescu and Simionescu (1976) and may prove to be a better option. The methods developed during the course of this study and the results gained have provided a basis by which purification of the enzyme could be achieved relatively easily. Both the size of the protein and its isoelectric point have been determined and hence these can be used in the selection of an appropriate protocol employing size-exclusion and ion-exchange chromatographies. Furthermore, an assay has been developed which would allow the measurement of specific activity and hence the progress of the purification could be monitored.

Although a direct comparison of the kinetic properties of the K2 tannin acylhydrolase and the fungal enzymes was not possible, an investigation of the physical properties in Chapter 6 revealed significant differences. It was therefore concluded that the K2 enzyme possibly represents a new, bacterial tannin acylhydrolase. Further characterisation demonstrated that the expression of tannin acylhydrolase activity was increased in K2 cells grown in the presence of gallic acid, tannic acid and GAME compared to cells grown in the absence of phenolics or in the presence of the non-gallate-containing phenolics, ferulic acid and catechin. These results

provide further evidence that this enzyme may have a role in the biodegradation of gallotannins and may represent the first example of bacterial gene expression regulated by tannins. The regulation of tannin acylhydrolase gene expression in K2 requires further investigation. The results of such work should provide more insight as to the true role of this enzyme in this bacterium and hence will indicate whether or not this organism is involved in the process of tannin detoxification. To this end, the cloning of the tannin acylhydrolase gene must be regarded as a priority. Initially, an analysis of the gene sequence may provide information on the true role of the enzyme because it may contain similarities to known proteins, in particular microbial non-specific esterases. Subsequently, primer extension experiments can be conducted to determine the transcription initiation site (TIS). Once identified, the region surrounding the TIS can be examined for regulatory motifs by comparison to sequence databases and by conducting gel-mobility shift assays using protein extracts derived from induced and uninduced cells. Although the production of fungal tannin acylhydrolases is also induced by esters containing gallic acid, none of these genes have been cloned and hence there is currently no information on tannin control of gene expression which could be used as a basis for comparison.

Defining the location of the tannin acylhydrolase in the cells of K2 will also help to provide an explanation for the role of this enzyme in this organism. Experiments presented in Chapter 6 of this thesis indicate that the enzyme is most probably located either in the periplasm or the cytoplasm of the cell. With this in mind, it is necessary to determine the exact size range of galloyl glucose esters able to: 1) be hydrolysed by the purified tannin acylhydrolase; and 2) support growth of the bacterium. The distinction between these questions is important. A periplasmic or cytoplasmic location of the enzyme would mean that the substrate needs to cross the outer membrane and presumably there is a limitation in the size of galloyl glucose esters able to do this. It therefore follows that there must also be a limit to the size of the galloyl glucose esters capable of supporting growth. In contrast, the fungal tannin acylhydrolases are located extracellularly, both bound to the mycelia and secreted into the growth medium, and they are

capable of hydrolysing the largest galloyl glucose esters. It is apparent that the fungal enzymes are immune to the protein precipitating properties of such large galloyl glucose esters; however, the mechanism by which this is achieved has not been investigated. If the purified K2 tannin acylhydrolase is also able to hydrolyse very large esters, this may not fit into the context of an intracellular location. In addition, if the enzyme is truly intracellular, its role in the biodegradation of tannins would need to be questioned.

A variety of techniques would need to be employed to define the location of the tannin acylhydrolase in K2. Initially, scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM) could be conducted on whole cells to look for the presence of putative membrane vesicles as described in Chapter 6. Such studies could be conducted on cells washed with Triton X-100 and unwashed cells to determine the effect of this detergent on membrane integrity in *Selenomonas ruminantium*. This would then help to explain the cellular location from which the K2 tannin acylhydrolase was extracted by Triton X-100. Alternatively, the enzyme could be purified and then used to raise polyclonal antibodies which could be used in immunogold labelling studies in conjunction with transmission electron microscopy to determine the cellular location of the enzyme. In addition to microscopy, a detailed cell fractionation procedure would need to be developed for this bacterium. This would require the development of a method for separating the outer and cytoplasmic membranes, perhaps using the work of Gong and Forsberg (1993) on *Fibrobacter succinogenes* as a basis.

