A POSSIBLE MECHANISM FOR ENZYMIC DEPILATION OF SKINS

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ABSTRACT

Streptomyces fradiae is a bacterium which has been previously found to produce extracellular enzymes which are capable of wool degradation and skin depilation. *Streptomyces fradiae* 3739 and other strains of *Streptomyces* were found in this study to be able to degrade a keratin source (wool) to a considerable degree. However according to the evidence of SEM micrographs presented here the highly keratinised spindle cells of the paracortex are fairly resistant to protease attack, and it is the cementation material which binds these cells together which is initially degraded by the proteases. A large degree of correlation was found with the strains of *Streptomyces* studied, between the ability of the individual strains to degrade wool and the ability of their extracellular proteases to reduce the depilation load of sheepskins.

With further analysis *S. fradiae* 3739 was found to produce at least one amylase and four or more proteases. The proteases as a group had maximal proteolytic activity in the 8.0-9.0 pH unit range, and were considerably thermostabilised by the inclusion of calcium ions into the reaction solution. The protease group was found to cause depilation of merino sheepskins.

For comparative purposes a protease produced by a strain of *Proteus vulgaris* isolated from a staling hide with hair slip (natural depilation) was studied. The protease activity was maximal in the alkaline region between 8.0-9.0 pH units. The protease appeared to be a single enzyme with a molecular mass of approximately 44 000 daltons. The protease was maximally active at 40^{o}C, although it was only thermostable to 30^{o}C. The enzyme was ineffectual as a depilant except when the skin was pre-treated with a strong alkali, preferably including sodium sulphite in the protease preparation.

One of the most important differences between the extracellular proteases of *S. fradiae* and *P. vulgaris* was that the former were greater in variety and caused a greater decrease in the depilation load of sheepskins than the latter. Further research with mixtures of commercial proteases provided evidence that a synergistic depilatory effect occurs when proteases of

complementary bond specificities are used in conjunction in enzymic depilatory preparations.

Some form of strong alkali treatment of skins was found to be necessary to produce leather of the prerequisite quality when the skin was depilated by proteases, otherwise the skin was found to be depleted and stiff. Calcium hydroxide alone was found to be inadequate for this task, probably owing to the fact that it is less alkaline than the lime-sulphide mixture. The calcium hydroxide (lime) must therefore be used in conjunction with sodium hydroxide (which makes the solution as alkaline as that of the lime-sulphide solution) to produce leather comparable to that produced by the lime sulphide treatment.

A combination of the information provided by the present research and that gleaned from the relevent literature allows for the construction of a model to represent the possible mechanism of enzymic depilation of skins, in which depilation is caused by the disruption of the basement membrane at the dermal-epidermal junction by the degradation of its constituent molecular components by general proteases, resulting in the removal of the epidermis and its associated wool or hair.

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A) INTRODUCTION

There is considerable interest in the leather industry in the possibility of replacing the liming process used in the manufacture of leather with an enzymic process that would produce a comparable end product, thereby avoiding some of the present disadvantages of the liming process. Although some success has been achieved in this direction there may be limits in the effectiveness of solely enzyme based processes. The present study examined the possible use of enzymic depilation. The present liming process will be reviewed before the enzymic process is considered.

I) The Liming Process

In the conversion of skins and hides to leather products a number of modifications in skin structure must occur. One of the initial and most important processes the skin undergoes is the liming process, which occurs in the beamhouse. The position of this process in the general scheme of leather manufacture processes is depicted below (fig. 1). The liming process itself contains a number of steps (fig. 2).

The liming step involves a treatment of skins and hides with a mixture of 3% lime ($Ca(OH)_2$) and 3% sodium sulphide (Na_2S), which modify the skin in numerous ways:

1) Depilation:

The epidermis and wool (or hair) consists mainly of keratin (14). Keratin proteins are usually found here as dual and triple helices of α-helical protein; the triple helices (protofibrils) are inter-bonded with disulphide bonds to form microfibrils in the hair or sheets in the skin. Some of the sodium sulphide added in the liming process forms hydrosulphide ions (116) which react with the disulphide bonds and thereby disrupt the keratinous tissues, i.e. the epidermis and the hair (123). This allows for the easy mechanical removal of the epidermis and the hair from the skin,

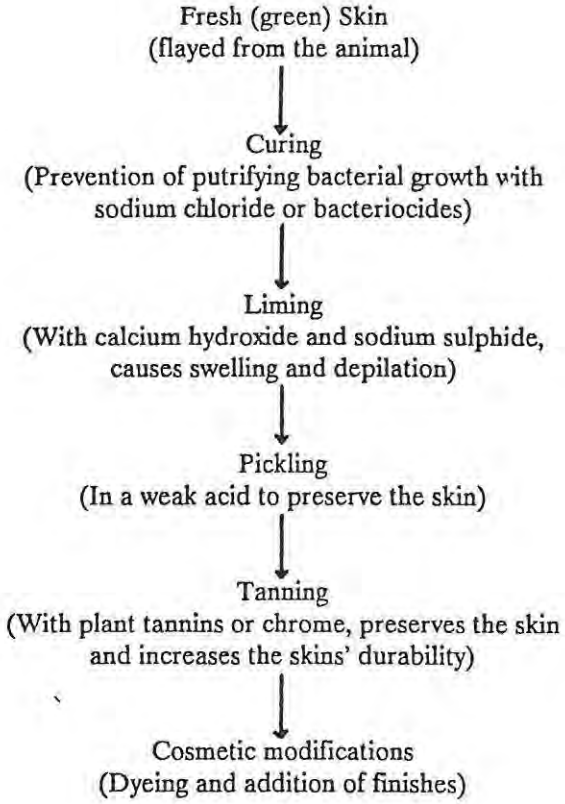


Fig. 1: Sequence of processes in leather manufacture.

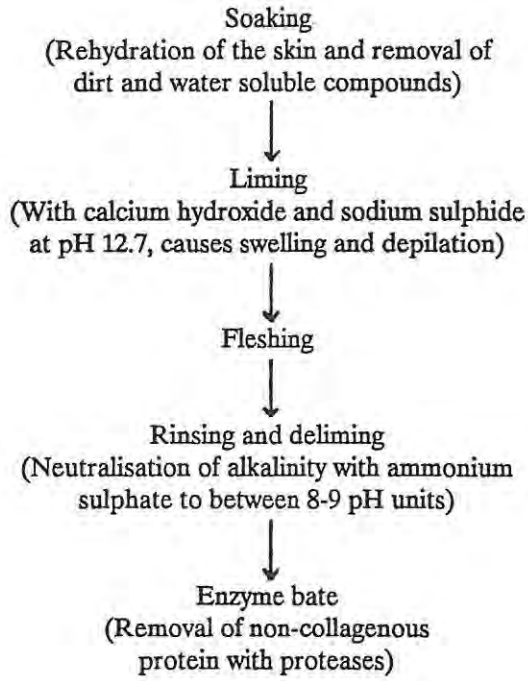


Fig. 2: Sequence of steps in the liming process.

leaving the top layer of the grain, which is known as the enamel surface, exposed. Exposure of the skin to sulphide for an extended period eventually results in the conversion of the keratin into a gel-like sludge.

2) Swelling:

Exposure of skins to extremes of pH causes swelling of the skin (19), visibly increasing the thickness of the skin. The swelling is the result of the hydration of the collagen fibres which plump and expand in diameter. In the lime-sulphide step the swelling effect is caused by an extremely alkaline pH of about 12.7 pH units. Later neutralisation of the pH with ammonium sulphate to about 8-9 pH units causes the skin to deflate, however considerable denaturation of the skin proteins has occurred and the skin after liming has different properties compared to its original state.

3) Removal of skin components:

Proteoglycans, ubiquitous in the skin, are removed. Soaking alone is sufficient to remove the hyaluronic acid, while dermatan sulphate proteoglycans are partially removed during liming and especially enzyme assisted liming (3). Non-collagenous proteins are removed during the bating step (116). The extreme pH and use of high salt concentrations in the beamhouse will obviously disrupt numerous non-covalent bonds between proteins within the skin.

The aim of the liming step is therefore to remove proteins other than collagen from the skin, and also to modify the collagen bundles by alkaline swelling and splitting (7). This yields a clean, hairless product with resilience and flexibility. Above all this process should damage the collagen matrix as little as possible as it is this matrix which constitutes the main component of the leather. After removal of the lime and sulphide, the skin is then treated with a pancreatic protease preparation which removes the remaining proteinaceous debris from the skin, a step which is referred to as bating. The resultant more open structure of the skin allows for a greater flexibility and is necessary for the proper penetration by the later processing liquids.

The disadvantage with this process is that it produces an industrial wastewater with a high

alkalinity and ionic strength, and sulphur (in various states of oxidation) at toxic levels. The sulphur also converts in part to hydrogen sulphide (H_2S), which is very toxic and is released into the work environment causing a health risk and unpleasant working conditions. Moreover the degradative effect of the sulphide on the wool prevents the use of these fibres in the textile industry and causes the release of insoluble solids into the wastewater.

II) Enzymic Depilation

1) Advantages and disadvantages

The advantages of enzymic depilation over the lime-sulphide process can be enumerated as:

a) The toxic sulphide could be replaced completely by enzymes, improving working conditions and decreasing the cost of the wastewater treatment.
b) There is little degradation of the wool fibre, the wool is therefore still intact and useful.
c) As the wool is not significantly degraded it can be trapped and retained, thereby resulting in a reduction in the amount of insoluble solids released into the wastewater.
d) The use of proteases for depilation means that in most cases they remove the same components from the skin as the bating enzymes do; therefore with enzymic depilation processes an additional bating step is not required.

However the enzymic depilation process can also have disadvantages such as:

a) The high cost of enzyme preparations may make them uneconomical for industrial purposes.
b) Enzymes themselves may constitute a health hazard if they are absorbed into the body, e.g. by inhalation of airborne dust.
c) In industrial trials enzymes have not always yielded a leather of as good a quality as those produced by the traditional lime-sulphide process. Complaints of uneven colour, residual fine hairs, and hardness of the leather have been noted.

The first objection is rapidly becoming invalid, the flourishing biotechnology industry has produced cheaper enzyme preparations; while the wastewater treatment costs have increased

dramatically recently, making the originally cheap lime sulphide process less cost effective. The second disadvantage can be avoided by presenting the enzymes as bound to granular materials such as sawdust, now a common practice. The third problem is the most difficult to overcome, in part owing to the lack of knowledge of enzyme action during depilation. More recently however certain depilatory processes involving enzymes have yielded good leather products (53).

2) Sources of Depilatory Enzymes

The depilatory enzyme sources are usually categorised as: animal, botanical, and microbial. Until fairly recent analysis of enzyme activity and specificity was initiated, mainly in the second half of this century, this crude categorisation of depilatory enzymes was the only one available and is still the one most commonly used. It does not, of cause, take into account the nature and specific mechanism of the enzyme preparation, and is therefore of little use in predicting the possible depilatory activity of an enzyme preparation.

a) Animal sources:

The organ which is richest in active proteases is the pancreas; in the leather industry these enzymes are used mainly for the purpose of bating. The proteases trypsin and chymotrypsin are extracted from the pancreas and they are optimally active at pHs between 8.0-10.0 and 7.5-8.0 pH units respectively. Pepsin, a protease which is isolated from the gastric juice, and cathepsins which are isolated from the spleen, are active in the acidic pH range and are optimally active at 1.5-2.5 and 3.5-5.0 pH ranges respectively.

b) Botanical sources:

Enzymes from plants are hard to extract and generally more difficult to use than animal enzymes (1) and are therefore unpopular in the leather industry. Papain, a protease extracted from the latex of the mammoth tree, has been used in depilation trials (120), and is optimally active in the 5.0-8.0 pH range. The protease bromelin, from pineapples, has also been assessed for its depilatory activity (22). More recently a protease was isolated from the pulped fruit of *Adenepus breviflorus* (1,2) which is optimally active in the 7.0-8.0 pH unit range. The pulped

fruit of *A. breviflorus* is used to cause depilation in a unique process developed in Nigeria.

c) Microbial sources:

The microbial enzymes used are usually extracellular proteases, released by microbes in submerged cultures. This makes them considerably easier to purify than those from plant or animal sources. They also have the immense advantage that they can be produced on demand in fermentors and therefore availability does not depend on slaughter numbers or on crop yields.

Microbial sources can be broken down into two broad categories:

i) Bacterial sources:The bacterial sources of enzymes used for depilation appear to be mainly species of *Bacillus* and *Streptomyces* (13, 85, 105).

ii) Mycoidal sources: These are usually species of *Aspergillus* and *Penecillium* (13, 85, 105). *Aspergillus oryzae* produces at least three different proteases (25, 69).

The individual enzymes of both these groups (i and ii) vary widely in their pH optima and specificity and are therefore an excellent source for various potentially useful enzymes.

3) A Historical Review of Enzymic Depilation

Depilation by means of enzymes can be said to originate in prehistoric times. Skins left in warm, moist conditions are a suitable substrate for bacteria (115), many of which produce proteolytic enzymes. While the skin is often damaged by the bacteria during this procedure, the wool itself is left intact and hence the method is still used for recovery of merino and fine cross-bred wools where the market price far exceeds that of skins themselves. The method requires 2-8 days and is therefore unpredictable and time consuming (116).

The possible use of partially purified enzymes to produce a more rapid and reliable depilation was considered as long ago as the turn of the century (41). Isolated enzymes were first successfully used for depilation by Otto Röhm in 1910 when he found that if the skins were initially swollen in alkali (a process referred to as pre-alkalisation) followed by neutralisation with sodium bicarbonate, then the normal pancreatic proteases usually used for bating would

now depilate the goat skins. The system was not widely used and was discontinued and replaced by less expensive non-enzymic methods (106).

During the mid 1950s interest in depilatory enzymes revived (16). In one trial nine different enzyme preparations were tested on hides and it was found that although most of the hair was removed there was some fine hair still remaining (22). It was also noted that there was an inconsistency of results in different trials conducted with the same enzyme preparations. Others have also noted fine hairs remaining after treatment with depilatory enzymes (42, 56). Moreover the enzymically depilated leather was of a different physical quality to the usual leather, however these differences disappeared when a pre-alkalisation step was used (45). It has been suggested that the fine hairs remaining on the enzyme depilated skins could be removed by a sulphide containing reliming liquor, or perhaps with a suitable depilating machine (42).

Pseudomonas is considered to be a principle hair slip causing organism (116). Between 1-3 different proteases were produced and released extracellularly by various strains of *Pseudomonads* isolated from sheep fleeces (57) and strains of *Pseudomonas maltophilia* were generally capable of degrading a wide range of components found in the skin (83). It was noted that skins cured in 10% NaCl could be depilated by a *Pseudomonas* strain (39).

Proteases from *Proteus vulgaris* have been reported to have a strong depilatory action (62). Gillespie (40) found that most strains of *Proteus vulgaris* and some strains of *Pseudomonas fluorescens, Flavobacterium esteroaromaticum*, and *Bacillus subtilis* yielded cultures which caused depilation only if disulphide bond splitting compounds were included in the depilatory enzyme liquor. *Aspergillus parasiticus* and *Aspergillus oryzae* also yielded enzymes with depilatory activity, the latter being notable in that its enzymes did not require the addition of any disulphide bond splitting compounds to cause depilation (40). The same worker also tested other enzyme preparations including papain, trypsin, pepsin, amylase, lipase, and chymotrypsin, but found them all to be devoid of depilatory activity. It was noted by others that crystalline trypsin had little depilatory activity (125, 128). One researcher claimed to have depilated sheepskins with papain, but reported that the preparation caused degradation of the skins,

possiby owing to bacterial contamination (120). The extracellular proteases of some species of *Streptomyces* bacteria were found to be depilatory and required neither addition of disulphide bond splitting compounds nor pre-alkalisation of the skin to be effective (45).

As the epidermis and wool are mainly composed of keratinised cells it was initially felt that a keratin degrading enzyme would be of immense value as a depilant. It was found that in wool containing basal salt cultures inoculated with the bacteria *Streptomyces fradiae* 3739 the wool was decomposed and the enzymes responsible were therefore considered to be keratinases (82).

An extracellular cell free filtrate of *S. fradiae* 3739 also caused the degradation of the wool but did not cause the release of soluble sulphydral compounds such as those that had been detected in the cell cultures (20). Further studies were conducted, but no conclusions could be drawn as to whether there was an extracellular disulphide reductase associated with the *S. fradiae* 3739 bacteria (20). An extracellular protease was isolated from the culture fluid, crystallised and characterised, and found to have strong keratinolytic activity which was maximum at 9.0 pH units (78, 79). A patent was taken out on processes of keratin breakdown using this bacterium and enzyme (77).

Later doubt was cast on the necessity of keratinolytic activity for depilation (119) and even on the above enzyme's ability to degrade hard keratin (29). However it was noted that crude extracellular protease solutions of *S. fradiae* 3739 were capable of depilation of hides and skins, and this crude enzyme product became commercially available as a depilatory enzyme preparation under the trade name M-zyme (85). Other organisms with keratinous activity were also reported (82); one of which was *Streptomyces griseus* which produces the extracellular protease mixture which is sold commercially under the trade name Pronase (74).

Another group (67) proved that more than one extracellular protease was released by *S. fradiae*, and that these proteases could be induced in the presence of protein substrates other than wool. Five proteases and two exopeptidases were separated, isolated, and characterised according to their properties and specificities. The five proteases were all inhibited by

diisopropylphosphofluoridate (DFP), an inhibitor of serine proteases, while being only slightly inhibited by ethylenediaminetetraacetic acid (ETDA), suggesting that they may all be serine proteases. The enzymes as a group had high activity against not only keratin, but also against elastin and casein, indicating that they were general proteases and not specifically keratinases. In later papers the enzymes were shown to have very broad specificity of proteolytic activity (68-71). Further research was carried out on these enzymes and their ability to degrade wool and modified wools (90), feather keratin (129), and enhanced degradation of wool by inclusion of reducing agents (32); in which these substrates were degraded to various degrees, particually in the latter case.

M-zyme was also reported as being used in various enzymic depilation trials (45, 49, 95, 121) and was reported to yield resultant leathers which were comparable to those produced by the lime-sulphide process providing that there was an alkalisation step included in the enzymic treatment.

Other organisms have been reported to be keratinolytic, but the wool was denatured in at least some of these cases by sterilisation treatments (91). Keratin has also been reported to be degraded by extracellular proteases from *Trichophyton rubrum*, (a fungus) (9), *Candida albicans* (another fungus) (75), and a mammalian enzyme isolated from cow snout epidermis (111).

4) Activity Necessary for Depilation

With the discovery that enzymic solutions from various sources could induce depilation came the need to be able to grade the depilatory activity of the different preparations and also to search for better depilatory enzyme systems. To do this the type of activity which causes depilation had to be ascertained. Enzyme preparations, pure and crude, characterised and uncharacterised, were screened for their depilatory activity and assayed for their activity against the major molecular components of the skin. The aim of these experiments was to enble a correlation of depilatory activity with the degradation of specific enzyme substrates and thereby elucidate the mechanism of depilation. The results of these studies include the following:

a) Hyaluronate: Neither of two groups of workers (16, 126) found activity against this glycosaminoglycan to be depilatory. It was later shown that most of the hyaluronate was removed from the skin in the pre-lime soak stage (3).

b) Dermatan sulphate (Chondroitin B): This proteoglycan is closely associated with collagen (98-101), and although it is removed by both the lime-sulphide treatment and by depilatory enzyme treatments it is likely that the removal of this component is important in the opening up of the fibre structure (3) but not in depilation (128).

c) Elastin: Histological studies have shown that the elastin fibres of the skin are interwoven with the collagen fibres and are predominant around the hair follicles (63). The elastin network extends throughout the grain, but is most dense one third of the distance down from the grain surface into the grain in cattle hide (63). Various workers have shown from histological and biochemical studies that changes in the elastic tissue are incidental to and not an integral part of the depilation process (23, 39, 113, 128). However elastin is degraded during bating with pancreatic enzymes, and may therefore beneficially affect the condition of the final leather (5, 63).

d) Keratin: As keratin is the component removed by sulphide in the liming step it was initially considered that the epidermis and the hair could only be removed by enzymes that had keratinase activity. However it has been shown that loosening of the hair is possible even before the immature keratin of the hair bulb is substantially degraded (119). Only the anchorage of the hair root in the hair papilla need be enzymically loosened, and then the hair can be removed from the papillae with the hair root still intact.

It was in fact found that although many good depilatory enzyme preparations had keratinolytic activity , it was not a prerequisite for a depilatory effect. Even enzymes which degrade wool fibres degrade the cells of the medulla only and not those of the cortex (104). During one experiment it was found that four enzyme preparations recommended for the tanning industry had no effect on the hard keratins, but they did have considerable degradative

activity on the soft or immature keratins (113) such as that of the young hair of the bulb which can be attacked by bacteria (27).

e) Collagen: As far as possible the integrity of the collagen matrix should be maintained as it is this matrix which constitutes the basic material of the leather. Proteases used as depilants appear to slightly degrade the collagen, increasing its solubility (17) by breaking some of the inter-molecular cross-links of collagen (114); but these can be replaced by tanning agents which form new cross-links. Collagen may be attacked by general proteases (11) and more specifically by collagenases (18, 46, 94).

f) Carbohydrates: Although some reports have suggested a role for amylytic enzymes in depilation (16) it was pointed out that many crude α-amylase preparations also exhibit considerable protease activity, and may therefore be depilatory for this very reason (125); pure α-amylase was found to have no depilatory activity (125). Moreover no relationship was found between sugar release and depilation with a diastase preparation (21) or with starch breakdown (126).

g) Non-structural proteins: These proteins are generally readily susceptible to proteolytic attack. In one study it was found that the proteolytic enzymes used had little or no effect on the fibrous proteins: viz. collagen, elastin, keratin, and reticulin, while the same enzymes readily hydrolysed non-fibrous or globular proteins (13). The involvement of peptide bond hydrolysis in depilation is well established, as is the link between increasing strength of proteolytic enzyme solutions and increasing depilation (128). Evidence has been presented that suggests that general broad specificity proteolytic activity of the endopeptidase type is the only activity necessary for depilation (128). This activity may be present in a group of enzymes (127) such as the commercial preparation Pronase (75).

Curing with sodium chloride partially removes the globular proteins which not only encourage bacterial growth but are also undesirable for leather manufacture (115). Soaking in brine can enhance enzymic depilation (31, 56) and results in hair loosening (56).

Thus the primary activity necessary for depilation appears to be general proteolytic activity against non-structural globular proteins. However this activity against any particular protein is in practice not necessarily an indication of its depilatory efficacy.

III) The Mechanism of Enzymic Depilation: A Synthesis of Previous Work

From the above information it appears that although broad spectrum proteolytic action is probably the only enzyme activity required for depilation, the specific proteins that have to be hydrolysed for depilation to occur are as yet uncertain. Recent research has given possible answers to this question.

Beneath the epidermis is the dermal-epidermal junction. Bacteria which cause staling tend to reproduce prolifically at this junction, growing dense in numbers after 24 hours, and by 72 hours the epidermis separates from the dermis with general hairslip (depilation) occurring (27, 73). It has also been observed that only the *stratum germinatium* and the basal cells of the hair bulb and not the wool itself is degraded during enzymic depilation and that sometimes the hair may be removed from the skin with the follicle sheaths still attached (119). In some cases, after enzyme or bacterial action, the epidermis can be detached in continuous sheets with undamaged hair still attached (16, 97). Thus there is a breakdown of the dermal-epidermal junction during enzyme depilation (93). Using a periodic acid-schiff stain which stains neutral polysaccharides, mucoproteins (proteoglycans), and glycoproteins (39, ,72, 103), it is possible to visualise a thin layer of connective tissue called the basement membrane at the dermal-epidermal junction. During depilation with proteases it was shown that the basement membrane, initially well defined by the stain lost its ability to stain, an effect which was most obvious in the areas where the epidermis had separated from the dermis (39). The region was not affected by α-amylase, hyaluronidase, or half saturated $Ca(OH)_2$ solutions (103).

It is then probable that the mechanism of depilation by enzymes involves the attack of non-fibrous proteins in the basement membrane by general proteolytic action. The dissolution of the

globular proteins which anchor the cells and the connective tissue matrix to one another would disrupt the adhesion of the epidermis to the dermis. A similar state of affairs has been noted in tissue culture experiments where trypsin has been shown to be slow and incomplete in its dispersion of mammalian tissues, while the mixed enzyme system of Pronase on the other hand has been found to rapidly and completely digest tissues to produce monodisperse cell suspensions (43). In the case of trypsin the cells remained strung together by fibres, suggesting that many cross-links between the matrix polymers and the cells remained.

IV) The Site of Action

1) The Basement Membrane

Recent work on the basement membrane has yielded a detailed picture of this region and its component molecules.

The basement membrane is not a lipid membrane but a thin matrix of type IV collagen linked to the epidermis above and the dermis below by glycoproteins (fig. 3). It is continuous with the dermal-epidermal junction. The following is a résumé of the current knowledge about this region and its component molecules.

a) Type IV Collagen

Type IV collagen is present throughout the entire thickness of the basement membrane (61), and is unique to basement membranes. Unlike type I collagen, type IV collagen does not form fibrils by lateral aggregation, but individual molecules aggregate end to end to form a net-like structure cross-linked by disulphide bonds (64). It is a very weak structure and there is evidence from transmission electron micrographs that it is removed in the early stages of pre-tanning (52).