Much information on the role of the tannin acylhydrolase in K2 and the possibility of a link between this enzyme and the ability of feral goats to thrive on diets high in tannins could be gained by ecological studies. Specifically, the distribution of this enzyme among *Selenomonads* and among rumen bacteria in general needs to be determined. This will reveal the frequency of this enzymatic activity within the rumen microbial population. Preliminary results presented in Chapter 6 showed that among *Selenomonads*, the enzyme activity is comparatively rare, with weak activity being detected in only 4 of 19 strains tested; however,

little is known about the activity in other species of bacteria. Previous reports have described tannin acylhydrolase activity in strains of tannin-resistant *Streptococci* (Osawa, 1990; Nelson *et al*, 1995), however, the evidence presented was indirect and no assays for tannin acylhydrolase were conducted. In the first case (Osawa, 1990), the tannin-resistant *Streptococcus bovis* Biotype I produced zones of clearing surrounding colonies on an agar medium containing a tannic acid-protein precipitate and these clearing zones were claimed to be evidence of tannin acylhydrolase activity. However, in Chapter 4 it was shown that *S. ruminantium* K2 produced no such clearing zones and in fact was unable to grow on this medium. Furthermore, *S. bovis* Biotype I displayed no tannin acylhydrolase activity when tested using the enzyme assay developed in Chapter 5. It was concluded that clearing of tannin-protein precipitates was not a result of tannin acylhydrolase activity and was probably due to lactic acid-mediated hydrolysis. In the second case, Nelson *et al* (1995) reported the isolation of a *Streptococcus* capable of degrading tannic acid from a goat fed a high-tannin diet. Evidence was provided by gas chromatography-mass spectrometry and thin layer chromatography of the production of pyrogallol from tannic acid. The authors proposed that this was achieved by the hydrolysis of the galloyl glucose ester bonds and the intergallate depside bonds catalysed by a tannin acylhydrolase, followed by decarboxylation of gallate catalysed by a gallate decarboxylase, resulting in the production of pyrogallol. No assays for either of these two enzymes were conducted. The authors also noted that their isolate was not able to grow on tannic acid as a sole energy source. Bearing this in mind, it is not obvious how this organism can release gallate from galloyl glucose esters and intergallate depside bonds without liberating glucose which could be readily fermented by this bacterium. It may be possible that the enzyme responsible for hydrolysis of these esters does not remove all gallate groups attached to the central carbohydrate core. This would suggest fundamental differences between this enzyme and the tannin acylhydrolase described in *S. ruminantium* K2. The relationship of the Nelson isolate to the *Streptococcal* isolates reported by Brooker *et al* (1994) and Osawa (1990) is unknown however evidence suggests the three are closely related. Since it has been shown in Chapter 6 of this thesis that neither the Brooker (*Streptococcus caprinus* 2.2) nor Osawa (*Streptococcus bovis* Biotype I) isolates express tannin acylhydrolase activity, the presence of

tannin acylhydrolase activity in the Nelson isolate should be examined. This could be achieved either by employing the tannin acylhydrolase assay described in this thesis or by probing Northern and Southern blots of the *Streptococcus* RNA and DNA with the K2 cloned gene.

As well as determining the frequency of tannin acylhydrolase activity among rumen bacteria in general, ecological studies must focus on determining whether there is a correlation between the presence of bacteria expressing this enzyme and an animal's ability to metabolise tannin-rich feeds. At this point in time, we do not know if domestic ruminants already harbour this organism, or, as with *Streptococcus caprinus*, it is only found in feral ruminants consuming tannin-rich material. Until the results of such experiments are known, we cannot say whether the transfer of this bacterium to domestic ruminants will benefit the livestock industries. Currently, this information could be easily gained by plating dilutions of rumen fluid taken from feral and domestic ruminants consuming tannin-rich and tannin-free diets onto non-selective media and then screening the resulting colonies for tannin acylhydrolase using the overlay procedure described in Chapter 7. Alternatively, if the gene coding for the tannin acylhydrolase was cloned it could be used as a probe to screen organisms for this gene.

Depending on the outcome of the ecological studies, it may be beneficial to conduct transfer experiments. For example, if it is shown that there is a correlation between presence of tannin acylhydrolase-positive organisms and ability to digest tannin-rich forage, the effect of transferring to domestic ruminants these organisms alone and in conjunction with tannin-resistant *Streptococci* could be examined. Various traits such as dry matter intake, digestibility, live-weight gain, nitrogen balance and wool production could be measured in animals consuming tannin-containing and control diets. Based on the results of such experiments, the possible benefits to the industry arising from bacterial transfers to domestic ruminants will be known.

Appendix

Media, Buffers and Reagents:

Media:

A1. modified M10 (mM10) /100ml

Tryptone (Oxoid)	0.20g
Yeast Extract (Oxoid)	0.05g
cysteine.HCl	0.05g
resazurin (0.1% stock solution)	0.10ml
Hemin Solution	0.01ml
VFA Solution	0.31ml
B&B Mineral Solution I	3.80ml
B&B Mineral Solution II	3.80ml
Na_2CO_3 (8% w/v stock solution)	5.0ml

Note pH of medium adjusted to 6.8 with 10% NaOH and then made up to volume with RO H_2O.

A1.1 Hemin Solution

Dissolve 2.0g hemin in 100ml of a 1:1 solution of 100% ethanol : 0.05M NaOH

A1.2 VFA Solution (Ogimoto and Imai, 1984)

	ml
glacial acetic acid	17.0
propionic acid	6.0
n-butyric acid	4.0
iso-butyric acid	1.0
n-valeric acid	1.0

iso-valeric acid	1.0
D-L-α-methyl butyric acid	1.0

Note pH adjustment and sterilisation are not necessary.

A1.3 B&B Mineral Solution I (Bryant and Burkey, 1953)

	g/l
K_2HPO_4	6.0

A1.4 B&B Mineral Solution II (Bryant and Burkey, 1953)

	g/l
NaCl	12.0
$(NH_4)_2SO_4$	12.0
KH_2PO_4	6.0
$CaCl_2$	1.2
$MgSO_4.7H_2O$	2.5

A2. <u>mBHI</u> /100ml

Brain Heart Infusion (Oxoid)	3.7g
hemin solution	0.05ml
cysteine.HCl	0.05g
resazurin (0.1% stock solution)	0.05ml

A3. <u>NB</u> (Nili and Brooker, 1995) /100ml

NB Mineral Solution I	6.0ml
NB Mineral Solution II	6.0ml

Trace Element Solution	0.5ml
Hemin/napthoquinone Solution	1.0ml
VFA Solution	0.33ml
Na_2S (5% Solution)	1.5ml
resazurin (0.1% stock solution)	0.05ml
NH_4Cl	0.37g
cysteine.HCl	0.05g
Na_2CO_3 (8% solution)	5.0ml
Vitamin Solution	4.0ml

Note Before adding Na_2CO_3 and Vitamin Solution the pH is adjusted to 7.7-7.8.

A3.1 NB Mineral Solution I	g/l
K_2HPO_4	11.84

A3.2 NB Mineral Solution II	g/l
KH_2PO_4	7.08
NaCl	1.78
$MgSO_4.7H_2O$	3.75
$MnCl_2.4H_2O$	0.20
$CoCl_2.6H_2O$	0.02
Na_2SO_4	8.3
$CaCl_2.2H_2O$	3.20

Note $CaCl_2.2H_2O$ is dissolved separately, mixed and the solution made to volume.

A3.3 Trace Element Solution	mg/100ml
$ZnSO_4.7H_2O$	10.0
H_3BO_3	10.0
$Na_2MO_4.2H_2O$	10.0
$NiCl_2.6H_2O$	5.0

$CuSO_4.5H_2O$	5.0
$FeSO_4$	10.0
$Al(SO_4)_3$	2.0

A3.4 Hemin/napthoquinone Solution

Dissolve 50mg hemin in 5.0ml 1.0M NaOH, add 95ml H_2O and 10mg 1,4-napthoquinone.

A3.5 Vitamin Solution	mg/300ml
biotin	2.5
folic acid	2.5
p-amino benzoic acid	2.5
cyanocobalomin	2.5
Ca pantothenate	20.0
nicotinamide	20.0
riboflavin	20.0
thiamine.HCl	20.0
pyridoxamine	20.0
lipoic acid	2.0

A4. H_2S Test Medium	/100ml
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.02g
$Na_2S_2O_3.5H_2O$	0.02g
mBHI medium	100.0ml

A5. LB (Luria Bertani) Medium /100ml

Tryptone	1.0g
Yeast Extract	0.5g
NaCl	1.0g

Note pH adjusted to 7.5 with NaoH then volume made up to 100ml with RO H_2O.

A6. SOC Medium for electroporated *E.coli*

	g/100ml
Tryptone	2.0
Yeast Extract	0.5
NaCl	0.06
KCl	0.02
$MgCl_2$	0.20
$MgSO_4$	0.25
Glucose	0.36

Note Autoclave or filter-sterilise and divide into 5-10ml aliquots.

Buffers and Solutions:

A7. Towbin Buffer /4.0l

Trizma base	12.1g
Glycine	57.7g
methanol	804.0ml

Note Dissolve dry ingredients in 2.7l H_2O, add methanol then make up to 4.0 litres with H_2O. Stable at 4°C for several months.