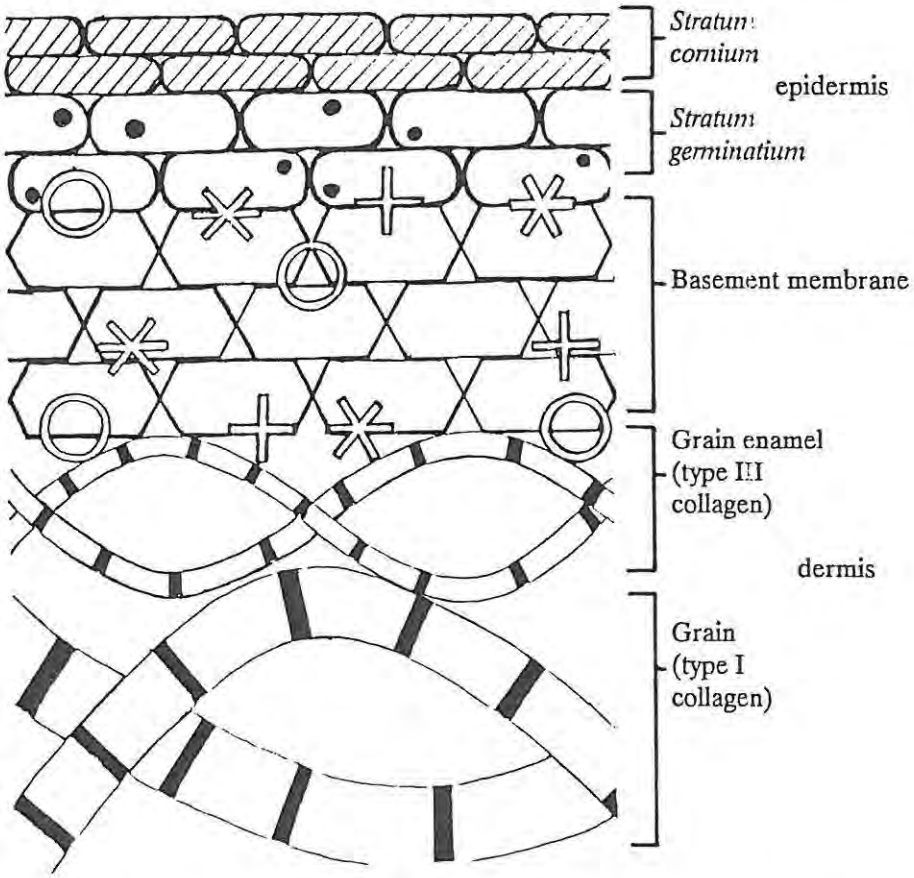


Fig. 3: Schematic cross-section of the dermal-epidermal junction. At the junction of the dermis and the epidermis is a layer of connective tissue referred to as the basement membrane. Type IV collagen forms a network throughout the basement membrane. Laminin (+), entactin (o), and heparan sulphate proteoglycan (*) are found mostly in the boundaries of the basement membrane (the *laminae lucidae*), but some is found in the interior (the *laminae densa*). Other components of the basement membrane are not depicted. After Kemp (52) and Martinez-Hernandez (61).

Type IV collagen is far more prone to enzymic attack than is type I collagen. *In vitro*, on exposure to pepsin, type IV collagen is degraded (60). Aggregates of type IV collagen readily disperse when treated in this way and the collagen molecules undergo considerable fragmentation, suggesting multiple attack sites (64). Type IV collagen can also be degraded by endogenous enzymes such as human neutrophil elastase (89) and a specific neutral protease (26).

b) Laminin

Laminin is the adhesion glycoprotein specific for connection of epithelial and epidermal cells to the type IV collagen of basement membranes (107, 117). Laminin also binds heparan sulphate proteoglycans, which are present in substantial amounts in basement membranes (124).

Laminin contains disulphide bonds which link its component subunits together and it can be degraded by proteases (109, 92). Although laminin can be cleaved by proteases not all cleavages will have the same effects. Thrombin's cleavage of the B subunit (the elongated arm of laminin's cruciform shape) does not affect its binding to type IV collagen, while pepsin and cathepsin G, which degrade the shorter arms of the A subunit, do inhibit this binding (92, 108). The protease insensitive domains of each arm of laminin are separated by protease sensitive α-helical regions. The B subunit, which binds heparan sulphate, is particularly sensitive to protease action (124).

c) Heparan Sulphate

Heparan sulphate is the main proteoglycan to be found in basement membranes (55). Owing to their large polyanionic and hydrated substituted carbohydrate side chains, they can inhibit the diffusion of other macromolecules across the basement membrane (38). Heparan sulphate proteoglycan binds to specific binding sites on laminin and types I, III, and IV collagen (38).

Other components of the basement membrane are nidogen (a glycoprotein), entactin (which may yet be proven to be identical to nidogen), the bullus pemphigoid antigen, and a few other minor components (110). At least three of the components of the basement membrane contain disulphide bonds (viz: type IV collagen, laminin, and the bullus pemphigoid antigen) which is perhaps a reason why disulphide bond reducing agents assist in enzymic depilation, possibly acting in a synergistic manner with proteases (102).

2) The Grain Enamel

Directly below the basement membrane is the surface of the dermis, which is referred to in the leather industry as the grain enamel (fig. 3). It is this surface which is seen in grain (but not suede) leather, therefore any damage inflicted on the enamel would blemish the leather.

It has been suggested that grain damage during enzymic depilation treatment is caused by contaminating collagenases present in the enzyme preparations (48). However the surface enamel of the grain consists of type III collagen which differs from type I collagen, which is prevalent in the rest of the dermis, in a number of ways. The fibrils formed by type III collagen are thinner than those formed by type I collagen, and unlike type I collagen type III collagen is more susceptible to proteolytic attack by general proteases (52). Not only is type III collagen degraded by high levels of protease activity, but is also vulnerable to high levels of alkali or sulphide (52) or to staling (53). This damage is greatly enhanced by staling of the hide resulting from lack of or incomplete curing (53).

This then is an important consideration when depilating skins with enzymes, as the enzymes must be able to degrade the basement membrane but leave the surface enamel intact. Normally enzymes can depilate at concentrations which are far below those that would cause observable grain damage (3). In a series of investigations (34-37) it was discovered that pre-alkalisation of pigskins prevented the enzyme preparations used from causing the grain damage which otherwise occurred. It was noted that "green" skins were less susceptible to

grain damage than were salt cured or brined skins, particularly where the original cure was faulty. However it was discovered that pre-alkalisation of calfskins did not protect them from enzyme related damage (37).

3) Transport of Enzymes to the Basement Membrane

The epidermis is designed to be generally impermeable to bacteria and enzymes, therefore in normal conditions entry is only possible via the flesh side of the flayed skin. In the case of bacteria they enter the skin via the flesh side and proceed through the dermis to colonise the dermal-epidermal junction where they cause hair slip (depilation)(27, 44, 73).

As the epidermis is impenetrable to enzymes unless it is pre-alkalised (122) enzymes too enter the skin via the flesh side and migrate to the basement membrane by diffusion (127). It has been noted that enzymes entering from the flesh side did not initiate depilation until a four hour delay had passed, this was followed by a quick reduction in the depilatory load, which in turn was followed by a slower rate of decrease until a depilation load of near zero was attained (16). It appears that the initial four hours are required for penetration of the enzymes to the basement membrane, and that this period can be reduced by mechanical action on the skin (3, 4).

4) Autolytic Enzymes

It has been suggested that where hair slip occurs readily it may be attributed to possible action by autolytic enzymes, with subsequent presentation of evidence of lysosomal enzymes in the skin which can cause hair slip (66). Where hides had been frozen or equilibriated to an acid pH of about 3.5 lysosomes were ruptured within the hides and acid conditions were favourable for the activity of the enzymes released, especially cathepsin D (66). Cathepsin G was found recently to degrade laminin's A subunit and thereby destroy laminin's binding sites for type

IV collagen (108). Another cathepsin, cathepsin L, was found to be very active in the degradation of the glomerular basement membrane of the kidneys; its optimal release of hydroxyproline from the type IV collagen was at 3.5 pH units (10). These enzymes may assist in the *Lactobacillus* based depilatory process (97), which operates at 3.5 pH units but appears to produce little proteolytic activity in itself.

V) Research Objectives

1) Initially the extracellular proteases of *Streptomyces fradiae* 3739 were chosen as possible depilatory enzymes because of their high keratinolytic activity. However as keratinolytic activity has been shown by other workers to be incidental and not a prerequisite for depilation it need not follow that the bacterium with the highest keratinolytic activity will be the most proficient depilant. The first research objective was therefore to compare keratinase activity of bacterial cultures with the ability of their extracellular enzymes to cause depilation and determine if there is in fact any relationship between the two.

2) Wool is not the best medium for bacterial growth. The second research objective was therefore to induce protease production from *Streptomyces fradiae* 3739 when cultured submerged in a non-keratin medium. The proteases produced would then be isolated and studied.

3) A third research objective was to isolate a bacteria capable of protease production from a hide undergoing hair slip and to determine the characteristics of the extracellular enzymes and their capabilities as depilants. These results could then be compared to the characteristics of the enzymes from *Streptomyces fradiae* 3739, a known depilant, and this could possibly yield further insight into the nature of enzymic depilation.

4) During the progress of this study a fourth objective arose from the results of the third objective. The aim here was to determine the effect of combining various commercial proteases for use in depilation experiments and determining if any synergistic effect occurred with these combinations compared to using single enzymes. This data could give futher information on the nature of enzymic depilation.

5) Based on the findings of this study a possible mechanism of enzymic depilation of skins could then be considered.

B) METHODS AND MATERIALS

I) Organisms:

A freeze dried culture of *Streptomyces fradiae* I.M.R.U. 3739 was obtained from Rutgers State University's Waksman Institute of Microbiology. *Streptomyces rimosus* and *Streptomyces* TK 64 were obtained from the John Innes Institute, Norwich. *Streptomyces griseus* was obtained from the NCIB (Aberdeen) collection 8056. *Streptomyces S.* 5058 was obtained from Liverpool University. The strain of *Proteus vulgaris* used was collected from a hide which exhibited malodor and hair slip by Mrs. G. A. Thompson of LIRI, and was then isolated on MacConkeys agar No. 3 (Oxoid).

II) Equipment and Materials:

Centrifugation was performed on a MSE high speed 18 centrifuge with a No. 69197 rotor. Spectrophotometric determinations were performed on a Beckman ACTA CII spectrophotometer. Scanning electron microscopy (SEM) micrographs were taken using the JEOL JSM 840 scanning electron microscope. Depilation experiments were performed on plugs of salt cured merino skins. Wool degradation experiments involved chloroform sterilised merino wool. CM Sephadex C-25, DEAE Sephadex A-50 and Sephadex G-75 were supplied by Pharmacia Fine Chemicals. KMB chromatography columns were used for all chromatographic separations. Novo Unhairing Enzyme No. 1 (NUE), Neutrase, Alcalase, and Pancreatic Trypsin Novo (PTN) protease preparations were supplied by Novo Industries (Denmark), and α-chymotrypsin was supplied by BDH (England). Pronase (B grade) was supplied by Cal Biochem (USA). Pellican (PTTK 047 10) ultrafiltration discs used in the ultrafiltration cell were supplied by Millipore.

III) Methods:

1) Wool Degradation and Depilation Experiments

a) Wool degradation

Freeze dried cultures of the various strains of *Streptomyces* bacteria were grown on either YEME (Table A1, appendix) or on nutrient agar slants. After 5 days of growth at 35°C these slants were used to produce spore suspensions (50). Aliquots (1 cm^3) of these spore suspensions were inoculated into 100 cm^3 of wool/ basal salt media (82) (Table 1) in a 300 cm^3 conical flask. The pH of the media after autoclaving was 7.7 pH units.

Table 1: Formulation for Wool/ Basal Salts Medium

Composition	Concentration $g.dm^{-3}$
$K_2H_2PO_4$	1.50
$MgSO_4.7H_2O$	0.05
$CaCl_2$	0.05
$FeSO_4.7H_2O$	0.015
$ZnSO_4.7H_2O$	0.005
Sterile merino wool (added after autoclaving)	0.85

The cultures were grown at 30°C for 30 days on a rotary shaker set at 150 rpm. After incubation was completed the wool remains were separated from the culture broth by vacuum filtration through pre-weighed Whatman No. 1 filter paper. The wool and accompanying bacterial mycelia were dried in an oven at 40°C for 20 hours and then weighed. The extent of wool degradation was expressed as the percentage loss of mass from the original 85 mg of wool. No microbial growth was observed in the control flask (with no inoculum included) and less than 1% of the wool in this flask was solubilized.

b) Electron microscope studies:

The wool fibres were visualised by both light microscopy and SEM. The wool required no preserving procedure for SEM and was therefore gold spray coated directly without any prior preservation. The gold spray was applied by the vacuum evaporation of gold ions which covered the sample evenly.

c) Wool Sterilisation:

Merino wool was clipped from salt cured skins. The discoloured tips were removed and the white wool was washed repeatedly in distilled water. After being dried in an oven at 40^{o}C for 20 hours to remove the moisture, the wool was washed repeatedly in chloroform to remove the wool grease (lanolin). The wool was then kept in fresh chloroform for one week to ensure sterility. Residual chloroform associated with the wool after decantation was removed under vacuum in a UV-light sterilised vacuum desiccator. This method of sterilisation had no apparent effect on the integrity of the wool structure (82).

d) Depilation Load Measurement:

This involved incubating 5 cm diameter merino skin plugs in 100 cm^3 of culture filtrate. These solutions were incubated at 32^{o}C while being shaken on an orbital shaker for 20 hours at 150 rpm. After this period had elapsed the skin plugs were removed and clamped in such a way as to pinch two opposite edges of the plug together with the flesh sides touching. A second clamp was attached to the tips of a staple of wool near the centre of the skin plug (at the edges the wool tends to be loosened). Attached to the lower clamp was a tray, upon which weights were applied until the wool was pulled from the skin (fig. A1, appendix). The pulled wool was then cut to a set length and dried on filter paper in an oven at 40^{o}C for 6 hours and subsequently weighed. The applied mass (M) in grams divided by the dry mass of the pulled wool (m) in milligrams per cm length of wool removed (l) was taken to be related to the enzymic loosening of the wool, as shown in the formula:

$$\text{Depilation load} = \frac{M}{m/l} \quad \text{in g.cm.mg}^{-1} \ (\text{or g.cm}^{-2})$$

If the number of wool fibres per area of skin is reasonably constant this formula allows for a comparison of the force required per unit area to depilate the skin, thereby giving an indication of the efficacy of the depilatory agent (62, 116).

2) The Production of Extracellular Enzymes by Submerged Cultures of *Streptomyces fradiae* 3739 in Soya Based Media

Spore suspensions of *S. fradiae* were inoculated into basal salts media as described above (1a) except that the wool was replaced with soya meal (10 $g.dm^{-3}$ unless stated otherwise).

a) The Effect of Limitation of Nitrogen and Carbon Sources on Extracellular Protease Production.

Various grades of soya meal were used as the sole carbon and nitrogen source of the bacteria to determine what effect the availability of these two elements had on the amount of protease release from the bacteria. The grades of soya meal used were: Soyatone (Merck & Co.) a predigested soya meal, soya flour, and soya meal sieved and graded into three different sizes after being ground from soya beans with a mortar and pestle viz. below 355, between 355-500, and between 500-1 400 µm diameter grains. The larger the grains the lower the area to volume ratio, thereby resulting in reduced digestability of the meal, and consequently reduced availability of nitrogen and carbon. In a parallel set of experiments 20$g.dm^{-3}$ of soluble starch (M&B) was included into the media to determine the effect of the presence excess carbon source on extracellular protease release. Each culture (50 dm^3 in a 300 dm^3 conical flask) was aerated by shaking on an orbital shaker at 150 rpm while being incubated at 30°C for 48 hours. The cultures were then vacuum filtered through Whatman No. 1 filter paper and assayed for alkaline protease activity as described below (section 5.a). Soya flour was chosen as a good nitrogen source from

the results of this experiment.

b) Production of Alkaline Protease and Amylase by *S. fradiae*

A 1 dm^3 airlift submerged culture of *S. fradiae* in basal salts medium with 1.0 $g.dm^{-3}$ soya flour included was incubated at 30°C for 48 hours. At intervals 15 cm^3 samples of culture fluid were withdrawn under sterile conditions and stored frozen. When the period of culture incubation was completed the samples were thawed and individually filtered through Whatman No. 1 filter paper and assayed for alkaline protease and amylase activity which was recorded as enzyme activity as a function of incubation time. The starch degrading enzyme was not extensively characterised, and therefore it may later be renamed after futher investigation. This and all other cultures used were monitored for contaminant bacterial growth by streak plating samples of culture on nutrient agar plates which were incubated overnight at 30°C.

c) Production of Protease from *S. fradiae* for Purification

A 1 dm^3 airlift submerged culture of *S. fradiae* in basal salts media including 10 $g.dm^{-3}$ of soya flour was incubated at 30°C for 48 hours. This culture was then vacuum filtered through Whatman No. 1 filter paper to remove the bacterial mycelia and insoluble solids, and then used in the purification process as described below (section 3).

3) Partial Purification of Extracellular Protease from Cultures of *S. fradiae*

This procedure for purification was adapted from that used by Morihara *et. al.* (67), and involved the following sequence of procedures:

a) Ammonium sulphate (60% saturation) was added to the filtered culture fluid for 12 hours at 4°C.

b) The precipitated protein was pelleted by centrifugation at 6000 rpm.

c) The pellets were resuspended in 50 cm^3 of 0.05 M sodium dihydrogen phosphate buffer, pH 6.0.

d) The procedure as described in points a-c was repeated.

e) To the resultant solution was added 0.5 volumes of acetone on ice, the solution was centrifuged at 10°C at 6 000 rpm, and the resultant pellet was discarded. The volume of the acetone was increased to 2.0 volumes, the solution was centifuged, and the pellet resuspended in 50 cm^3 of 0.05 M phosphate buffer pH 6.0.

f) The enzyme solution was dialysed for 24 hours at 4°C against 8 dm^3 of distilled water and then freeze dried.

g) Some of the freeze dried material (100 mg) was dissolved in 5 cm^3 of the phosphate buffer and applied onto a cation exchange column (CM Sephadex C-25) previously equilibrated with the same buffer. The dimensions of the column were 2.4 cm diameter and a height of 30 cm. The flow rate was maintained at 1 $cm^3.min^{-1}$ using a Pharmacia P3 peristaltic pump, with an ionic gradient of 0-0.1 M NaCl in 0.05 M phosphate buffer pH 6.0. Fractions of 10 cm^3 were collected and assayed for alkaline protease, keratinase, and elastase activity, and for protein content. The protease positive fractions were then pooled and kept at 4°C until used.

If the proteases had a molecular mass of below 30 000 daltons but above 10 000 daltons, as suggested in the literature (67), then a useful purification procedure would involve excluding molecules with molecular masses above 30 000 daltons and subsequently retaining all molecules with molecular masses of below 10 000 daltons by using the appropriate ultrafiltration membranes. A separate batch of *S. fradiae* culture medium was vacuum filtered through Whatman No. 1 filter paper, and the crude filtrate was ultrafiltered through a magnetically stirred ultrafiltration cell using a 30 000 dalton retention ultrafiltration membrane (Millipore, Pellican

PTTK 047 10).

4) Identification of *Proteus vulgaris* and Partial Purification of its Extracelluar Protease

4.1) Isolation and identification of *P. vulgaris*

The bacteria from a hide exhibiting malodor and hair slip were transferred onto an agar plate containing casein, which was used to determine the presence of proteolytic activity. The bacteria was then serially transferred on MacConkey agar No. 3 (Oxoid) plates to preferentially enhance the growth of this bacteria and inhibit the growth of contaminating bacteria. The isolated bacteria was thereafter maintained on nutrient agar plates (Oxoid CM 3).

The bacterium was categorised with an API 20E bacteria identification system.

4.2) Production of Protease in *P. vulgaris* cultures

Two separate submerged airlift cultures of 1 dm^3 volume each were inoculated with 1 cm^3 suspensions of *P. vulgaris*. The cultures were incubated at 30°C for 24 hours in a soya based media (Table 2), and the pH after autoclaving was 7.2 pH units.

Table 2: Formulation of the Culture Medium for *Proteus vulgaris*

Composition	Concentration $g.dm^{-3}$
Soyatone (Merck)	5.0
NaCl	5.0

4.3) Partial Purification of Protease from *P. vulgaris* Cultures

a) The cultures produced in section 4.2 were combined to yield 2 dm^3 of solution.

b) The cultures were then centrifuged at 6 000 rpm and the solution was filtered through 0.45 µm retention filters on a Millipore Sterifill Aseptic System to yield a clear dark yellow solution.

c) This solution was precipitated with 60% saturation of ammonium sulphate overnight and centrifuged at 6 000 rpm for 15 minutes.

d) The pellets were resuspended in 100 cm^3 of 0.1 M Tris buffer pH 7.2. This solution was both reduced in volume and washed with the same buffer in an ultrafiltration cell with a 30 000 dalton retention membrane (Millipore, Pellican PTTK 047 10), to yield a final volume of 45 cm^3.

e) A volume of 20 cm^3 of this proteolytic concentrate was applied onto an ion exchange column (DEAE Sephadex A-50), which had been previously equilibriated with 0.1 M Tris buffer pH 7.2. The dimensions of the column packing were 2.4 cm diameter and 18 cm high. Elution was performed with the Tris buffer incorporating a saline gradient of 0-0.5 M NaCl and a flow rate of 1 $cm^3.min^{-1}$. Fractions of 10 cm^3 volume were collected.

f) The fractions were assayed for alkaline protease activity and the protease positive fractions were pooled. The pooled fractions were reduced in volume from 80 cm^3 to 21 cm^3 on the 30 000 dalton retention ultrafiltration membrane.

5) Characterisation of Enzymes

a) Alkaline Protease Assay

This assay was modified from Boethling (12). A 1 cm^3 aliquot of a suitable dilution of enzyme

solution was prewarmed with an equal volume of distilled water for 5 minutes to 37°C. A 1 cm^3 aliquot of casein solution (10 $mg.cm^{-3}$ casein in 0.05 M Tris buffer pH 9.0, dissolved at 70°C), prewarmed separately, was added to the diluted enzyme solution. After exactly 15 minutes of incubation at 37°C the reaction was terminated by addition of 2 cm^3 of 10% trichloroacetic acid. The reaction tube was immediately placed in a 0°C water bath for a duration of at least one hour to allow the precipitation of insoluble denatured protein to occur.

The solution was then filtered through Whatman No.1 filter paper and the filtrate was assayed spectrophotometrically at 550 nm for protein content (measured as tyrosine concentration) by the Folin-Lowry method (59) (fig A2, appendix). Controls of each tube were treated in the same manner except that the 10% trichloroacetic acid was added prior to addition of the casein solution.

b) Protein Assay

The protein content of solutions was measured spectrophotometrically at 550 nm by the Folin-Lowry method (59), and relating the results to an albumin standard curve (fig. A3, appendix).

c) Ionic Stability

This measured the proteolytic activity of the proteases in environments of different salinities. The assay was performed in a similar manner to that of the alkaline protease assay except that the 1 cm^3 of distilled water was replaced with 2 cm^3 of dilutions of a 2.8 M NaCl stock solution.

d) Thermostability

Here 1 cm^3 of enzyme solution plus 1 cm^3 of distilled water was incubated at one of a range of temperatures (30-80°C) for precisely 10 minutes. At the end of the incubation period the tubes were rapidly cooled in a 0°C water bath. The tubes were then assayed in the usual manner for alkaline protease activity with the addition of casein solution and incubation at 37°C. A variation of this assay was carried out wherein the enzyme solution was incubated at the set temperature with 1 cm^3 of 0.2 M $CaCl_2$ instead of distilled water. This allowed assessment of the stabilising effect of divalent ions on the enzymes.

e) Temperature Optimum

This assay was similar to that of the alkaline protease assay except that the incubation temperature was preset at various levels (20-60^{o}C).

f) pH Optimum.

This assay was similar to that of the alkaline protease assay except that the pH of the solution was varied by the use of buffers of different pHs. Between 7.0-9.0 pH units a 0.1 M Tris buffer was used, and between 9.0-11.0 pH units a 0.1 M carbonate/ bicarbonate buffer was used to effectively buffer the casein solution instead of the 0.05 M Tris buffer used in other cases.

g) Amylase Activity Assay

This method was adapted from Fairbairn (33). Prewarmed solutions of 0.5 cm^3 of enzyme solution were added to 0.5 cm^3 of 1% soluble starch (M&B) in 0.05 M Tris buffer pH 7.5. After incubation at 37^{o}C for one hour the reaction was terminated by placing the tube in a boiling water bath for 10 minutes. The amount of maltose released was measured spectrophotometrically at 540 nm by the Somogyi-Nelson method (76) against a standard curve (fig. A4, appendix). Control tubes were treated similarly except that they were boiled immediately after addition of the substrate.

h) Elastase Activity Assay

This method wass adapted from that according to Morihara *et. al.* (67). To 20 mg of powdered elastin (Sigma Co.) was added 1 cm^3 of distilled water plus an equal volume of 0.1 M Tris or 0.1 M carbonate/ bicarbonate buffer. To this was added 1 cm^3 of enzyme solution. Both solutions had been prewarmed to the incubation temperature of 40^{o}C before mixing. Incubation proceeded for one hour with occational shaking and was terminated by placing the reaction tube in a boiling water bath for ten minutes, followed by addition of 2 cm^3 distilled water. The solution was then filtered through Whatman No. 1 filter paper and the filtrate was assayed for soluble protein according to the Folin-Lowry method. Control tubes were treated similarly except that they were boiled immediately after addition of enzyme solution.

i) Keratinase Activity Assay

This was according the the method of Nickerson *et. al.* (78). To 200 mg of chloroform sterilised wool, cut into 1 mm length sections, was added the following: 5.0 cm^3 of 0.05 M Tris buffer (9.0), 0.5 cm^3 of 10^{-3} M $MgCl_2$, 1 cm^3 enzyme solution, and 3.5 cm^3 distilled water to give a total volume of 10 cm^3. The mixture was incubated for 3 hours after the timed addition of the enzyme solution at 37°C. At the end of this period the reaction was terminated by filtering the reaction solutions through Whatman No. 1 filter paper. The solubilised protein in the filtrate was assayed according to the Folin-Lowry method at 550 nm. For each assay the following controls were included: (a) reaction mixture with enzyme solution replaced with an equal volume of buffer, and (b) reaction mixture without inoculation of the wool substrate. From the absorbance values of the reaction mixture was subtracted the sum of the absorbance values of the two different controls. The corrected absorbance value was referred to as the A_{550} value.

j) Molecular Mass Determination

Partially purified enzyme solutions from *S. fradiae* and *P. vulgaris* were applied onto a gel exclusion column (Sephadex G-75, fine grade) of 1.6 cm diameter and packed 65 cm high. Elution was performed with 0.05 M Tris buffer pH 8.0 at a flow rate of 15 $cm^3.h^{-1}$. Fractions of 4 cm^3 were collected and assayed according to the alkaline protease assay. The column was calibrated according to Andrews (6), using molecular mass markers (fig. A5 and Table A2, appendix).

k) Depilation Experiments with Partially Purified Protease Concentrates

An ammonium sulphate precipitated protease concentrate from *P. vulgaris* was incubated with merino skin plugs (2.5 cm diameter). The concentrate was sufficiently active to degrade 90% of the casein in the alkaline protease assay. Other solutions of the enzyme were incubated with merino skin plugs with an inclusion of 0.1 M sodium sulphite as a reducing agent, where the skin plugs were either untreated or pre-alkalised with 0.2 M NaOH overnight. Untreated skin plugs which had not been pre-alkalised were pre-soaked in distilled water overnight. The plugs were incubated with enzyme solution at 32°C for 18 hours. After this period the skins were depilated according to the method described in point 1d of section III of this chapter.

6) Depilation Experiments Involving Combinations of Commercial Proteases.

6.1) Depilation with Single and Dual Enzyme Preparations

Solutions of 100 cm^3 enzyme stock solutions of Alcalase, Novo Unhairing Enzyme No. 1 (NUE), Neutrase, and Pancreatic Trypsin Novo (PTN), were prepared individually by dissolving 100 mg of enzyme powder in 0.05 M Tris buffer pH 8.0. These solutions were then filtered through Whatman No. 1 filter paper to remove the insoluble supports. A 0.1 $mg.cm^{-3}$ solution of crystalline α-chymotrypsin was made up in the same buffer.

Merino skin plugs (2.5 cm diameter) were pre-soaked in distilled water for 12 hours and incubated with 5 cm^3 of enzyme preparation at 32°C. The enzyme preparations were either individual stock enzymes or combination of two stock enzyme solutions in volume ratios of 1:1 as depicted below (fig. 4). When the incubation period was completed the plugs of skin were measured for depilation load as described in point 1d of section III of this chapter. The average reduction of depilation load caused by the single enzyme preparations was compared to that caused by the dual enzyme preparations.

6.2) Depilation with Multiple Enzyme Preparations

Merino skin plugs (2.5 cm diameter) were pre-soaked in distilled water for 12 hours and then incubated with 5 cm^3 of an enzyme preparation for 12 hours at 32°C. When the incubation period was completed the plugs were measured for decrease in depilation load as described in point 1d of section III of this chapter. The enzyme preparations consisted of mixtures of enzymes in equal volumes (Table 3), thereby giving an increasing variety of enzymes, but maintaining the total concentration of enzyme.

A parallel experiment was run where the skin plugs were pre-alkalised for 12 hours in 0.2 M NaOH prior to enzyme treatment.

6.3) Depilation Followed by Various Alkalisation Treatments

To determine the effect of different treatments on skin quality sections of salt cured cross-bred

merino skin (4.5 x 7 cm) were soaked in distilled water for 12 hours and then treated in one of the following ways:

i) Incubated at room temperature in 3% lime plus 3% sodium sulphide for 12 hours.

ii) Incubated at 32°C for 12 hours in a solution of 20 cm^3 100 mg/ 100 cm^3 commercial depilatory enzyme (from CPC) and then either placed in distilled water, 100 cm^3 3% CaOH, or 100 cm^3 2% CaOH plus 1% NaOH for 12 hours.

All skin sections were delimed with 3% ammonium sulphate and then dehydrated with acetone and dried at 40°C to yield raw leather.

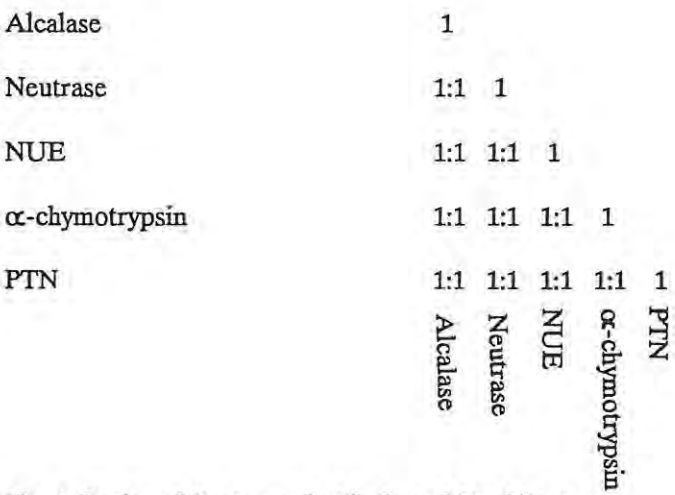


Fig. 4. Ratios of Proteases in Single and Dual Protease Preparation Depilation Experiment

Table 3: Composition of Multiple Protease Preparations

Component Enzymes	ratio
1. A	1
2. A + B	1:1
3. A + B + C	1:1:1
4. A + B + C + D	1:1:1:1
5. A + B + C + D + E	1:1:1:1:1

Where A = Alcalase, B = Neutrase, C = NUE, D = α-chymotrypsin, E = PTN.

C) RESULTS

I) Wool Degradation and Depilation Experiments

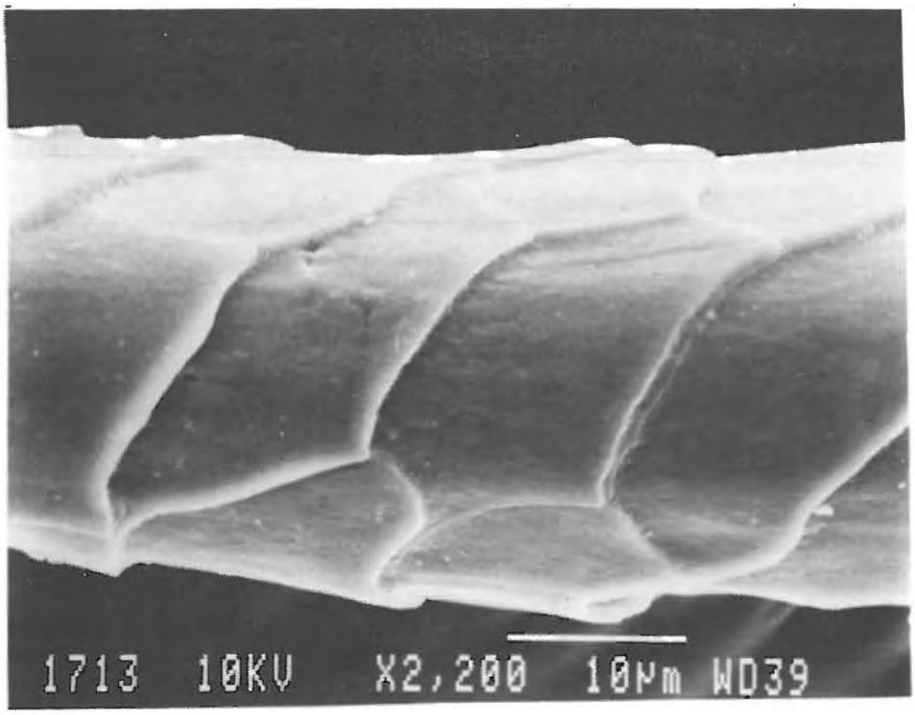
1) Wool Degradation

During the incubation period wool degradation with some of the *Streptomyces* strains became evident. When separated from the basal salts medium and dried the partially degraded wool could be seen to have lost its lustre, and compared to the wool from the control flask it looked dull when viewed under low magnification light microscopy. The more powerful magnification afforded by scanning electron microscopy (fig. 5-9) showed the reason for this. The original wool fibre (fig. 5) was intact, however initial enzyme attack on the wool fibre caused loosening of the scales (fig. 6) which leads to dulling of the wool and to felting of the wool as the raised scales interlock. Eventually the scales of the wool cuticle were completely lost and the cortex of the wool shaft was exposed and the spindle cells were released (fig. 7). The enzymes appeared to degrade the material binding the spindle cells together (fig. 8), until finally only individual spindle cells remained (fig. 9).

2) Depilation Related to Wool Degradation

The measured loss of wool mass caused by degradation by *Streptomyces* bacteria was recorded as percentages of the original wool mass. These figures paralleled the SEM micrograph observations in that loss of wool mass was highest in those cultures where wool degradation was most prominent. The results of the depilation experiments using the extra cellular cultures of *Streptomyces* bacteria incubated with the wool were compared to the wool degradation figures and the relationship between the two parameters was plotted (fig. 10). There is a clear correlation between the two parameters. As one of the cultures outside the curve (7 in fig. 10) is of the same strain as some of those inside the curve (e.g. 5 in fig. 10), i.e. *S. fradiae* 3739, it suggests that this may possibly be a case where the protease release from the bacteria has only recently occurred and although it can decrease the depilation load it has not yet had time to degrade the wool.

a)



b)

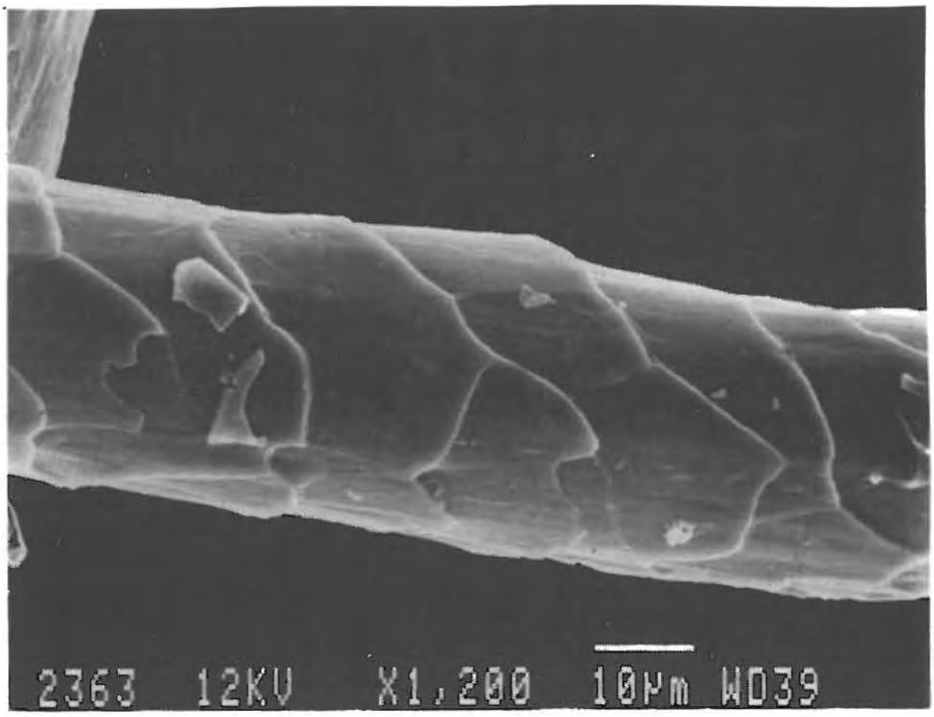
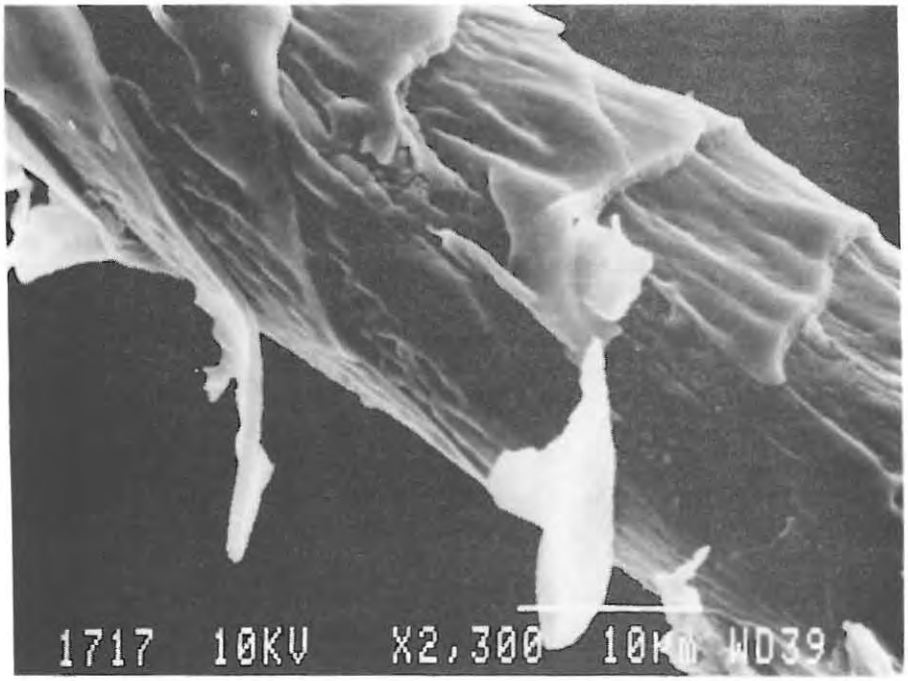


Fig. 5: SEM micrographs of intact wool, before (a) and after (b) incubation in basal salts solution.

a)



b)

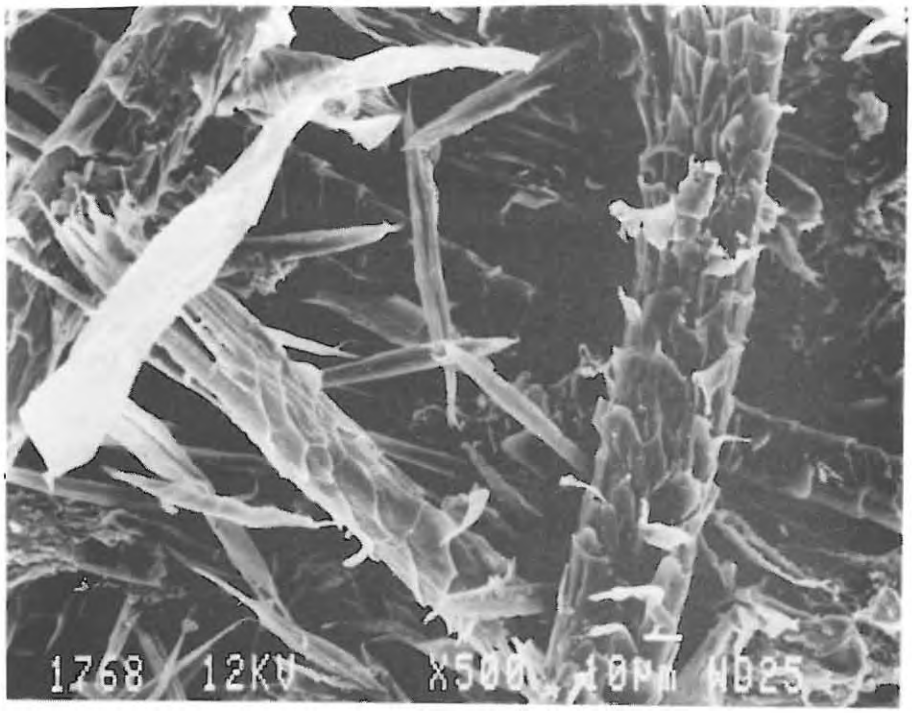
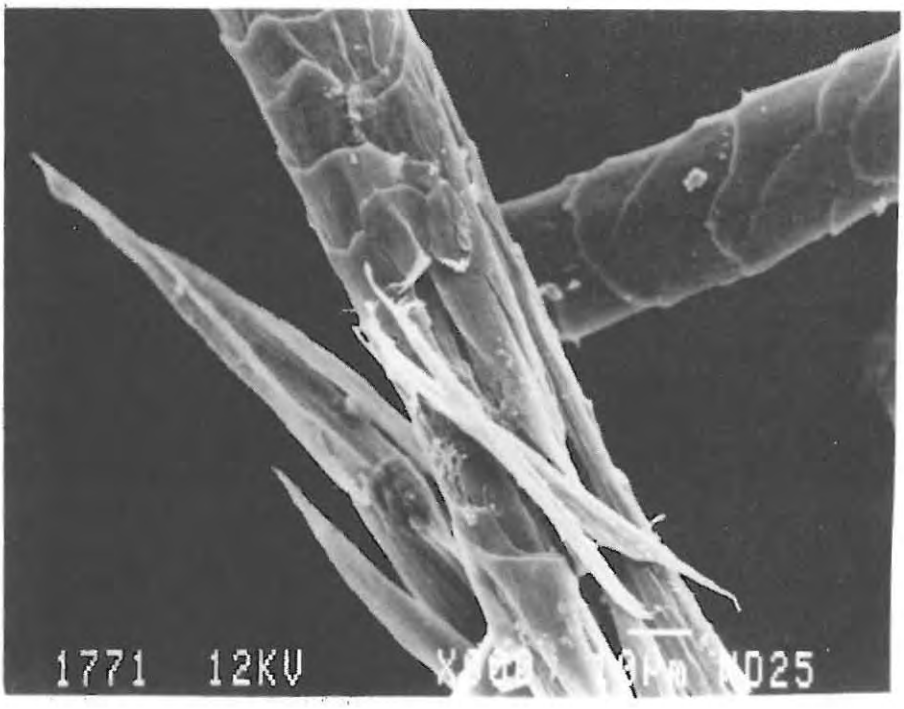


Fig. 6: SEM micrographs of wool degraded by (a) *S. griseus* and (b) *S. rimosus*. Initially the scale cells of the cuticle begin to pull away from the fibre shaft.

a)



b)

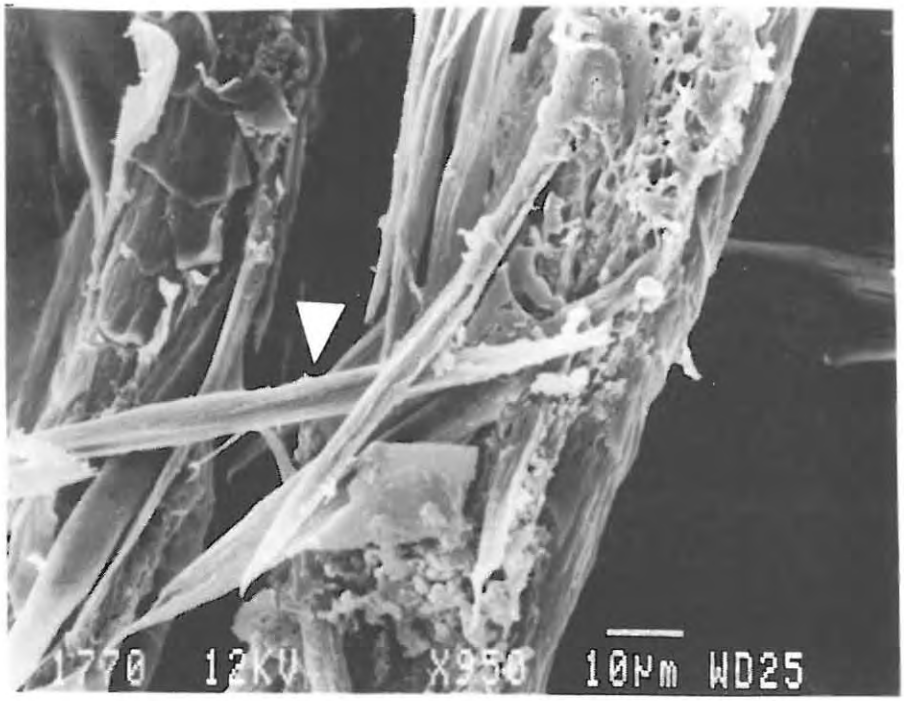
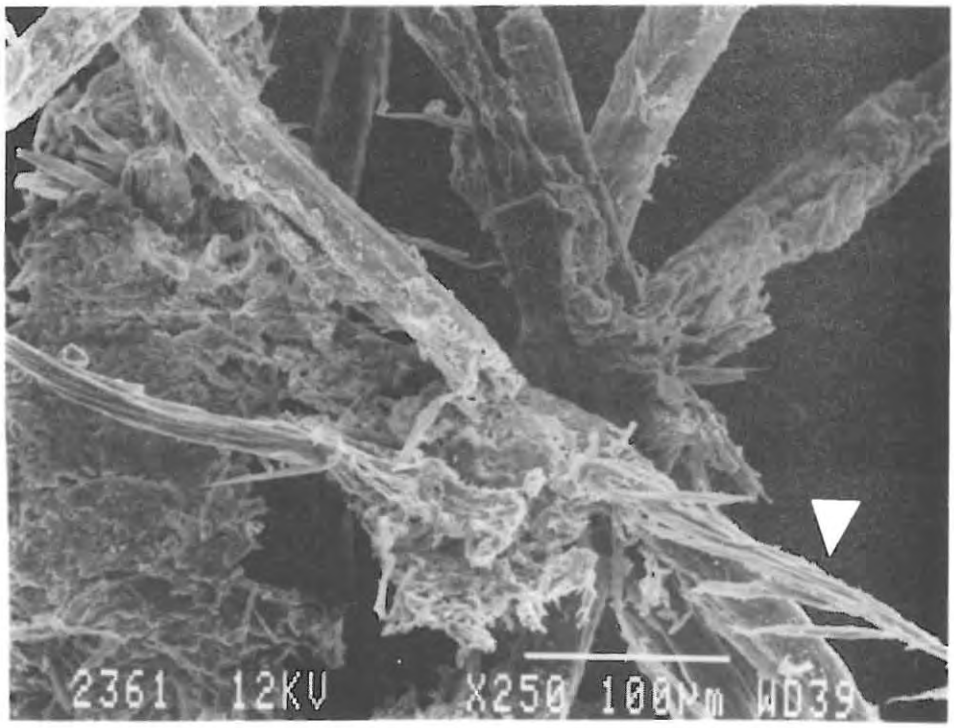


Fig. 7: SEM micrographs of wool degraded by *S. rimosus*. As the breakdown of the wool progresses the cortex of the wool is exposed and the spindle cells (V) can be observed (a). Eventually they too are removed (b).

a)



b)