A8. TNT Buffer /l

1.0M Tris.HCl pH 8.0	10.0ml
NaCl	8.77g
Tween 20	0.5ml

A9. Blocking Buffer

Add skim milk powder to TNT Buffer to 5% w/v.

Note Make up fresh for each use; do not store.

A10. SM Phage Dilution Buffer	/l
NaCl	5.8g
$MgSO_4$	2.0g
1.0 M Tris.HCl pH 7.5	50.0ml
Gelatin (2.0% w/v stock solution)	5.0ml

Note Autoclave or filter-sterilise in 5-10ml aliquots.

A11 Gram Staining Solutions (Ogimoto and Imai, 1984)

A11.1 Crystal Violet Solution	/100ml
Crystal Violet stain	2.0g
ethanol (95%)	10.0ml

A11.2 Oxalate Solution

Dissolve 1.0g oxalic acid in 100.0ml H_2O.

A11.3 Lugol's Solution	/300ml
resublimed iodine	1.0g
Potassium iodide	2.0g

Note Store in a light-proof bottle.

A11.4 Decolourising Solution 95% ethanol

A11.5 Safranin Solution	/100ml
safranin O stain	0.25g
ethanol (95%)	10.0ml

A12 VFA Analysis Solutions

A12.1 Analytical Standard

Stock VFA Mixture	1.5ml
Protein Precipitant	0.5ml
Internal Standard	0.5ml
H_2O	1.0ml

A12.2 Stock VFA Mixture	/100ml
acetic (1.0M stock)	10.0ml
propionic (1.0M stock)	2.5ml
iso-butyric (1.0M stock)	0.25ml
n-butyric (1.0M stock)	2.5ml
iso-valeric (0.1M stock)	5.0ml
n-valeric (0.1M stock)	5.0ml

Note Solution brought to 100ml final volume with H_2O.

A12.3 Protein Precipitant

Dissolve 375g metaphosphoric acid in 900ml H_2O, add 500ml of 100% formic acid and make up to 2.0l final volume with H_2O.

A12.4 Internal Standard

Dilute 10.5ml of 100% caproic acid to 2.0l with H_2O.

A13. Anaerobic Dilution Solution (Ogimoto and Imai, 1984)

	/100ml
B&B Mineral Solution I	3.8ml
B&B Mineral Solution II	3.8ml
resazurin (0.1% stock solution)	0.1ml
cysteine.HCl	0.05g
Na_2CO_3 (8% stock solution)	5.0ml

Note pH is adjusted to 6.8-7.0

A14 Paper Chromatography Solutions

A14.1 Solvent A Butanol-Acetic acid-Water (4:1:5)

H_2O	50.0ml
n-butanol	40.0ml
glacial acetic acid	10.0ml

Note This is a two-phase solvent system. The upper (organic) phase is used.

A14.2 Solvent B Butanol-Ethanol-Water (4:2:2.2)

n-butanol	48.8ml
ethanol (95%)	24.4ml
H_20	26.8ml

Note This is a single-phase solvent system.

A14.3 Aniline/Phthalate Reagent (Dawson *et al*, 1986)

Dissolve 0.93g aniline and 1.66g phthalic acid in 100ml water-saturated n-butanol.

A15. Gallic Acid Assay Solutions

A15.1 Methanolic Rhodanine solution

Dissolve 0.67g rhodanine (2-thio-4-ketothiazolidine, Sigma) in 100ml of 100% methanol. Store at 4°C.

A15.2 KOH solution

Make a 0.5N solution by dissolving 2.81g KOH pellets in 100ml RO H_2O.

A15.3 Gallic acid stock solution

Dissolve 10.0mg gallic acid (3,4,5-trihydroxybenzoic acid, Sigma) in 10.0ml of 0.2 N H_2SO_4. Store at room temperature.

A16. DNA Hybridisation Solutions

A16.1 Prehybridisation Solution	/5.0ml
20X SSC	1.5ml
skim milk powder	0.05g
Na pyrophosphate (10% w/v stock solution)	25.0µl
SDS (10% w/v stock solution)	0.25ml
salmon sperm DNA (sheared and denatured)	50.0µl

Note Make up to volume with RO H_20. Make fresh prior to use; do not store.

A16.2 Hybridisation Solution	/5.0ml
20X SSC	1.5ml
skim milk powder	0.05g
Na pyrophosphate (10% w/v stock solution)	25.0µl
Polyethylene glycol (40% w/v stock solution)	0.5ml
salmon sperm DNA (sheared and denatured)	50.0µl

Note Make up to volume with RO H_20. Make fresh prior to use; do not store.

A16.3 20X SSC (see Sambrook *et al*, 1989)

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