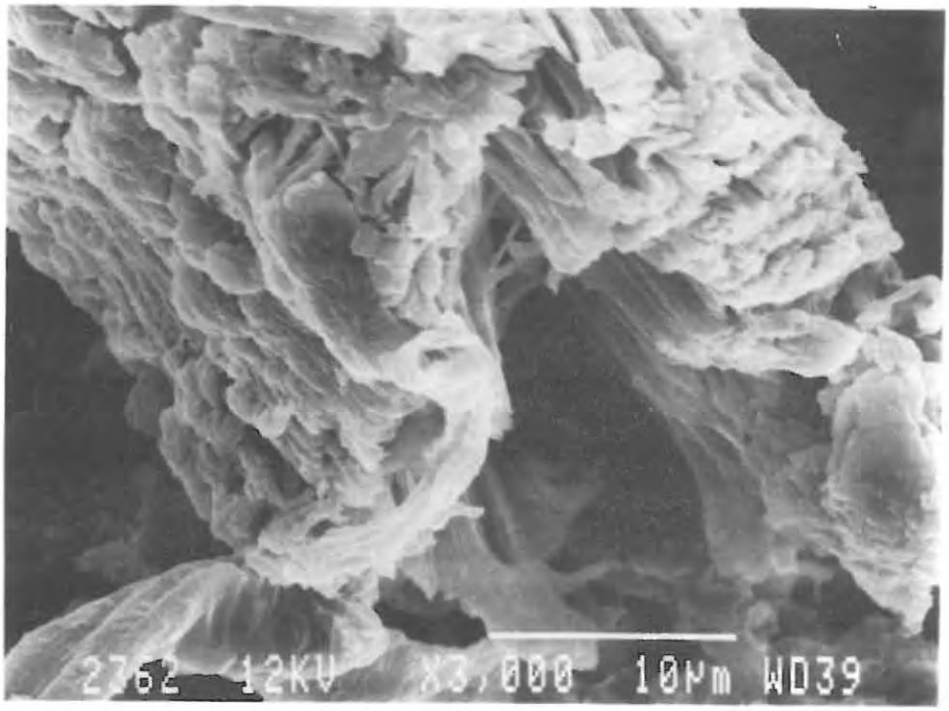
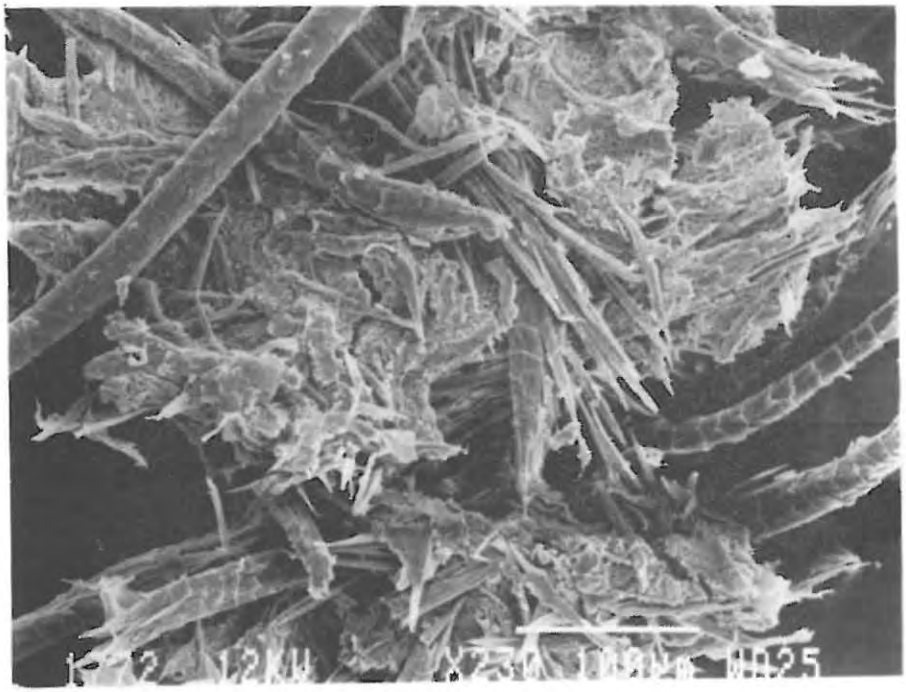


Fig. 8: SEM micrographs of wool degraded by *S. fradiae*. The spindle cells (V) persist when the rest of the wool fibre is completely degraded (a). Under higher magnification of the central region of fig. 8a it appears that the material between the spindle cells is preferentially degraded (b).

a)



b)

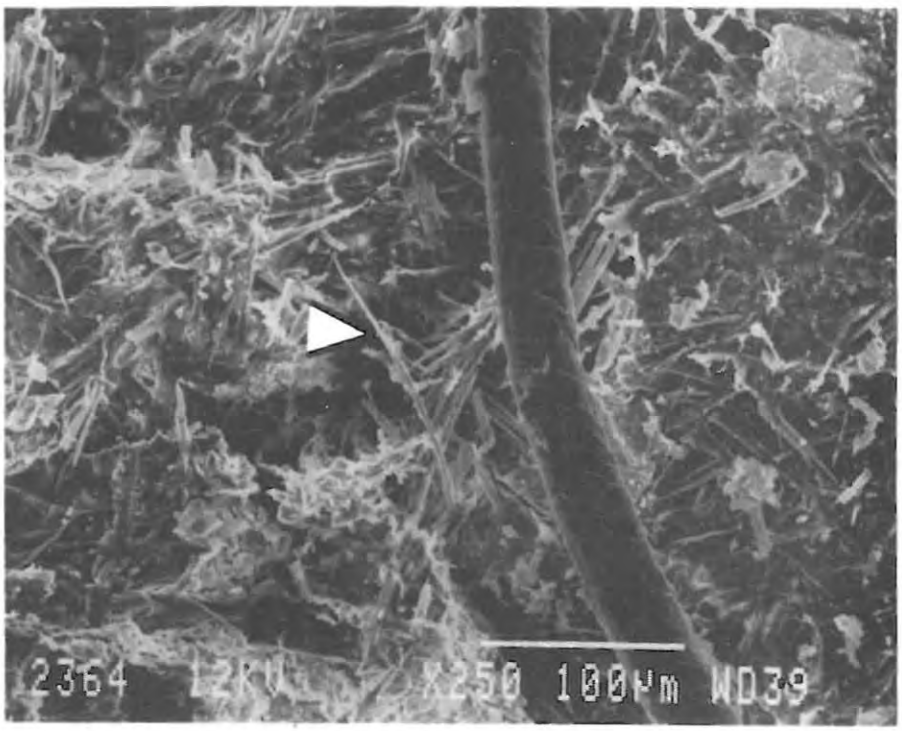


Fig. 9: SEM micrographs of wool degraded by *S. rimosus* (a) and *S. fradiae* (b). Even after the complete breakdown of the general fibre structure the spindle cells still persist (V), indicating that they are far more resilient to proteolytic degradation than is the intercellular material.

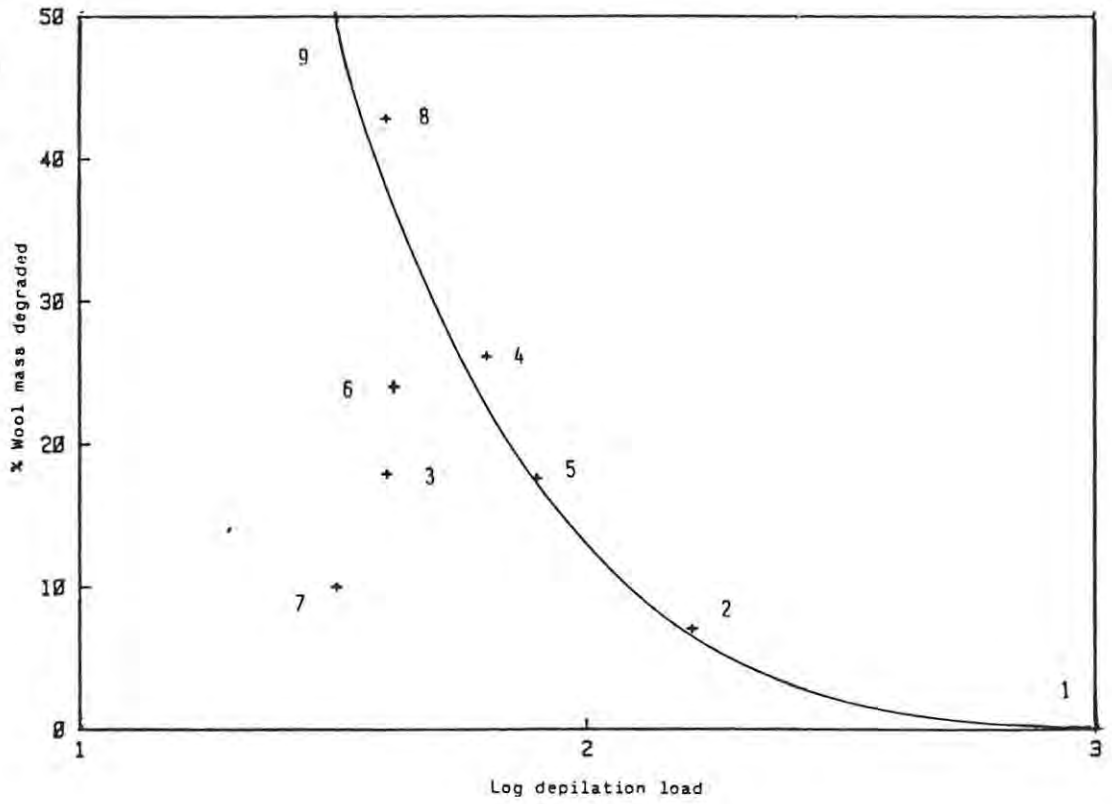


Fig. 10: The relationship between *Streptomyces* culture's ability to degrade wool and the culture filtrate's ability to decrease the depilation load of sheepskins. The cultures are numbered as such: 1) Control, 2) *Streptomyces.* 19, 3) *Streptomyces.* TK 64, 4) *Streptomyces.* 58, 5-7) *Streptomyces. fradiae* 3739, 8) *Streptomyces. griseus*, 9) *Streptomyces. rimosus.*

II) Production and Characterisation of Extracellular Enzymes of *Streptomyces fradiae* 3739

1) Bacterial Growth

Colonies of *S. fradiae* were grown on nutrient agar plates until sporulation occurred (usually within 3-5 days) as was evidenced by the opaque whiteness of the colonies and, if the colonies were separate, by the appearance of a dark lavender pigment in the centre of the colony. Some colonies were cryolised,sprayed with gold, and then visualised and photographed using the SEM. The micrographs (fig. 11) show the aerial hyphae to have formed spores at their terminal ends. The spore walls are smooth as is characteristic of this species (54).

2) Extracellular Enzyme Release

a) The Effect of Limitation of Nitrogen and Carbon Sources on Extracellular Protease Production

The amount of freely available amino acids and peptides in the medium did seem to have a noticeable effect on the release of proteases (fig. 12). When these elements were easily obtained, as with the partially predigested Soyatone, protease release was repressed. Protease release increased as the availability of the soya product decreased until a certain point, at which the trend reversed itself. The latter effect is probably due to the greatly reduced biomass observed in these cases, and therefore reflects a reduced protease release as a consequence of a reduced population and not a form of enzyme repression. Addition of soluble starch caused a reduction of protease release in certain cases (fig. 12) suggesting that this energy source is preferentially utilised by the bacteria over that of the protein. As the available nitrogen source becomes more limiting the bacteria is forced to release more protease. When the nitrogen source is extremely restricted, as in the case of the coarsely milled meal (1 in fig.12), the addition of soluble starch seems to enhance protease release to a limited extent.

a)

b)

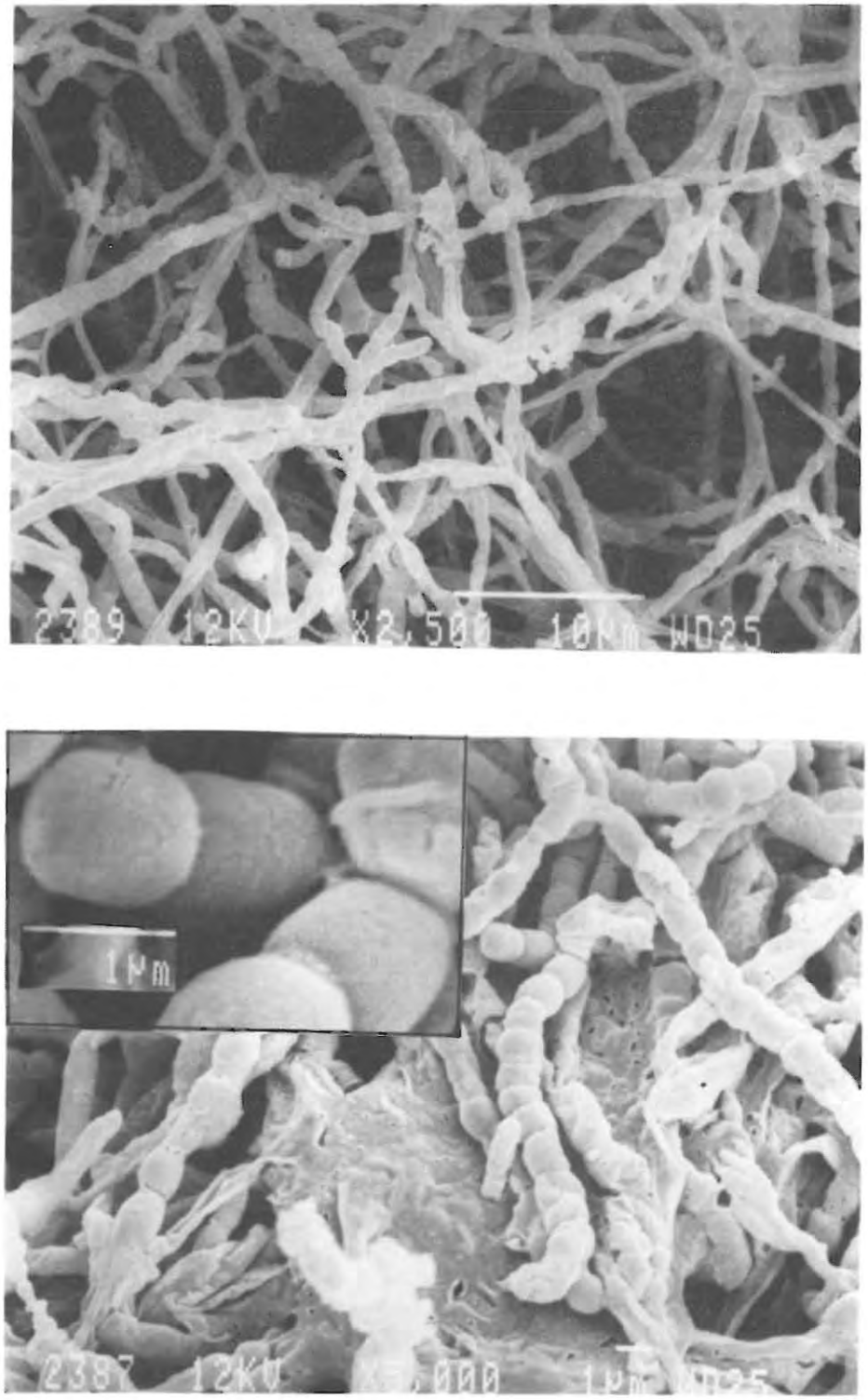


Fig. 11 SEM micrographs of the aerial mycelia of *S. fradiae* 3739. The complex network of hyphae (a), when viewed at greater magnification, can be seen to terminate in chains of spores (b) which have smooth walls and are about 1 μm in diameter (see insert).

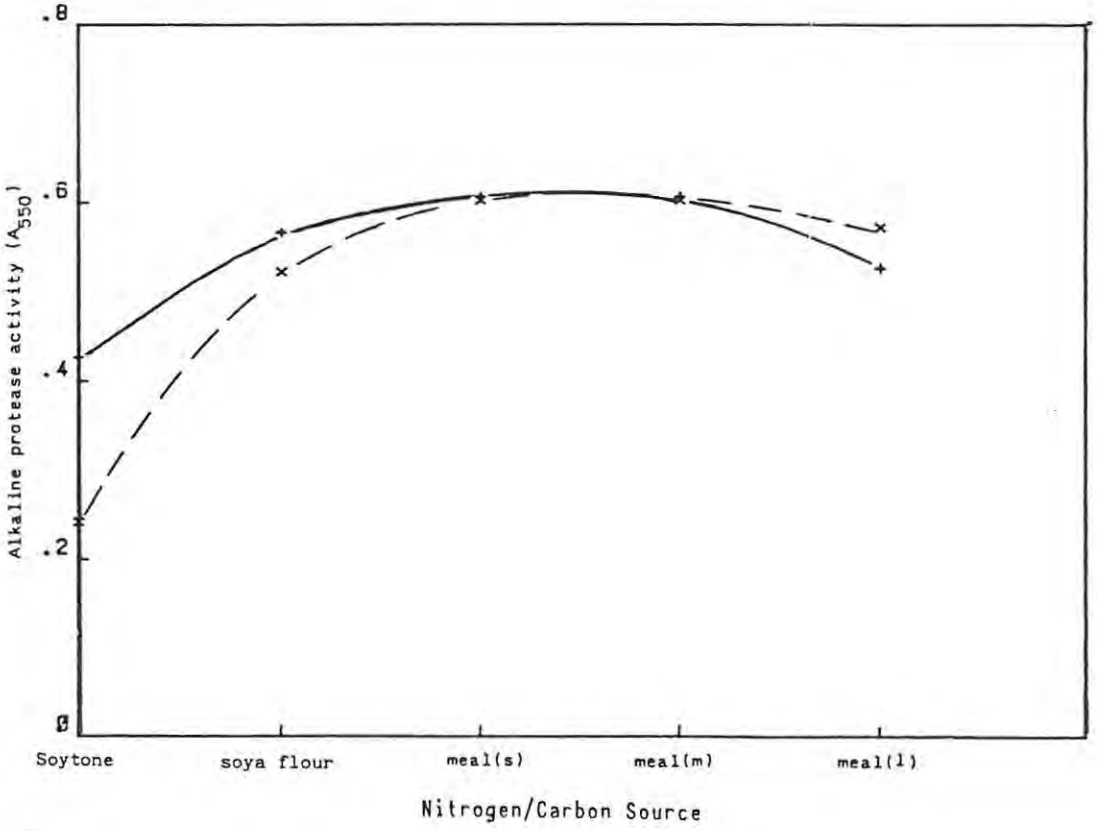


Fig. 12: The effect of limiting nitrogen and carbon sources on extracellular alkaline protease release from *S. fradiae*, without (—) or with (----) inclusion of 2% soluble starch. Soyatone is a pre-digested soya meal, soya flour is very finely milled, while the other meals were course with particle diameters of below 355 µm (s), between 355-500 µm (m), and 500-1 400 µm (l). These meals therefore represent nitrogen and carbon sources with serially decreasing availability.

b) Release of Alkaline Proteases and Amylase by *S. fradiae*

Alkaline protease release began after approximately 30 hours of incubation and reached a maximum after 40 hours and then remained constant (fig. 13). Amylase release began after 25 hours and reached its maximum level after another 10 hours of incubation time. Later amylase activity decreased again and then levelled off, possibly reflecting the presence of two different species of amylase enzyme, one of which being more stable than the other. The corresponding decrease in amylase activity with high protease release may reflect a species of amylase which is prone to proteolytic attack. This would have to be substantiated by further experimentation.

3) Purification of Extracellular Proteases from *S. fradiae* cultures

The results of the purification procedure can be seen in Table 4. The precipitations with ammonium sulphate and acetone each gave the same percentage yield in repeated purifications (i.e. 66-69% and 23-29% respectively). A second ammonium sulphate precipitation proved to be useful in that it doubled the specific activity while only causing an 8% decrease in yield as compared to the acetone precipitation which gave only a slightly greater specific activity and a 30-40% decrease in yield. Cation exchange on CM Sephadex C-25 purified the protease considerably (Table 4).

The protease exhibited poor binding to the cation exchange column but enough separation occurred to observe four partially separated peaks of activity, one of which was considerably smaller than the others (fig. 14), which was paralleled by keratinase (fig. 15) and elastase (fig. 16) activity levels. It is noteworthy that the overlap of the two protease peaks in fraction 5 appear to have a synergistic effect on the degradation of elastin and especially on keratin, i.e. in fractions where two peaks of alkaline protease activity merged enhanced substrate degradation occurred.

Unexpectedly both the protease and amylase components of the *S. fradiae* extracellular enzymes were retained by ultrafiltration filters of 30 000 dalton retention. During this procedure the

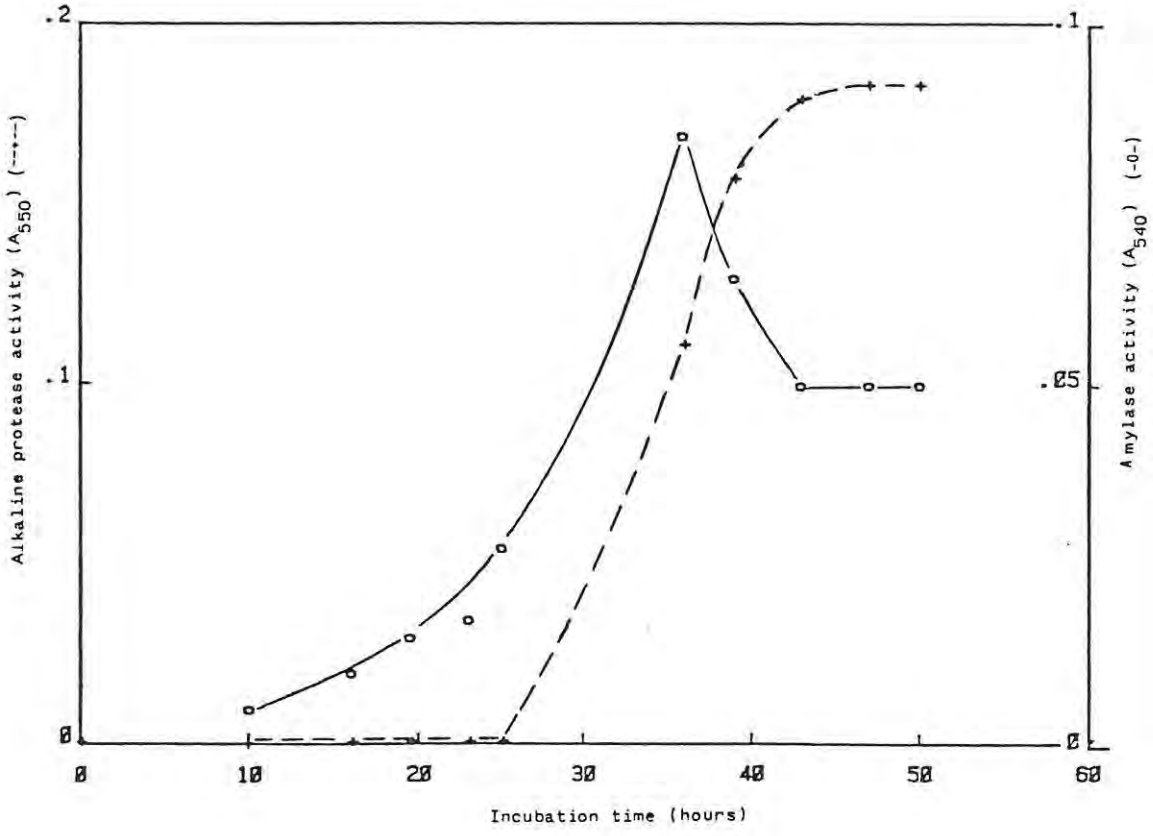


Fig. 13: Extracellular enzyme release from *S. fradiae* as a function of culture incubation time.

TABLE 4: Purification of Alkaline Protease from Cultures of *S. fradiae* 3739

Fraction	Total volume cm^3	Protein $mg.cm^{-3}$	Alkaline protease activity			
			Activity/cm^3 μmol/min/cm^3	Specific act. μ mol/min/mg	Total act. μ mol/min	% Yield
Crude culture	230	3.0	1.67	0.56	384	100
Ammonium sulphate precipitation	200	0.9	1.33	1.48	266	69.3
Ammonium sulphate precipitation (No. 2)	50	1.5	4.67	3.11	233	60.7
Acetone precipitation (0.5-2.0 volumes)	50	0.65	2.20	3.39	110	28.6
Cation exchange column	110	0.02	0.82	41.2	90.2	23.5

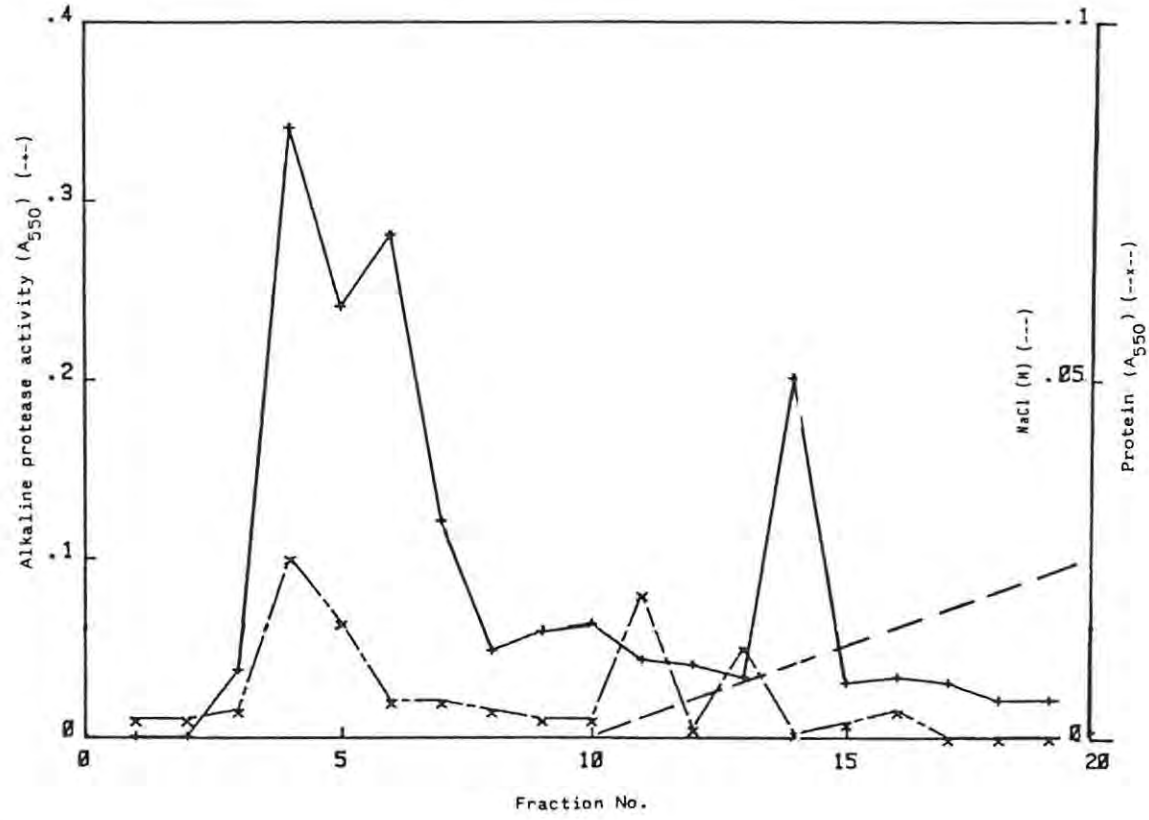


Fig. 14: Elution of alkaline protease of *S. fradiae* from cation exchange column (CM Sephadex C-25) equilibriated with 0.05 M phosphate buffer and eluted with a 0-0.1 M NaCl gradient in the same buffer at a flow rate of 1 $cm^3.min^{-1}$. Fraction volumes collected were 10 cm^3.

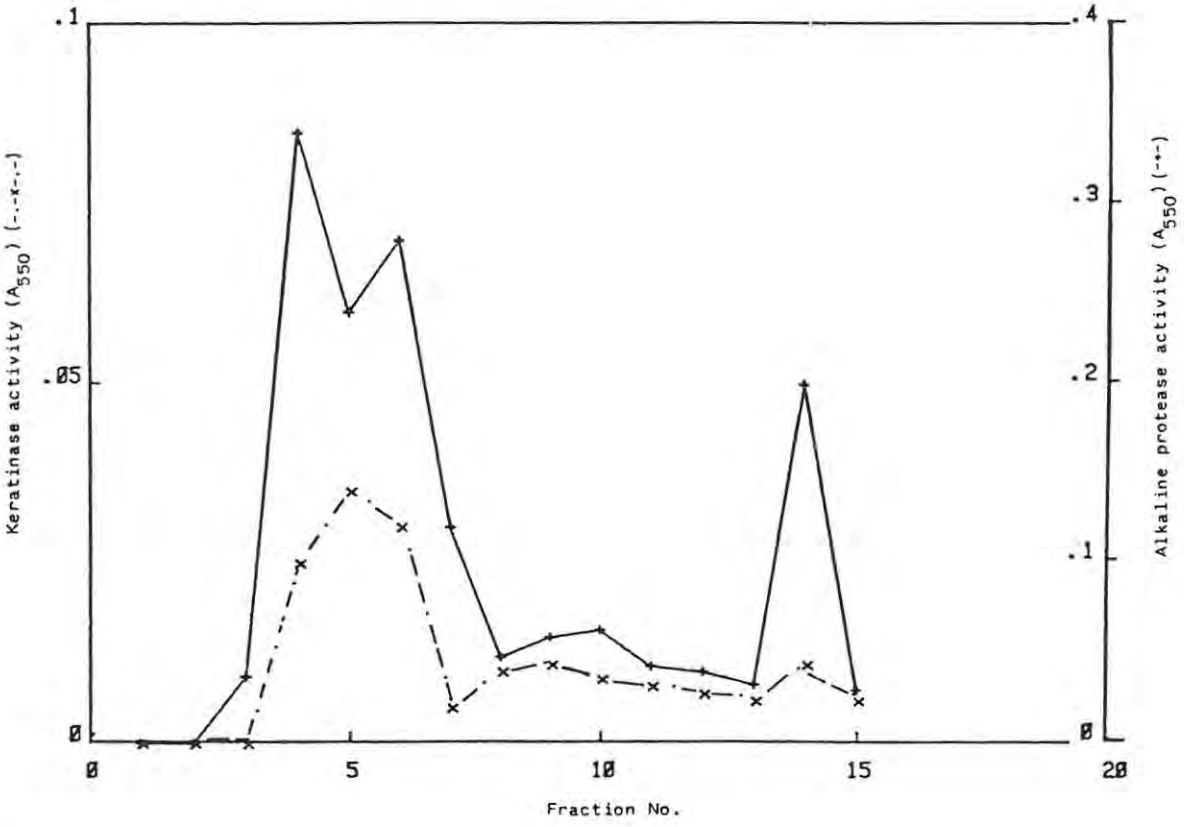


Fig. 15: Comparison of keratinase and alkaline protease activity of *S. fracliae* eluted from cation exchange column.

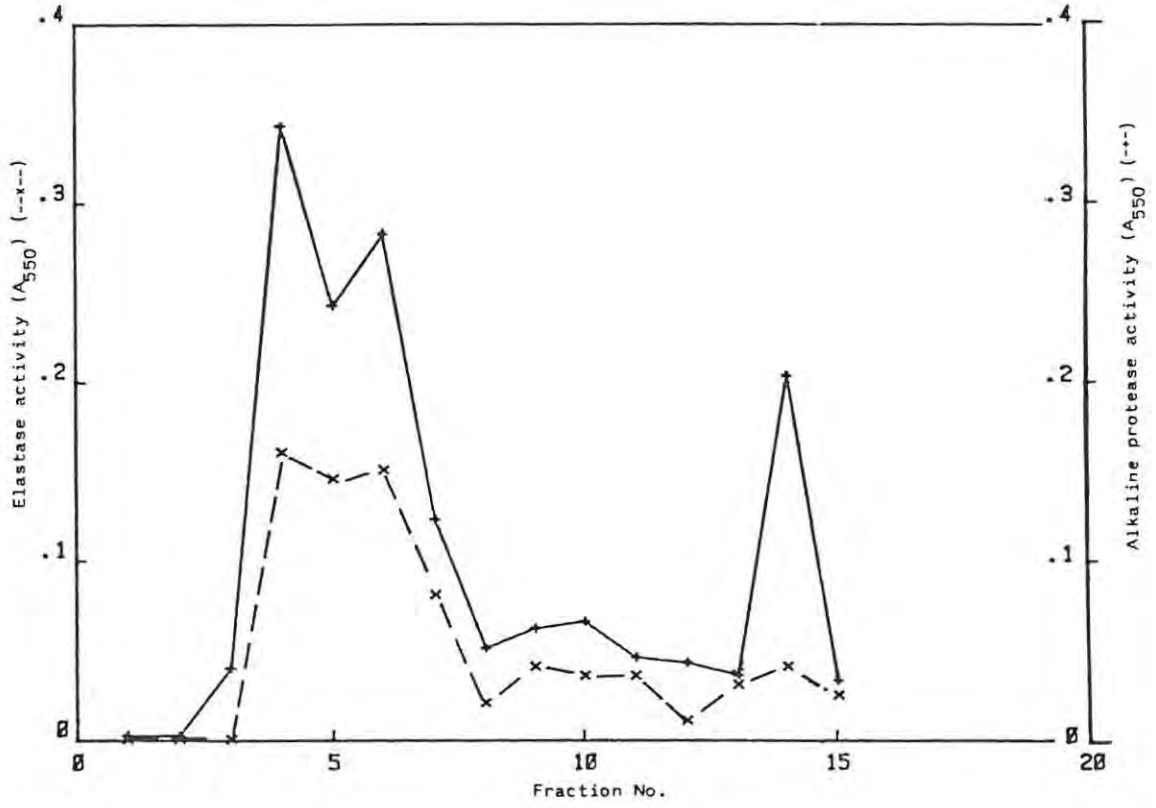


Fig. 16: Comparison of elastase and alkaline protease activity of *S. fradiae* eluted from cation exchange column.

amount of alkaline protease activity was reduced by approximately 1/3, probably as a result of a degree of enzyme denaturation, while the yield of amylase was reduced by nearly 90% (Table 5).

4) Characterisation of the Partially Purified Alkaline Proteases from *S. fradiae*

The alkaline protease positive fractions eluted from the cation exchange column were pooled in order to obtain sufficient material for further analysis. The analyses were performed on the partially purified fractions in order to obtain further information on the general characteristics of alkaline proteases from *S. fradiae*.

a) pH Optimum

The pH optimum for protease activity for this enzyme group with casein as the substrate was between 8.0-9.0 pH units (fig. 17). The same enzyme group was similarly most active with elastin as the substrate in the 8.5-9.0 pH range, giving a slightly narrower optimum range of activity than where casein was the substrate (fig. 18). The protease group as a whole then appears to be optimally active in the weakly alkaline range.

b) Thermostability

The enzyme group was inactivated completely by boiling for 10 minutes. At 80^{o}C there was a 70% decrease in proteolytic activity over 10 minutes. However divalent calcium ions proved to stabilise the enzymes considerably as the inclusion of 0.01 M $CaCl_2$ into the solution resulted in only a 10% decrease in proteolytic activity when the enzyme solution was maintained at 80^{o}C for 10 minutes.

c) Molecular Mass

The alkaline protease group when eluted from the gel exclusion column yielded two separate peaks of activity, a relatively sharp one of 39 000 daltons, and a broader one of between 13 000-17 000 daltons (fig. 19).

TABLE 5: Ultrafiltration of Extracellular Enzymes from Cultures of *S. fradiae* 3739

Fraction	Total volume	Protein	enzyme activity			
	cm^3	$mg.cm^{-3}$	Activity/cm^3 µmol/min/cm^3	Specific act. µmol/min/mg	Total act. µmol/min	% Yield
Alk. protease activity Crude culture	400	0.288	1.87	6.49	748	100
Filtrate	410	0.205	0.0	0.0	0	0
Retentate	10.5	0.117	45.3	387.0	476	63.6
Amylase activity Crude culture	400	0.288	0.12	0.417	48	100
Filtrate	110	0.205	0.0	0.0	0.0	0
Retentate	10.5	0.117	0.53	4.53	5.57	11.6

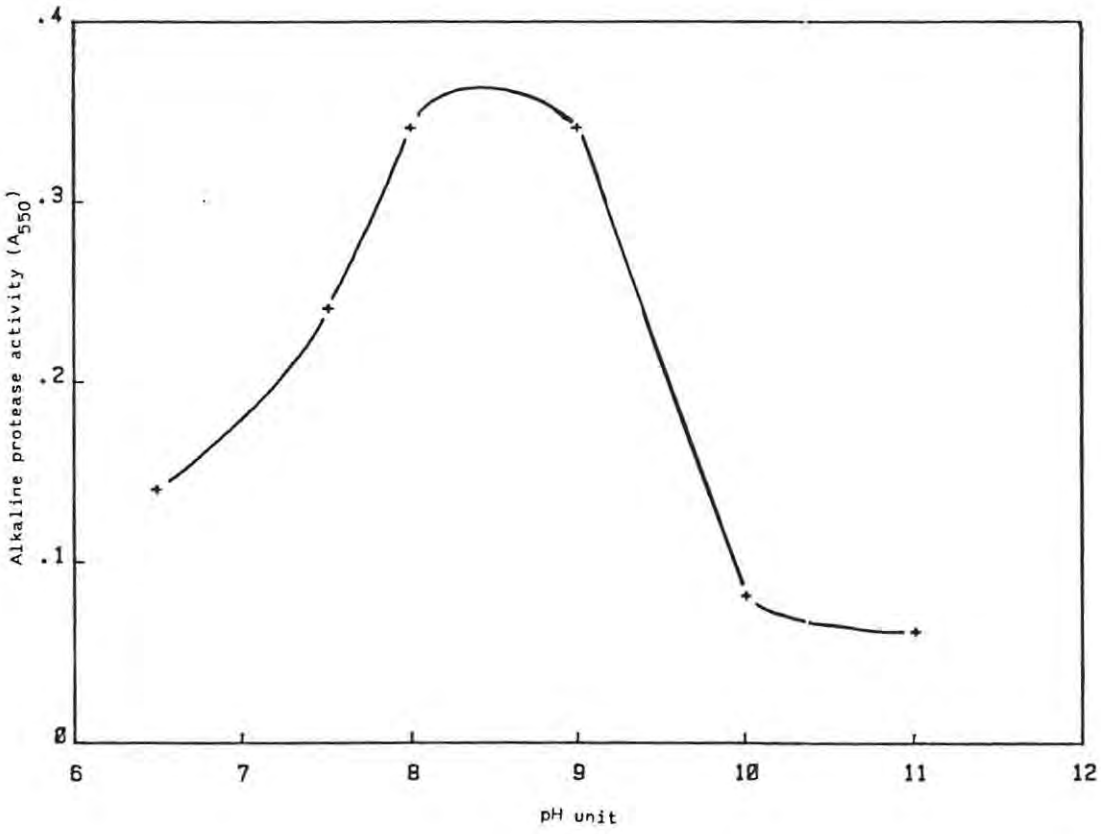


Fig. 17: *S. fradiae* extracellular protease activity as a function of pH.

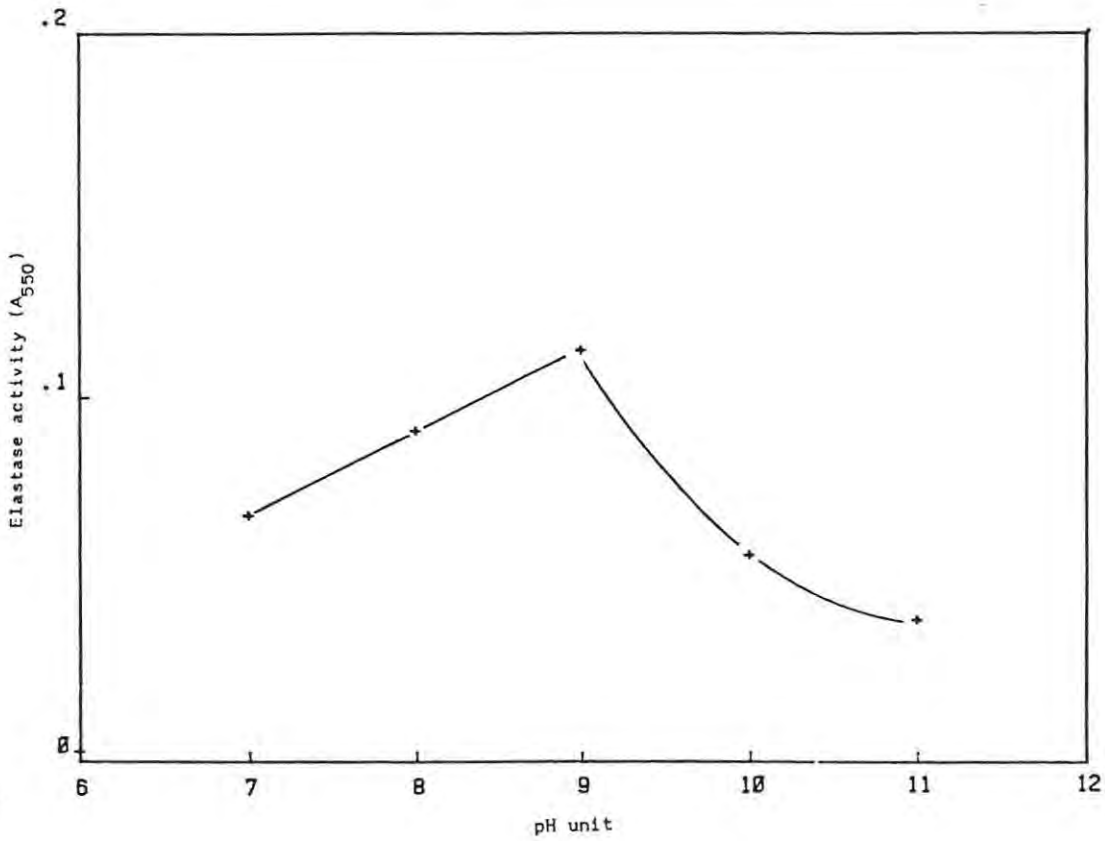


Fig. 18: *S. fradiae* extracellular elastase activity as a function of pH.

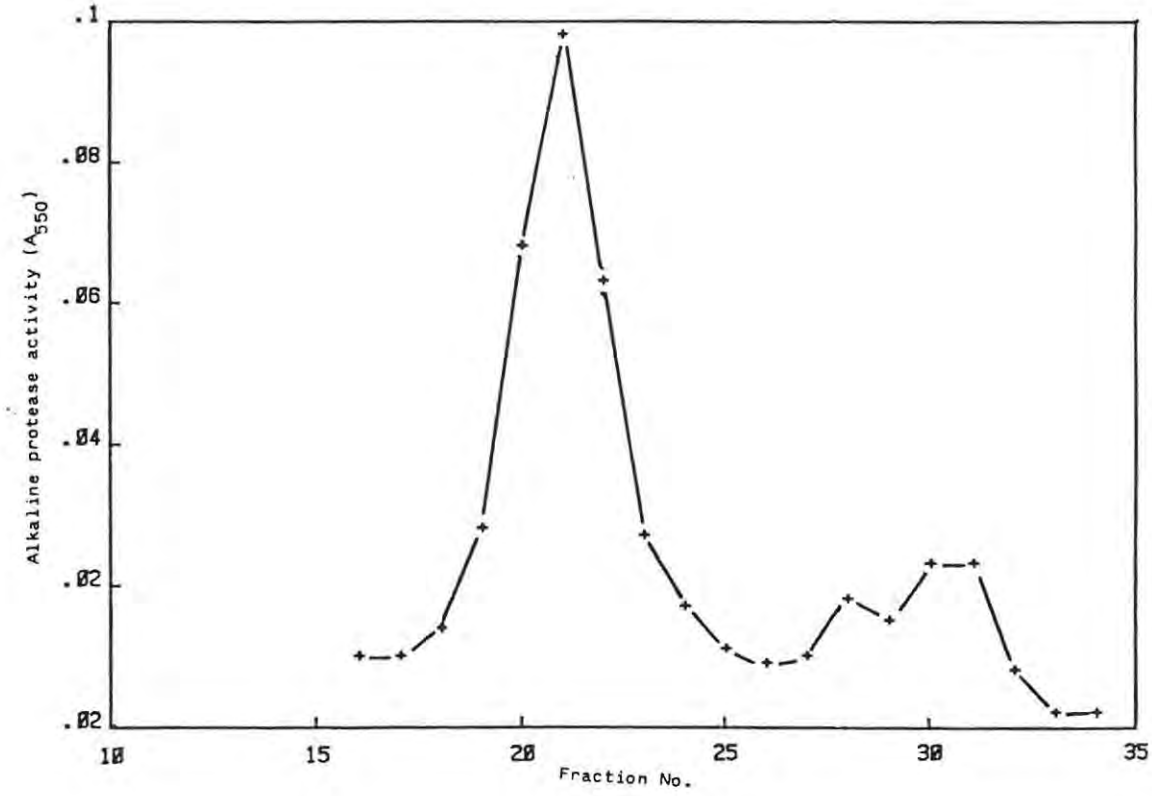


Fig. 19: *S. fradiae* alkaline protease gel exclusion elution profile from a Sephadex G-75 column.

III) Production and Characterisation of Extracelluar Proteases of *Proteus vulgaris*

1) Identification of a proteolytic bacterium (*P. vulgaris*)

The unidentified bacterium on casein-agar plates showed powerful proteolytic activity as evidenced by the clearing of the opaque media around the bacterial colony, while a control colony (*Escherichia coli*) had no effect on the media. The bacterium also showed powerful gelatinase activity when being identified by the API system, the results of which confirmed the bacterium to be a strain of *P. vulgaris* (fig. A6, Table A3 appendix). *P. vulgaris* had no degradative activity towards soluble type I collagen as was the case with *S.fradiae* (A.Brüning, personal communication) When grown on either the casein-agar plates (Table A4, appendix) or on nutrient agar plates the typical swarming of this genera occurred with *P. vulgaris*, i.e widely separated concentric rings of growth points could be seen (fig. A7, appendix).

2) Production and Isolation of the *P. vulgaris* Extracellular Protease

The protease was released into the media between 12 and 24 hours in the conditions of culture. The precipitation of the proteases with ammonium sulphate was most effective in the 55-65% saturation range (fig. A8, appendix), and this step of purification was very effective, giving a yield of above 80% and a purification of nearly 50 times over the crude preparation (Table 6).

Treatment with anion exchange resin was extremely mild, causing little loss in yield (less than 2%) and producing one peak (with a small shoulder) of alkaline protease activity which was free of most of the contaminating protein (fig. 20), giving a three fold increase in the degree of purification. The ultrafiltration steps, however, proved to be extremely detrimental to the enzyme activity causing a 71.5% and a 67.4% decrease in activity in the two different stages of enzyme purification respectively (Table 6), with very little increase in the degree of purification. .

TABLE 6: Purification of Alkaline Protease from Cultures of *P. vulgaris*

Fraction	Total volume	Protein	Alkaline protease activity			
	cm^3	$mg.cm^{-3}$	Activity/cm^3 µmol/min/cm^3	Specific act. µmol/min/mg	Total act. µmol/min	% Yield
Crude culture	1550	3.40	2.60	0.76	4030	100
Ammonium sulphate precipitation	100	0.9	33.30	37.0	3330	82.6
Ultrafiltration	45	0.45	21.00	47.0	945	23.5
Anion exchange column (combined fractions)	80	0.034	4.89	143.8	879	21.8
Ultrafiltration	21	0.042	6.00	142.0	284	7.1

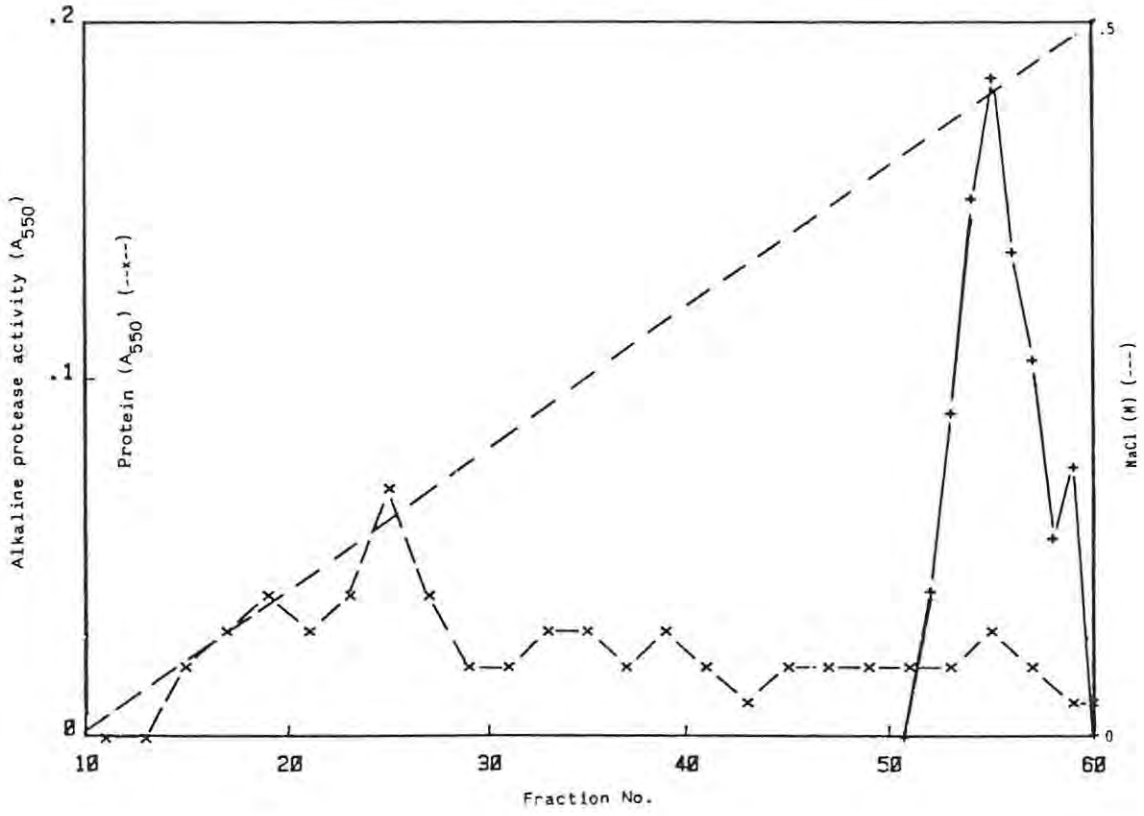


Fig. 20: Elution of *P. vulgaris* protease from anion exchange column (DEAE Sephadex A-50). The column was previously equilibriated with 0.1 M Tris buffer pH 7.2 and eluted with a saline gradient (0-0.5 M NaCl) in the same buffer at a flow rate of 1 $cm^3.min^{-1}$. Fraction volumes of 10 cm^3 were collected.

3) Characterisation of the Partially Purified Protease Activity from *P. vulgaris*

a) Ionic Stability

The enzyme was fairly stable at a wide range of salt concentrations (0-0.8 M), but between 0.8-1.4 M salinity the alkaline protease activity began to tail off rapidly (fig. 21) The enzyme appears to be most active at a 0.2 M concentration of NaCl, although this probably indicates a greater stability at near physiological salinity (0.15 M) at the incubation temperature of 37^{o}C rather than any potentiation of the enzyme (fig. 21).

b) Thermostability

The enzyme was not particularly thermostable with loss of enzyme activity occurring above 30^{o}C and proteolytic activity was completely destroyed by heating at 50^{o}C for 10 minutes. Inclusion of 0.01 M $CaCl_2$ in the reaction mixture afforded the enzyme only a small increase in thermostability (fig. 22).

c) Optimum Temperature

The optimum temperature for enzyme activity was seen to peak at 40^{o}C (fig. 23). The enzyme had very little activity at 10^{o}C, and it is rapidly inactivated at temperatures higher than 40^{o}C.

d) Optimum pH

The enzyme is optimally active in the 8.0-9.0 pH unit range. There is a rapid loss of activity on the alkaline side and a more gradual loss of activity towards neutrality (fig. 24). The enzyme can therefore be said to act optimally in the weakly alkaline range.

f) Molecular Mass

Only one peak of alkaline protease activity was eluted from the gel exclusion column when the partially purified extracellular protease of *P. vulgaris* was run through a Sephadex G-75 column.The molecular mass of the protease from *P. vulgaris* can be calculated to be approximately 44 000 daltons from this graph (fig. 25). This would explain the fact that the enzyme was completely retained by the 30 000 dalton retention ultrafiltration membrane.

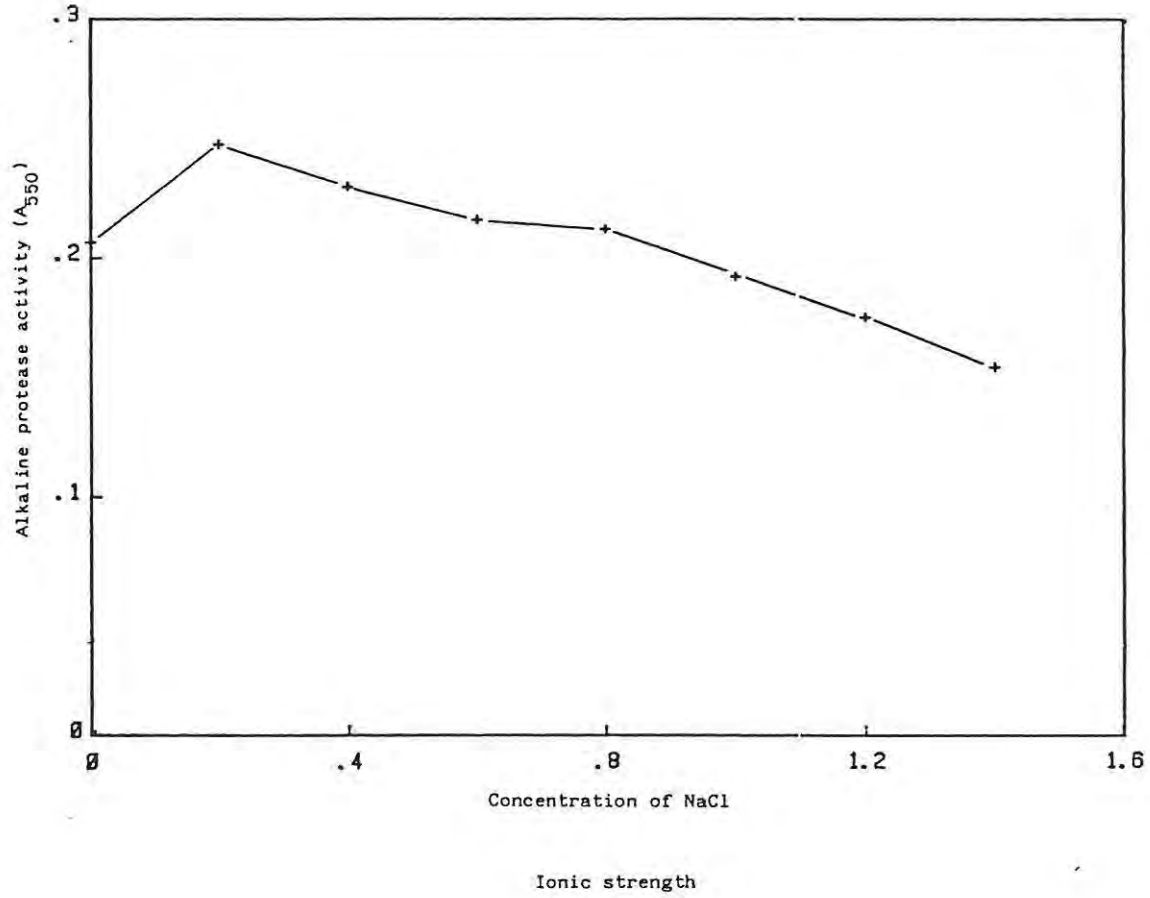


Fig. 21: Activity of *P. vulgaris* protease as a function of ionic strength.

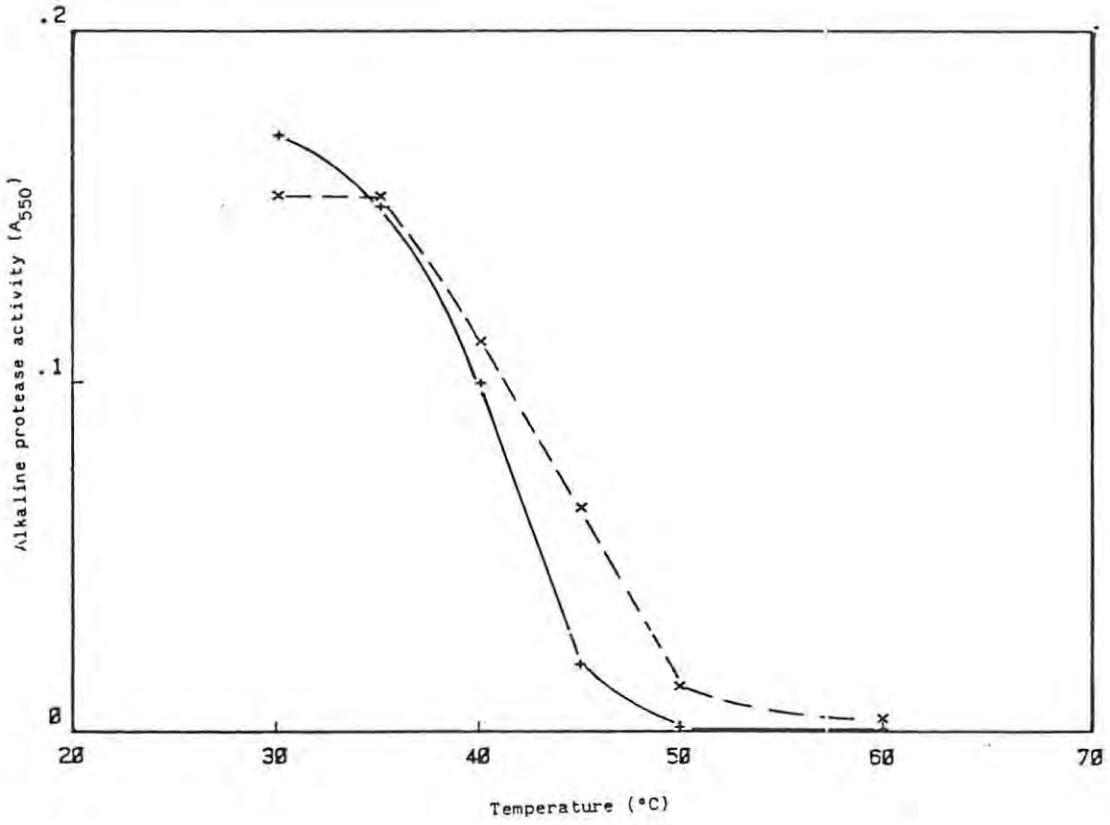


Fig. 22: Thermostability curve of *P. vulgaris* protease (—+—) and with the inclusion of 1 cm^3 of 0.2 M $CaCl_2$ (--x--).

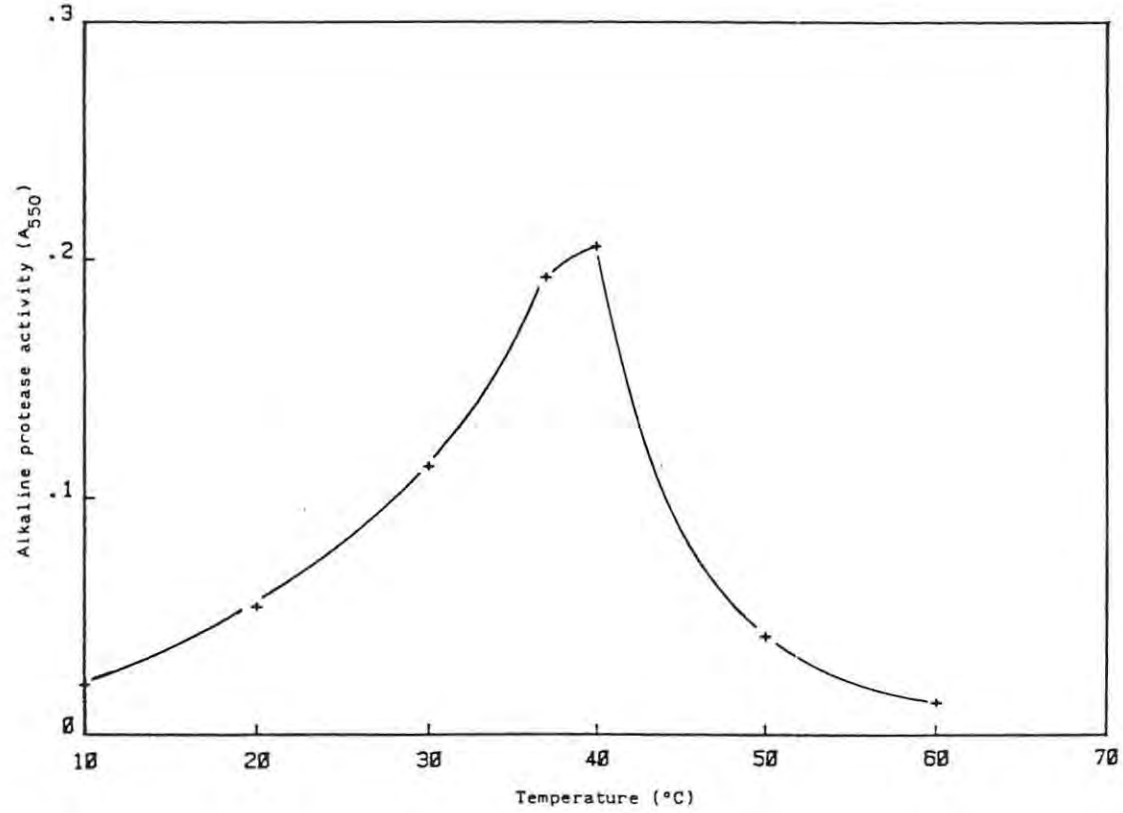


Fig. 23: Optimal temperature for activity of protease from *P. vulgaris*.

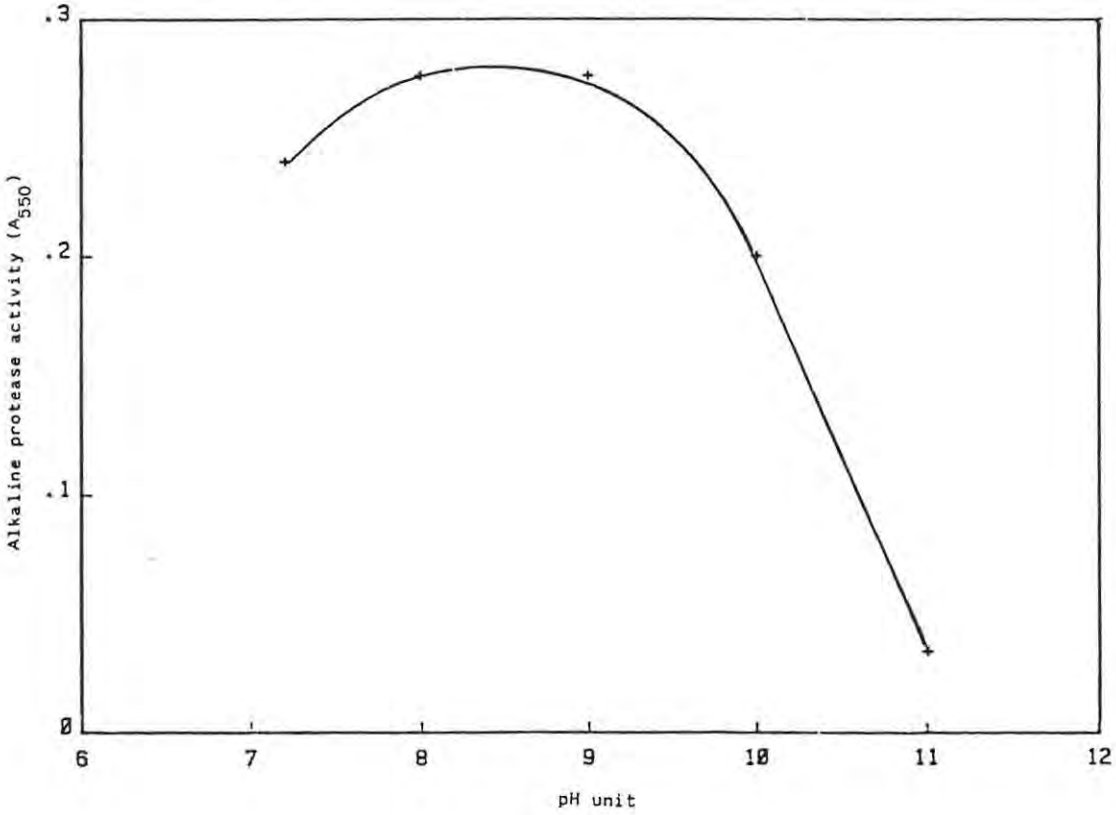


Fig. 24: *P. vulgaris* extracellular protease activity as a function of pH.

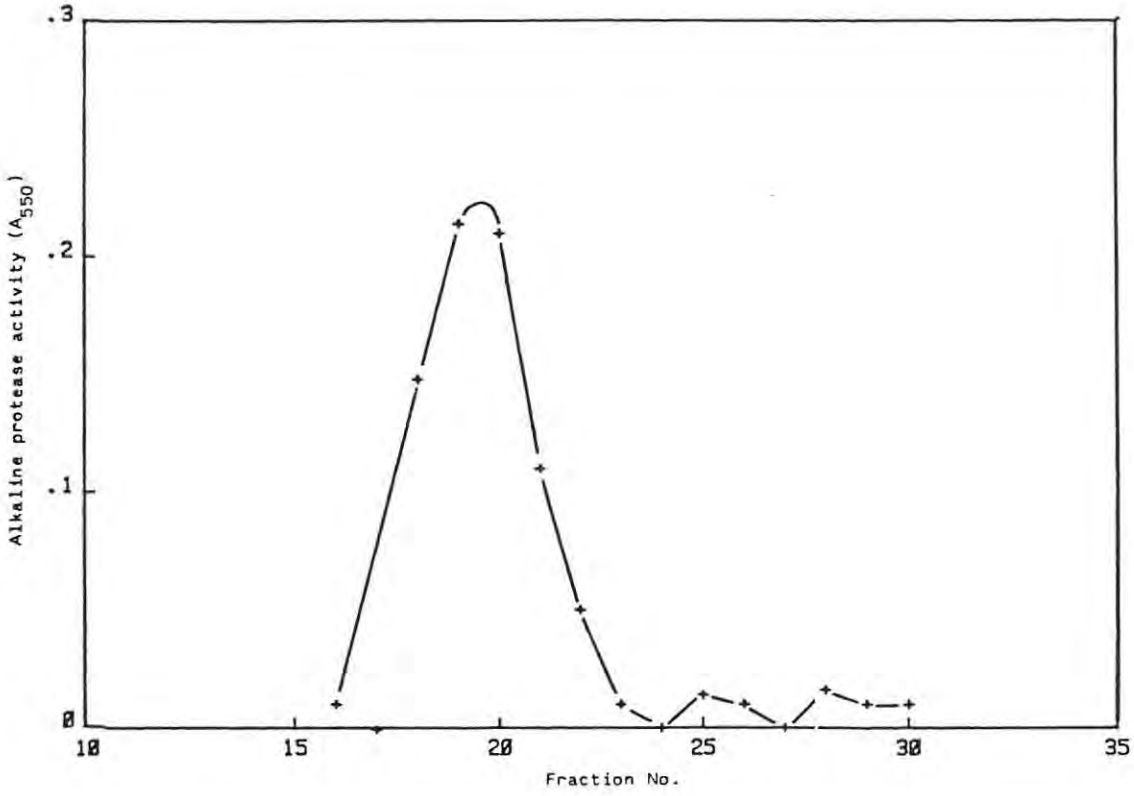


Fig. 25: *P. vulgaris* extracellular protease gel exclusion elution profile from a Sephadex G-75 column.

IV) Depilation of Merino Skins with Bacterial Proteases

1) Depilation with the Extracellular Proteases of *S. fradiae* 3739 and *P. vulgaris*

Partially purified alkaline protease solutions from *S. fradiae* and *P. vulgaris* (from cation and anion exchange columns respectively) and a commercial preparation of Pronase (as a positive control) were incubated with pre-soaked merino skins for 24 hours. Both the Pronase and the protease from *S. fradiae* reduced the depilation load to below 2 (at which the wool comes away freely from the skin), while on the sheep skin plugs treated with the *P. vulgaris* protease the wool was still quite firmly attached. All protease solutions were previously adjusted to pH 9.0 by addition of equal volumes of 0.1 M Tris buffer pH 9.0.

A crude culture of *P. vulgaris* concentrated approximately 50 times by ammonium sulphate precipitation (60%) and resuspended in 0.05M Tris buffer pH 7.2 was capable of depilation if the skin was pre-alkalised in 0.2 M NaOH, especially if 0.1 M sodium sulphite was included in the depilatory enzyme preparation (Table 7). Pre-alkalisation and sodium sulphite alone did not appear to reduce the depilation load to a large degree, but they did appear to increase the effectiveness of the protease.

2) Depilation Experiments with Combinations of Commercial Enzyme Preparations

Combinations of enzymes had a significant effect on the ability of the enzyme preparation to depilate. The average depilation load of the skins treated with a mixture of enzyme preparations was less than 60% of that of the single enzyme preparations, even though they contained the same concentrations of protease. Combining proteases therefore significantly increases the depilatory activity of the preparations without increasing the amount of enzyme required (fig. 26).

TABLE 7: Depilation of Sheepskins with Extracellular Enzymes from *F. vulgaris*

Treatment	Total mass applied in g (M)	Total mass wool pulled in $mg_{\bullet}cm^{-1}$ (m/l)	Depilation load $g.cm.mg^{-1}$ (M)/(m)
Untreated (control)	840	9.2	91.3
Enzyme alone	440	7.6	57.9
Pre-alkalisation + enzyme	100	16.0	3.2
Pre-alkalisation + sodium sulphite	740	9.0	82.2
Pre-alkalisation + sodium sulphite + enzyme	30	15.4	1.9

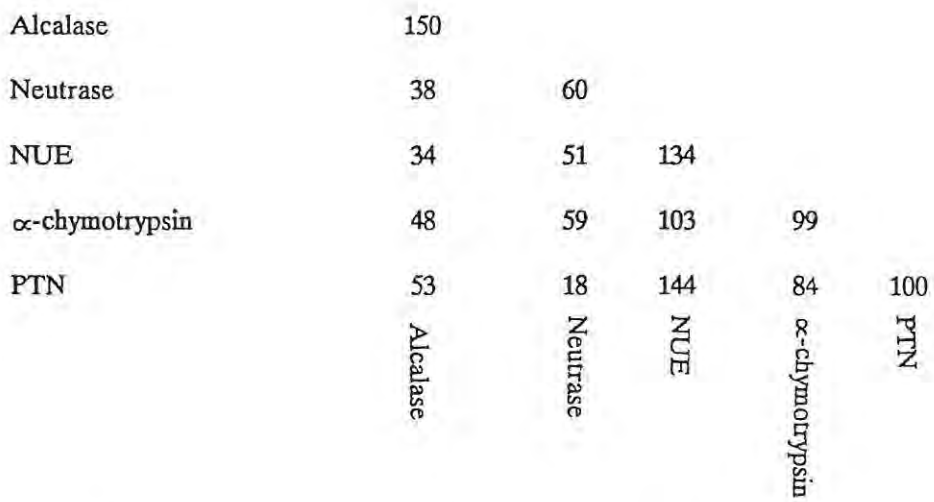


Fig. 26: Depilation loads of skins treated with single and dual protease preparations. The average depilation load for single enzyme preparations was 109, while that for dual protease preparations was 63.

3) Depilation with Multiple Enzyme Systems

The addition of a number of enzymes without increasing enzyme concentration once again showed that there is a synergistic effect between enzymes to reduce the depilation load (fig. 27). The pre-treatment with a strong alkali could be seen to reduce the number of enzymes required for complete depilation to occur.

4) Depilation Experiments with Various Alkalisation Treatments Included

The quality of the resultant raw leather was found to be decidedly dependent on the type or lack of an alkalisation treatment. Skin which was merely enzyme depilated had a 48.5% increase in area compared to the lime sulphide treated skin, but was very thin and cardboard like to the feel; it was also unsupple and could be permanently deformed. The skin sections treated with 3% $Ca(OH)_2$ or 2% $Ca(OH)_2$ plus 1% NaOH had an increase in area of 21% and 19% respectively as compared to the lime-sulphide treated control (although on full size skins smaller area increases are to be expected). Of the enzyme depilated skin sections the ones treated with the "sharpened" lime were the most supple, being better to the feel than those just treated with lime and far superior to those not treated with an alkali whatsoever. The skin sections treated with the NaOH "sharpened" lime after enzymic depilation had a comparable thickness and grain quality to those treated with lime and sodium sulphide. The lack of opening up of the enzyme depilated skins which were not alkalised or merely treated with 3% $Ca(OH)_2$ was evidenced by the fact that release of acetone soluble material into the acetone washes continued for a far greater number of serial acetone treatments than occurred with the lime-sulphide or "sharpened" lime treatments. This would conversely probably hinder the penetration of the raw leather by later process liquors and would therefore be disadvantageous.

All of the enzyme depilated skin sections retained a degree of fine short wool. Transverse sections of raw leathers, viewed by light microscopy, showed that the skin sections treated with lime-sulphide retained some debris of the wool fibre in the hair papillae, while those treated with enzyme showed completely debris free papillae.

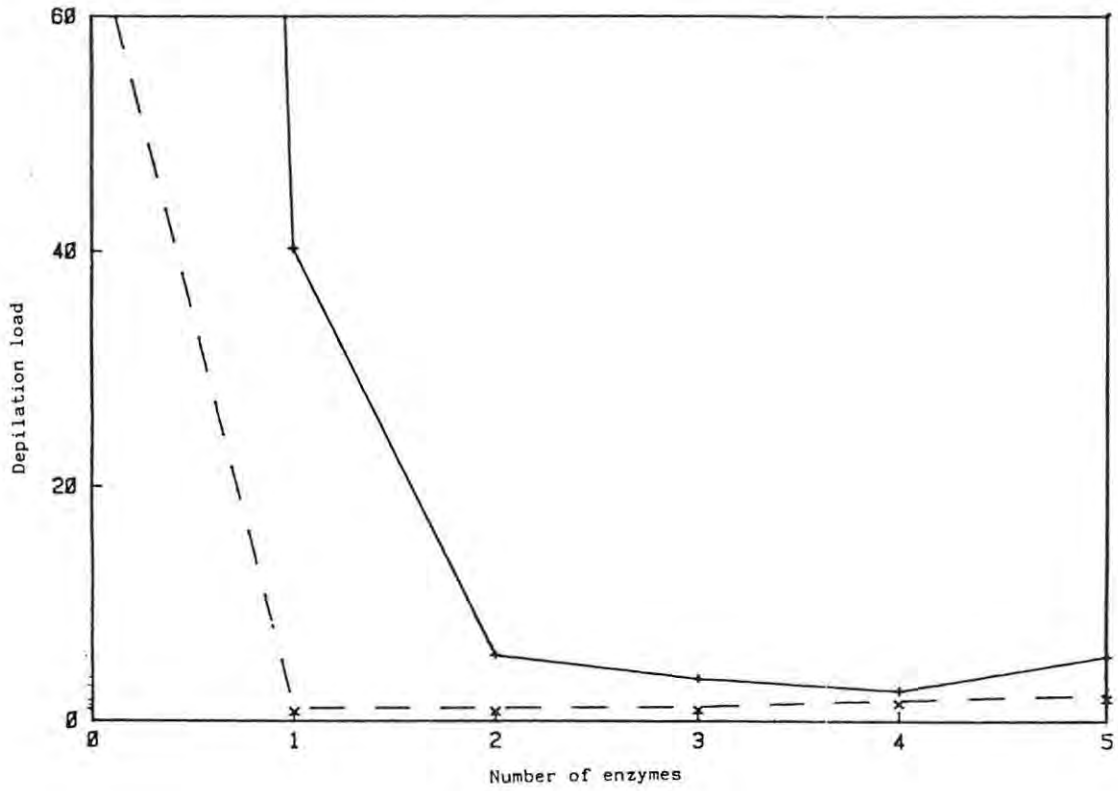


Fig. 27: Decrease in depilation load of skins after treatment with multiple protease preparations alone (— + —) or after pre-alkalisation (--x--).1 = Alcalase, 2 = Alcalase + Neutrase, 3 = Acalase + Neutrase + NUE, 4 = Alcalase + Neutrase + NUE + ∝-chymotrypsin, and 5 = Alcalase + Neutrase + NUE + ∝-chymotrypsin + PTN, all in equal ratios. The total concentration of protease was identical in each preparation.

D) DISCUSSION

I) Evaluation of Experimental Results

The results from this study will be discussed with respect to the objectives of this investigation as mentioned in the introduction, e.g.:

i) To investigate the possibility of a correlation between a bacterial strain's ability to degrade wool and its extracellular enzyme's capacity to cause depilation.

ii) To isolate and characterise the extracellular proteases of *Streptomyces fradiae* 3739, which are known to be depilatory enzymes.

iii) To isolate and characterise the extracellular proteases of a bacteria (*Proteus vulgaris*) which is probably linked to the occurrence of hair slip during hide staling.

iv) To determine if there is a synergistic effect between enzymes during depilation with mixed enzyme systems.

1) Correlation Between Wool Degradation and Depilation

According to the results obtained in this study there appears to be, at least within the *Streptomyces* family, a distinct correlation between their ability to degrade wool and the capacity of their extracellular enzymes to cause a decrease the depilation load of sheep skins. These results include and expand upon those already published (15). The assay method for depilation load, although simple, appears to give fairly accurate and reproducible results.

The extracellular enzymes of some *Streptomyces* strains are able, according to the scanning electron microscope studies, to degrade the binding proteins which hold the spindle cells together but not the "hard" keratin of the spindle cells themselves. This is in agreement with the results of other workers who pointed out that "hard" keratin structures such as mature human *stratum corneum* was resistant to a commercial preparation of keratinase from *S. fradiae* (29).

Although the proteases of *S. fradiae* 3739 have keratinolytic activity according to this and other studies (67, 78, 79, 82, 90), the enzymes are only partially keratinolytic and bring about slow and incomplete degradation of the "hard" keratins. When using these enzymes in depilation experiments, depilation proceeded before any visible damage to the wool occurred. In fact it was noted that parts of the root sheaths were still attached to the depilated wool in many instances, and that all the depilated wool fibres appeared to have intact roots when viewed under low power light microscopy.

It appears then that the main action of the extracellular enzymes of certain strains of *Streptomyces* bacteria is to degrade the immature or "soft" keratins and not the heavily cross-linked mature or "hard" keratins. The strong correlation between wool degradative and depilatory activity of these systems then has more subtle implications than the indiscriminate destruction of the wool and epidermis. Is the reason simply that only the bacteria that could degrade wool could multiply and produce proteases; or is there a similarity between the two processes, i.e. a synergistic effect between proteases in both depilation and wool degradation? The results of the present study suggest that the latter possibility may be correct.

2) Isolation and Characterisation of the Extracellular Proteases of *S. fradiae* 3739

It has been shown in this study that *S. fradiae* 3739 produces, in certain conditions, both amylase and alkaline protease extracellular enzymes. The amylase activity peaks before that of the alkaline protease activity, perhaps reflecting a greater initial requirement for an energy source than for a nitrogen source. It was also shown that if the nitrogen and carbon source was fairly readily available then the protease release was repressed; this is in agreement with the results of previous workers who pointed out that free amino acids or simple sugars could repress protease expression when included in the culture medium of *S. fradiae* (67, 82) similar to that seen in other bacteria (58), or that increasing the protein content of the media also caused repression of protease release (67).

Although the initial steps of purification of the alkaline protease of the extracellular proteases of *S. fradiae* 3739 were successful, the proteases failed to bind to the cation exchange column to any significant degree. Morihara et. al. (67) grew a strain of *S. fradiae* on a complex media and obtained binding to a cation exchange column (CM cellulose). However they stated that the product from *S. fradiae* 3739 that had been cultured in a wool/ basal salts medium had to be treated with an anion exchanger (DEAE cellulose) before binding to the cation exchanger could occur; a treatment designed to remove an acidic polymer associated with the enzymes (78, 79). By either process they obtained 4 well separated peaks of activity, one of which on further purification was found to contain two peptidases and two proteases, while the others contained one protease each. In the present study 4 peaks of activity were observed, but unfortunately they were not well separated. When more crude protease solution was added to the column resolution deteriorated. Numerous attempts at further purification of the enzymes proved unsuccessful.

Owing to the failure in effectively separating the individual proteolytic enzymes it was decided to analyse the group as a whole. This was in some ways more informative than looking at the individual enzymes as it is generally crude enzyme preparations and not highly purified enzymes that are used in the leather industry (85). The alkaline protease positive fractions from the ion exchange column were therefore combined and characterised as a group.

A sample of the combined fractions was run on the gel exclusion column and two peaks of activity, relating to the molecular masses of 39 000 and 13 000- 17 000 daltons, were obtained. Morihara *et. al.* determined the molecular masses of the proteases of *S.fradiae* designated as Ib, II, III, and IV to be 20 000, 17 700, 14 000, and 16 500 daltons respectively (69, 67). Another group gave the molecular mass ranges of the proteases to be between 15 000- 21 000 daltons (90) and that of the keratinase (II) to be 27 000 daltons (79). The acidic polymer which is suggested to prevent binding to the cation exchanger would contribute to the molecular mass of the proteases, possibly explaining the alkaline protease activity peak of 39 000 daltons, while the smaller peak of activity could represent a portion of the protease which was not associated with the acidic polymer.

The results of the characterization of the combined proteolytic fractions from the cation exchange column could be compared to those reported in the literature. The observed pH optimum of alkaline protease activity at between 8.0-9.0 pH units, the slightly sharper peak of optimal elastase activity in the same pH region, the considerable increase in thermostability of the enzyme with addition of calcium salts, the enzymes' ability to degrade wool, and their ability to cause depilation without pre-alkalisation, were similar characteristics to those found by other workers (67, 45, 95, 129), indicated that this group of enzymes was similar to that reported in the literature.

3) Isolation and Characterisation of a Protease from *Proteus vulgaris*

Apart from the harshness of the ultrafiltration process on the enzyme during the isolation of an extracellular protease from *P. vulgaris*, the purification of this protease proceeded without any major difficulties. From both the anion exchange column and the gel exclusion column only one peak of alkaline protease activity was eluted. The molecular mass of the protease was determined to be approximately 44 000 daltons according to the elution volume of the enzyme from the gel exclusion column.

The protease activity of this strain of *P. vulgaris* had a similar pH optimum to the protease from *S. fradiae*, being weakly alkaline. The pH curve was found to be similar for both purified and crude enzyme solutions (the latter not shown). The enzyme was not thermostable at temperatures above 35^{o}C and its thermostability only improved to a small degree with inclusion of calcium ions into the reaction mixture. The enzyme's thermostability limited its optimum temperature for proteolytic activity to 40^{o}C The enzyme was, however, quite stable to highly ionic environments; this is important as it allows the enzyme to function in the saline conditions of hides and skins that have been inadequately salt cured after some degree of staling has commenced. The enzyme was still active at salt concentrations of 1.4 M, which equates to an 8.1% saline solution.

In itself the enzyme did not appear to cause depilation unless the skin being treated had been

pre-alkalised, and the enzyme's depilatory activity was increased by the inclusion of a reducing agent (sodium sulphite) into the enzyme solution. Maxwell and Lennox (62) claimed successful depilation with extracellular enzymes from *P. vulgaris*, but they had exposed the sheep skin to sodium metabisulphite at pH 2.0 prior to enzyme treatment, therefore swelling and denaturation similar to that caused by pre-alkalisation had occurred. Gillespie (40) also used a strain of *P. vulgaris*, and found that its extracellular proteases only caused depilation if a reducing agent was added into the enzyme preparation. This suggests that the enzyme investigated in that study was a similar enzyme to that investgated in this study.

One investigation of an extracellular protease of *P. vulgaris* suggested that it could be precipitated with 40-60% saturation of ammonium sulphate and that, according to paper electrophoresis, it had only one proteolytic component (80). The enzyme had a optimal proteolytic activity at pH 9.0, and a range of proteolytic activity between 6.0-9.0 pH units. When purified the enzyme lost its activity when incubated at 60^{o}C for 15 minutes, although it was stable at 120^{o}C if left in the unpurified state (80). Again this enzyme appears similar to that described in the present study. Unfortunately there was insufficient material in the fractions eluted from the gel exclusion column in this study to yield definite bands on sodium dodecyl sulphate polyacrylamide electrophoresis so the number of proteolytic components could not be determined, however with a molecular mass of 44 000 daltons it is unlikely that any more than two components are present, and possibly no more than one.

In another study a protease of *P. vulgaris* was found to have optimal proteolytic activity between 9.0-10.0 pH units, and according to analytical ultracentrifugation had a $S_{20,w}$ of 3.7, which can be equated to a molecular mass of approximately 41 600 daltons. The enzyme showed greater proteolytic activity on denatured proteins than on native proteins; its proteolytic activity with haemoglobin as a substrate was greater than that of chymotrypsin but only one third of that of trypsin. It was said to require free -SH groups for activity (65). In the present study the protease appeared to require free -SH groups in the form of sodium sulphite for maximum depilatory activity to occur.

4) Synergism Between Enzymes During Depilation with Mixed Enzyme Preparations.

In the two bacterial enzyme systems studied the *S. fradiae* 3739 produced at least four different types of protease, while the *P. vulgaris* bacterium produced apparently no more than one type of protease. The former enzymes cause unassisted depilation, while the latter enzyme does not. Although the *S. fradiae* enzymes are keratinolytic and elastolytic it was pointed out in the introductory chapter that neither of these parameters are necessarily linked to depilatory activity. The main difference between the two enzyme systems, as far as depilatory activity is concerned, is the number of different proteases involved. It is probable that the *S. fradiae* extracellular proteases have a broader specificity of proteolytic activity than the single enzyme of *P. vulgaris*. This lends support to the theory that broad specificity proteolytic activity is the only activity required to cause depilation (128).

In this study and others (82, 125) Pronase has been shown to cause depilation. Pronase (a collective term for the extracellular proteases of *Streptomyces griseus*) is a group of extremely non-specific proteases, which have been noted to hydrolyse 87% of the total peptide bonds in ovalbumin and 75% of those in casein (74, 81, 112). The extracellular proteases of *S. fradiae* also exhibit a broad specificity of bond cleavage. Three of the extracellular enzymes from *S. fradiae* have been shown to be similar to chymotrypsin in bond specificity although each of them had a broader range of bond cleavage, with slightly different ranges of bond specificities from one another, while one has been shown to be similar to trypsin in its bond specificity (69, 70, 71, 68).

Thus a progression of depilatory activity can be seen:

i) Trypsin alone cannot cause depilation (40, 128).

ii) Trypsin plus chymotrypsin (pancreatic preparations) cannot cause depilation unless applied after pre-alkalisation (122).

iii) The extracellular enzymes of *S. fradiae* as a group have activity which includes tryptic, chymotryptic, and other activity. These enzymes are capable of depilating native skins.

Therefore with increasing broadness of specificity of peptide bond cleavage, it appears, comes increasingly depilatory activity. Yet a single example is not convincing, therefore in the final part of this study experiments were performed to determine if the above example could be extended to other protease preparations. In the first experiment involving a comparison between single and dual protease preparations a significant synergistic effect was seen in the dual protease preparations as compared to their single enzyme counterparts, even though the two groups contained similar concentrations of protease. In the second experiment it was seen that, up to a point, addition of different proteases while maintaining the overall protease concentration, produced increasingly better depilatory activity. Thus the rule of thumb which has previously been suggested (128) that increasing broadness of specificity of proteolysis parallels depilatory activity seems to have some validity. It would be unwise though to treat this rule of thumb as an indisputable fact until further studies have been performed with purified proteases on a wider range of skins.

II) A Possible Mechanism for Enzymic Depilation of Skins

The results of this and other studies have raised a number of important points, e.g.:

i) No activity against any skin component other than proteolytic actitivity against globular or non-fibrous proteins can be correlated with the depilatory activity of enzyme preparations.

ii) As a general rule increasing the broadness of the specificity of peptide bond cleavage of protease preparations leads to increasing depilatory activity but, from the results of the present study, with an ever diminishing percentage of depilatory load decrease as the specificity range increases.

iii) The basement membrane at the dermal-epidermal junction appears to be the site of depilatory action.

iv) The basement membranes consist of a network of globular glycoproteins, proteogylcans, and non-fibrous types of collagen. All of these components appear to be prone to cleavage by general proteases.

Taking these points into consideration it is possible to propose a mechanism for enzymic depilation of skins. Proteolytic enzymes may cause depilation of skins and hides by degrading the component globular and non-fibrous proteins of the basement membrane at the dermal-epidermal junction, which is continuous with the epidermis and underlies the hair follicles. As the basement membrane binds the epidermis to the dermis its disruption would lead to the removal of the epidermis and associated wool.

Globular proteins consist of non-repeating sequences of amino acids and therefore only a limited number of any one kind of peptide bond will be exposed. A protease with a very narrow range of bond specificities, trypsin for instance, may not cleave a sufficient number of bonds to disrupt the integrity of a globular protein well cross-linked with ionic, disulphide, hydrogen, and hydrophobic bonds. However the cleavage of further bonds at different points would cause the disruption of the protein; this would require additional proteases with complementary bond specificities (fig. 28). Yet a large number of proteases would overlap to a certain extent in their bond specificities and , moreover, multiple cleavages would be likely to occur near each other which would be unnecessary and wasteful as far as the depilatory process is concerned. Consequently there would be a limit to the number of proteases required to give maximum depilatory activity. One would therefore expect progressively reduced depilatory load with each complementary protease added to the depilatory preparation, but that with each complementary protease added there would be a smaller increase in depilatory activity than occurred with the last addition. Results obtained on the effect of the successive addition of multiple enzymes on depilatory load support this model.

Should the globular protein be denatured, by the strongly alkaline conditions of liming for instance, it would then require fewer cleavages to disrupt the integrity of the protein. Moreover it is likely that the denaturation will result in the exposure of more peptide bonds which were previously hidden in the hydrophobic centres of domains, making it more likely that a single protease with a narrow range of bond specificities would disrupt the integrity of the protein (fig. 29). This would result in depilation with less complementary proteases than would be required in the example of proteolytic degradation of native proteins. Again the results of the present study are in agreement with this proposal.

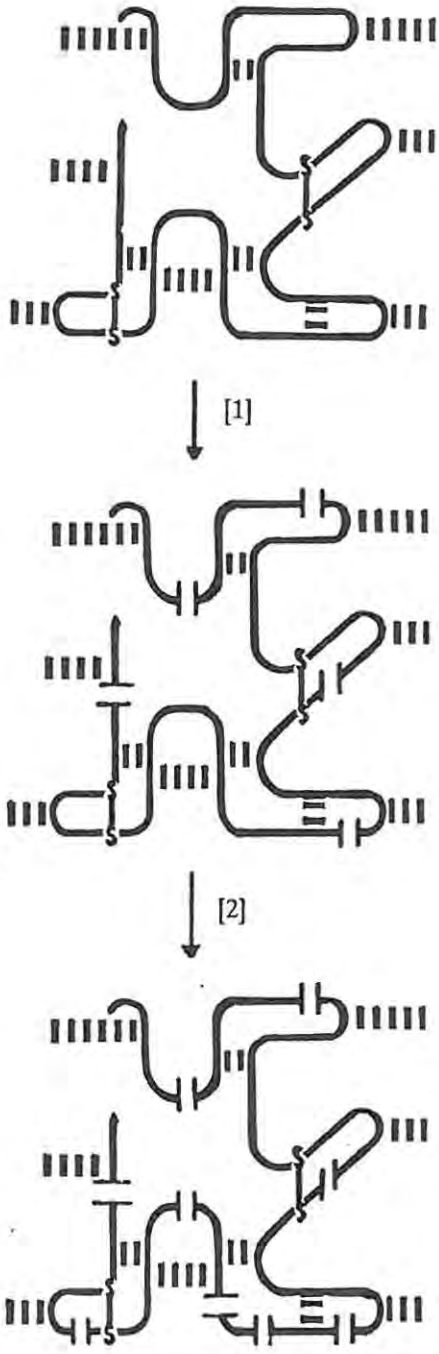


Fig. 28: Schematic representation of the degradation of globular proteins by multiple proteases. A globular protein stabilised by numerous intramolecular bonds (|||||) may maintain its integrity if it is cleaved at some points (||) by a protease [1]. By addition of further proteases with complementary specificities [2] the globular protein can be completely disrupted. However as more proteases are added it becomes increasingly likely that their specificities will overlap, or that they will act intensively on regions that need only a few cleavages for disruption.

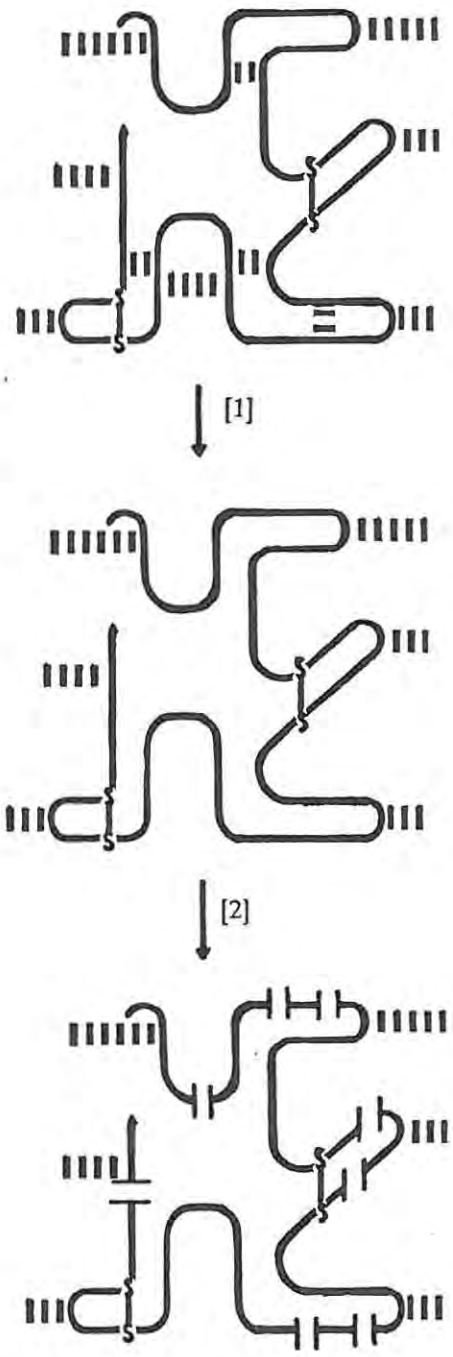


Fig. 29: Schematic representation of the degradation of denatured proteins by proteases. In the case of a denatured globular protein the effect of the denaturation [1] is the disruption of the intramolecular electrostatic bonds and the exposure of previously inaccessible cleavage points. Therefore it is easier for the denatured protein to be degraded by only a few proteases [2] than if the protein was still in its native state.

The same kind of model can be applied to the basement membrane as a whole. It may require a number of different proteases to degrade the different components of the basement membrane network (fig. 30). The model predicts that a narrow specificity protease would be able, with increasing concentration or time, to cause a decrease in the depilation load, but only up to a certain point and no further, at which it would of degraded all the components of the basement membrane that it was capable of degrading. In this study this effect was seen when incubating cured merino skin plugs with trypsin (not shown). The number of complementary enzymes required would decrease if several of the intermolecular bonds were disrupted or exposed by denaturation during liming.

Denaturatation of individual proteins may not be the the only way in which liming assists proteolytic degradation of the basement membrane. The heparan sulphate proteoglycans are found on the outer boundries of the basement membrane (61). Heparan sulphate proteoglycans, owing to their relatively large hydrated polyionic substituted carbohydrate side chains, can inhibit diffusion of macromolecules across the basement membrane (38). Heparan sulphate proteoglycans can bind to collagen, but glucosaminoglycan-collagen interactions at least are electrostatic since they can be disrupted with increasing salt concentrations (101). It has been noted that although some of the basement membrane proteoglycans can be removed by saline type solutions, the remainder can only be removed under denaturing conditions such as in 7 M urea (47). Moreover in strongly alkaline conditions dermatan sulphate proteoglycan undergoes cleavage between the glycosaminoglycan side chains and the protein backbone (84), while heparin and dermatan sulphate are also affected by strong alkali which causes β-elimination of the sugar moieties (51, 72). This leads to the possibility that alkali denaturation may remove or degrade the majority of the heparan sulphate proteoglycan from the basement membrane, thereby affording depilatory enzymes easier access into the basement membrane. The domains found in the protein backbones of proteoglycans (55, 86, 118) may be denatured in alkaline conditions, thereby allowing more complete degradation of these protein backbones by proteases which degrade them (96). The glycosaminoglycan side chains of proteoglycans can also be specifically degraded by enzymes such as those produced by some strains of *P. vulgaris* (30).

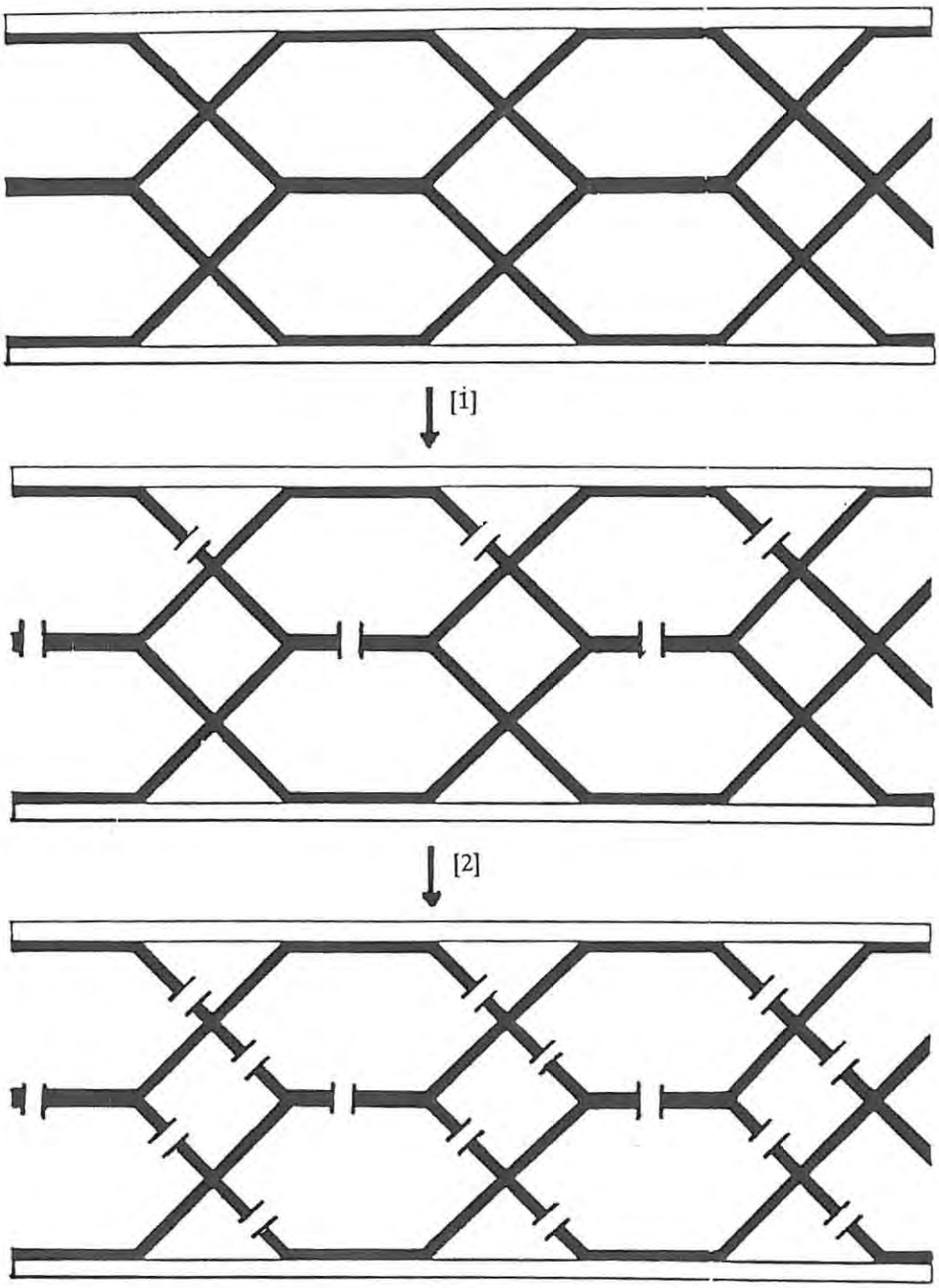


Fig. 30: Schematic representation of the degradation of networks of cross-linked proteins by proteases. In a network of varied proteins, such as that found in the basement membrane should any one type of component be cleaved by a particular kind of protease [1] the network may remain intact. However addition of proteases of complementary specificities [2] is likely to lead to increased disruption of the network.

III) Enzymic Depilation with Pre-alkalisation

It has been pointed out that alkaline pre-treatment is not necessary for depilation by some protease preparations (28), but in these cases a swelling treatment was necessary to produce leather comparable to that produced in by the lime-sulphide treatment. It was discovered that the liming step plumps up the collagen fibre bundles and thereby sets up pressure within the hide which opens up the weave pattern . Absence of plumping during an enzyme depilation treatment without an alkali treatment was reflected in the more compact structure of the weave (7). Enzyme treatment alone causes splitting up of the fibre bundles but fails to open up the weave pattern, this condition can be corrected by subsequent alkaline treatment (8).

Treatment with lime (calcium hydroxide) alone is insufficient for complete plumping. A 0.18% lime solution is saturated and any lime added beyond this will precipitate out and cannot contribute to the alkalinity of the solution. In the lime sulphide process the sulphide contributes to the free hydroxyl ions (116). If a sulphide free process is to be used then a more alkaline agent than lime must be incorporated into the lime (e.g. sodium hydroxide). It has been suggested that calcium ions are necessary for the optimum opening up of the fibre structure and that a combination of 1% NaOH and 2% $Ca(OH)_2$ is an ideal swelling agent (28).

In the present study it was found that the initial pH of the lime-sulphide solution (3% $Ca(OH)_2$ plus 3% Na_2S) solution was 12.8 pH units, while that of the 3% calcium hydroxide alone was only 12.3 pH units. The 2% calcium hydroxide "sharpened" with 1% NaOH gave an initial pH of 12.8 pH units, which is that of the lime-sulphide solution. Therefore it appears that the 0.5 pH unit difference between the two solutions is critical in the final condition of the leather, as was shown by the results of the present study.

Swelling of skins and hides can occur at the two extremes of pH. Acid swelling occurs between 1-4 pH units, and alkaline swelling occurs above 8 pH units (19). During the lime-sulphide

treatment the pH is initially about 12.7, falling to about 12.5 pH units as degraded protein exerts a buffering action (3). Pickling acid has been used to cause swelling (8), and pre-alkalisation and pre-acidification have been found equally effective in permitting pancreatic proteases to depilate (122). It has, however, been suggested that acid swelling is too difficult to control to be used in practice (45).

Alkaline pre-swelling has the added advantage in that it reduces the amount of enzyme required for effective depilation. The enzyme requirement has been reported to be reduced to between 50% (88) and 10-20% of that of that used to depilate native skins (49). The results of the present study also suggest that a smaller range of proteases within the depilatory preparation is required for pre-alkalised skins. With highly alkali stable proteases the alkali swelling could proceed simultaneously with enzymic depilation, an idea that has been adopted by certain companies such as Novo Industries of Denmark, although in this example the enzymes are used as a depilation assistant in the lime-sulphide liquor. Alkalinity causes chemical modifications of the wool proteins but does not appear to damage the wool fibre unduly (123).

IV) Unresolved Problems

1) Residual hair

It has been found that the action of enzyme preparations tested in trials is not always consistent, and the process often left fine hairs, especially on "tight patches" on the hide where the hair is difficult to remove. This problem was found in the study by Cranston *et. al.* (24) to be related to differences in responses between:

a) Hides from different breeds of cattle.

b) Hides from cattle slaughtered at different times of the year where the hair follicles were at different stages of the growth cycle according to the season.

c) Hides with varying amounts of fat or flesh resulting from inadequate fleshing.

The third problem can be avoided by more careful fleshing, but the first two problems are of a more fundamental nature. They may represent seasonal and breed dependent differences in the molecular composition of the basement membrane at the dermal epidermal junction. If this is the case then would a broader range of protease specificities help to alleviate that problem? What of the problem of the residual fine hairs, could these be adequately dealt with? There is obviously a need for further research into these problems.

2) The wastewater pollution

Although the use of proteases for depilation appears to be a viable alternative to the use of sulphides it does not negate the requirement for a highly alkaline treatment for the skins or hides. Therefore calcium hydroxide, sodium hydroxide, and ammonium sulphate (the deliming salt) will still be released into the wastewater and this problem will therefore require an independent solution. Perhaps alkali solutions containing alkali stable proteases could be reused by "rejuvenating" the solution with fresh alkali and protease, thereby reducing the amount of salts released into the wastewater.

CONCLUSIONS

In summation it appears that general proteases are capable of causing depilation of sheepskins. If the skins are in their native state it is necessary to use an enzyme preparation with a broad range of proteolytic specificities (as found in mixtures of proteases with complementry specificities) to cause depilation. However if the skin is pre-treated with strong alkali a preparation which includes proteases with a narrower range of specificities may be sufficient to cause depilation. Even in the former case a treatment with strong alkali is still necessary to convert the skin to a condition with similar properties to that of lime-sulphide treated skin. Enzyme based depilation methods can therefore lead to a wastewater free of sulphide and insoluble solids, but cannot negate the necessity for the use of alkalising agents which remain a pollutant. Before a completely sulphide free process can be instituted, however, the problem of remaining fine hair must be resolved.

More work needs to be done on the mechanism of enzymic depilation. One method which may produce informative results would be to stain the basement membrane of the dermal-epidermal junction with fluorescent dyes bound to monoclonal antibodies specific for each type of molecular component found in the basement membrane. If this were done to skins which had undergone sequentially increasing degrees of depilation then this method would yield a step by step account of the mechanism of enzymic depilation when cross-sections of these samples were viewed microscopically.

Although enzymic depilation has been slow to gain acceptance in the leather industry there is little doubt that it is the main alternative to the traditional process or that it will eventually be adopted by the industry.

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APPENDIX

TABLE A1: Yeast Extract/ Malt Extract Medium (YEME).

Composition	Concentration $g.dm^{-3}$
Yeast extract (Merck)	3.0
Bacto-peptone (Difco)	5.0
Malt extract	3.0
D(+)Glucose	10.0
Sucrose	340.0

After autoclaving add 2 $cm^3.dm^{-3}$ of 2.5 M $MgCl_2.6H_2O$.

(From Hopwood *et. al.*, Reference 50, pp.239).

Fig. A1: Equipment for measuring depilation load. Wool is clamped at the tips, while the skin is clamped at opposing edges. Weights are applied to the tray until depilation occurs. See text for details.

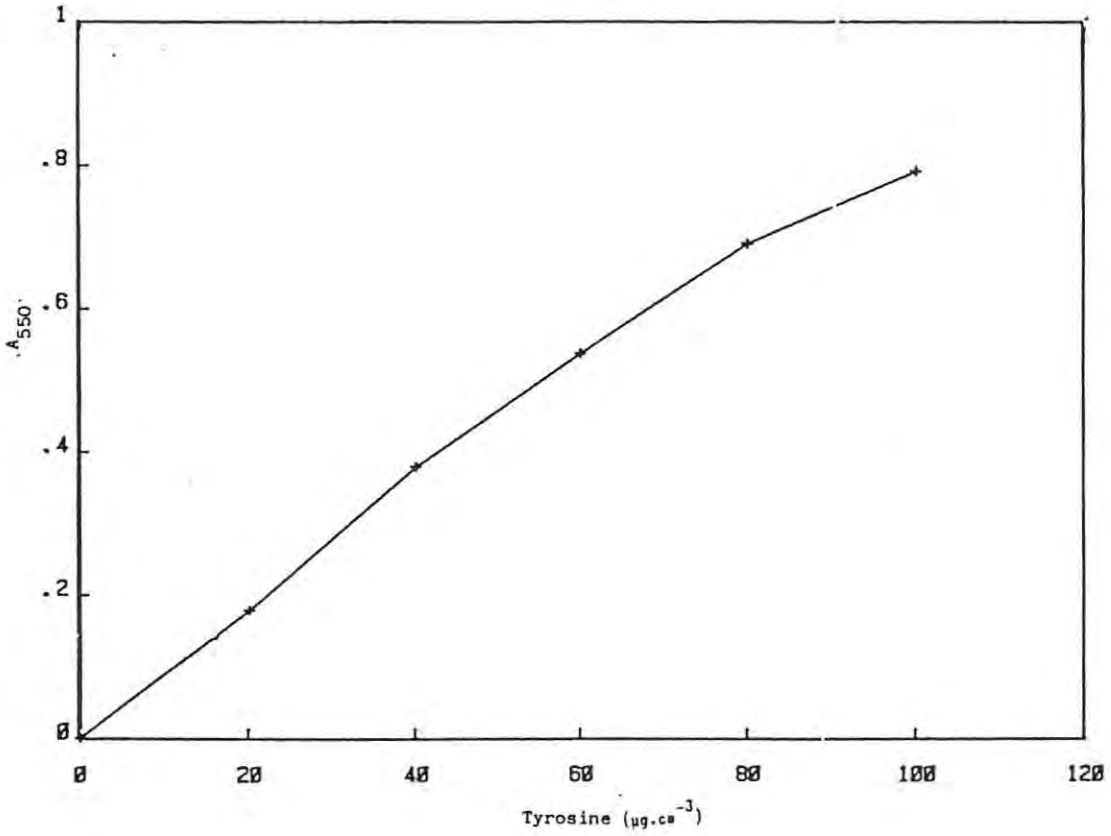

Fig. A2: Standard curve for determination of tyrosine by the Folin Lowry method.

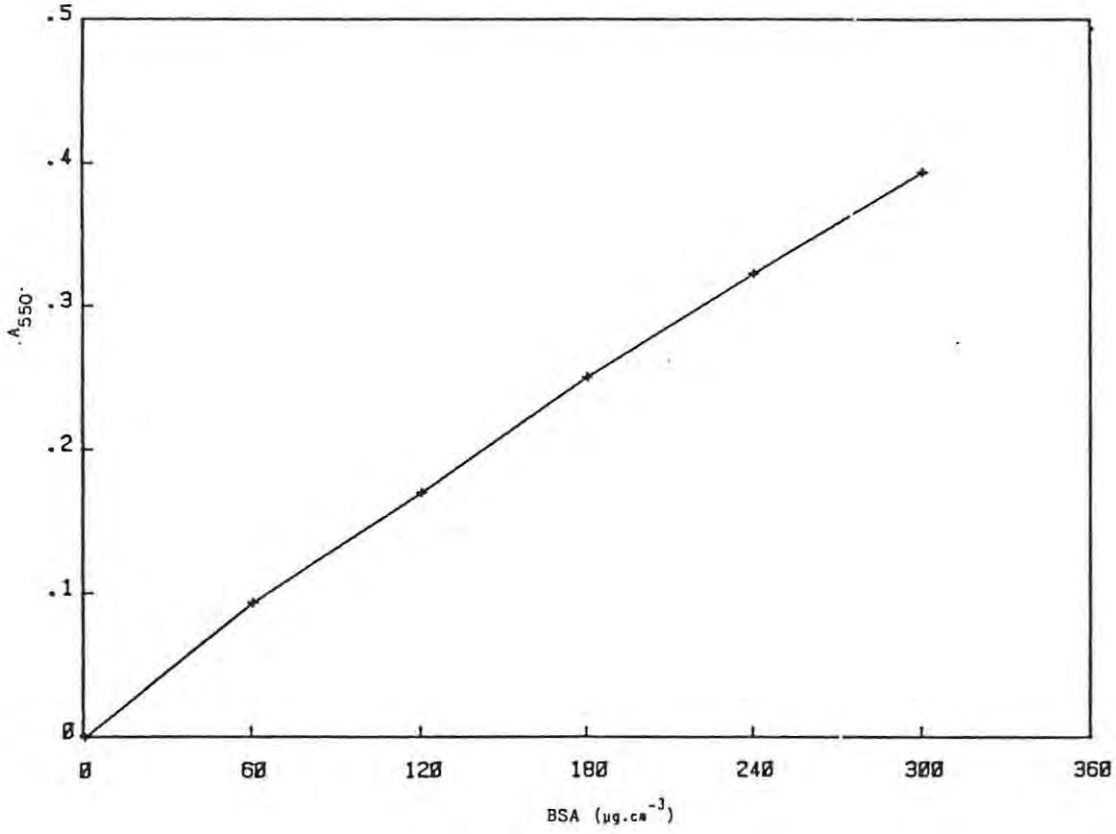

Fig. A3: Standard curve for determination of albumin by the Folin Lowry method.

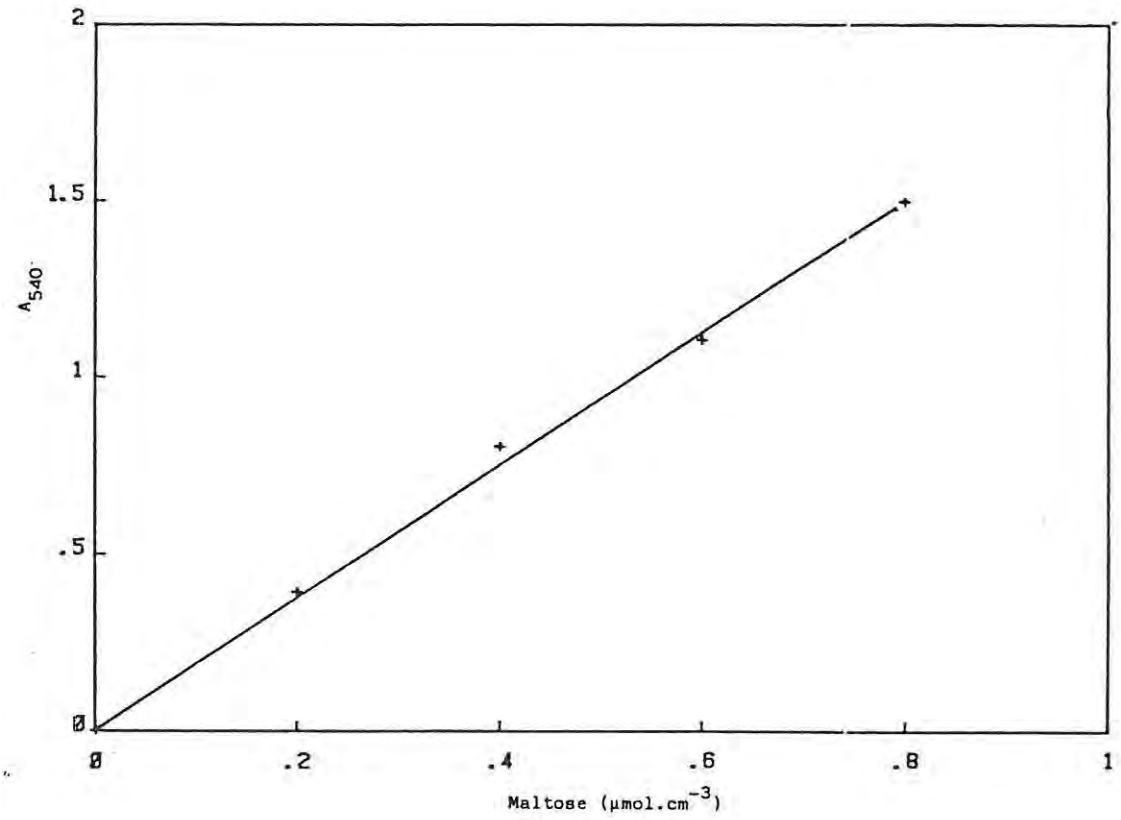

Fig. A4: Standard curve for the determination of maltose by the Somogyi-Nelson method.

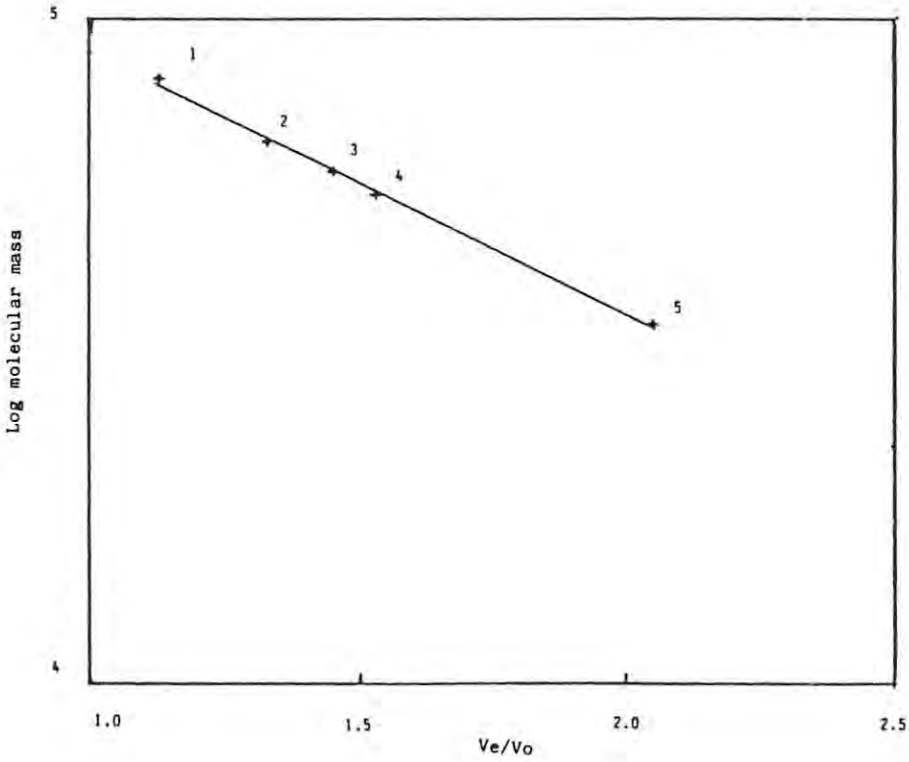

Fig. A5: Calibration curve for gel exclusion column (Sephadex G-75). Regression coefficient = 0.99861. 1 = Albumin, 2 = Ovalbumin, 3 = Pepsin, 4 = Carbonic anhydrase, and 5 = Cytochrome C.

TABLE A2: Molecular Masses of Proteins Used in the Calibration of the Sephadex G-75 Gel Exclusion Column.

Protein	molecular mass (daltons)
Albumin	66,000
Ovalbumin	43,000
Pepsin	35,000
Carbonic anhydrase	30,000
Cytochrome C	12,000

TABLE A3: Oxidation Fermentation (OF) Test Media (as used in the identification of *P. vulgaris*).

Composition	Concentration $g.dm^{-3}$
Bacto-Peptone from casein (Difco)	2.0
Yeast extract (Merck)	1.0
NaCl	5.0
K_2HPO_4	0.3
Bromothymol blue	0.08
Agar	2.5

A volume of 100 cm^3 is autoclaved and then 10 cm^3 of filter sterilised aqueous 10% solution of D(+)Glucose is added. Dispensed as 5 cm^3 into McCartney bottles, 50% of the bottles were overlayed 1 cm deep with sterile paraffin oil. The prepared medium had a green colour and a pH of 7.1 at 35°C. Incubation was at 35°C for 18 hours, with strains of *Escherichia coli* and *Pseudomonas fluorescens as +/+ and +/- controls respectively.*

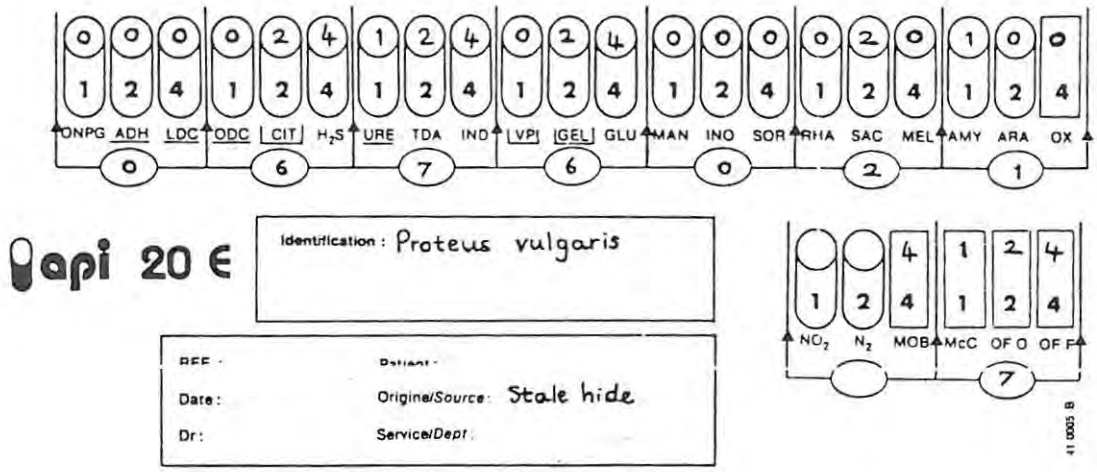

Fig. A6: Results of identification of *P. vulgaris* with an API 20E system.

TABLE A4: Casein-Agar Medium (after Thompson).*

Composition	Concentration $g.dm^3$
Peptone (Oxoid L37)	5.0
Meat extract (Oxoid L29)	3.0
NaCl	5.0
Casein (Hammastein)	2.5
$Ca(OH)_2$	0.15
$CaCl_2$	0.05
Agar (Oxoid L11)	20.0
Skim milk powder	10.0

Adjusted to pH 7.0 with NaOH. After autoclaving cool to 45°C and add 0.1 cm^3 of 1% 2,3,5-Triphenyltetrazolium chloride (TTC) (Merck) per 100 cm^3 of medium.

*Personal communication.

Fig. A7: *P. vulgaris* colony on a casein-agar plate. The concentric rings of growth caused by the bacteria swarming is visible, even though the concentration of the agar used is somewhat inhibitory to swarming (87).

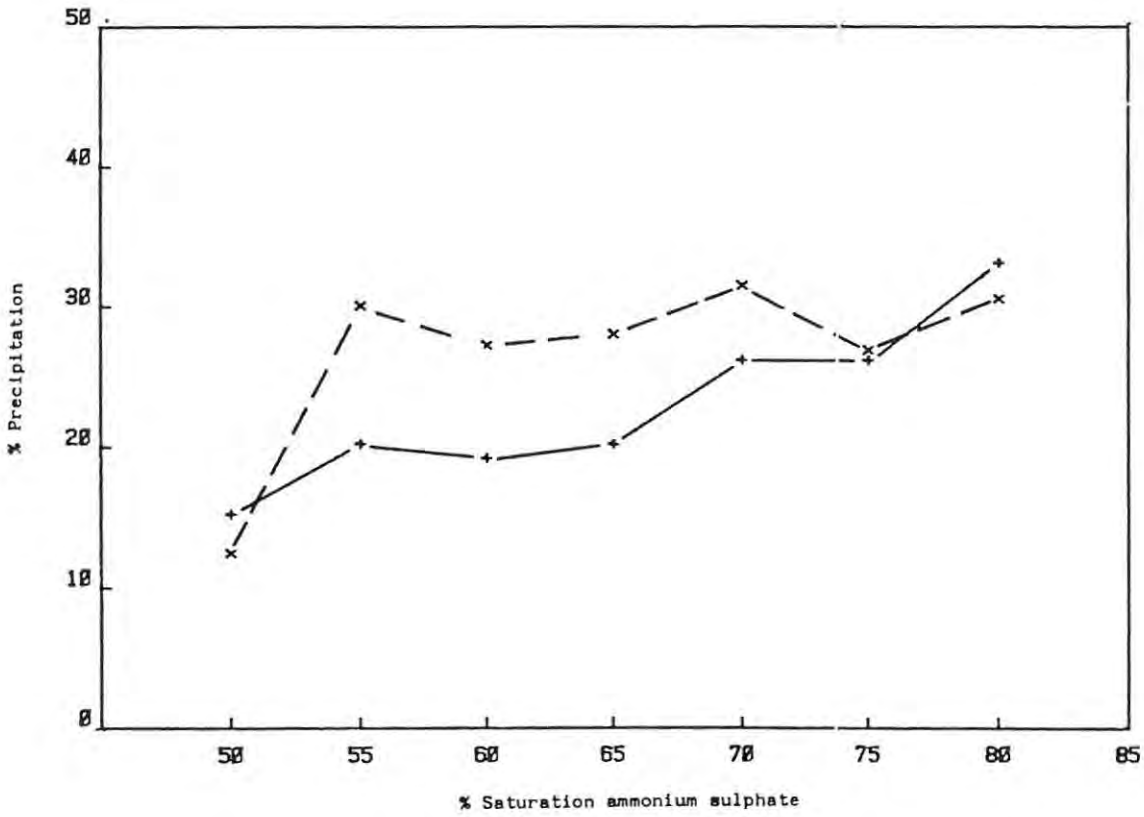

Fig. A8: Optimisation of ammonium sulphate precipitation of *P. vulgaris* extracellular protease activity (--x--) against protein (— + —) x 10.