Method for Screening Microgram Quantities of Proteolytic Enzymes for Depilation

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Abstract

A method was developed to investigate the efficacy of proteases in removing wool from raw sheepskin on a laboratory scale. We were particularly interested in being able to determine the effect of proteases on the wool bearing structures, as well as their effect on collagen VI, the retention of which is known to be important for leather quality. Initially, samples of raw skin were depilated using a protease known to have depilatory activity. Sections were prepared and examined using histological staining techniques, including immunohistology, to determine the microscopic characteristics of successful enzyme depilation. Single skin sections from raw skins were then treated with the same enzyme under identical conditions and assessed using the same histological techniques. This showed that "single section depilation" is a valid analysis technique. This technique was then used to determine if any of four commercially available enzymes (some in only microgram quantities) had the requisite specificities to achieve successful dewooling without damaging collagen VI. It was found that of the enzymes tested, dispase was the only one that exhibited little activity against collagen VI while retaining some dewooling activity. The results did confirm however that the depilatory activities of proteases could be satisfactorily assessed using this technique, meaning that the dewooling efficacy of proteolytic agents can be tested even if only very small quantities are available. Work is currently underway to test combinations of enzymes that will achieve depilation without damaging collagen IV.

Introduction

Enzyme unhairing and enzyme depilation is the subject of much interest (Cantera 2001; de Souza and Gutterres 2011; Dettmer et al. 2012; Saravanabhavan et al. 2005; Saravanabhavan et al. 2007; Sivasubramanian et al. 2008; Thanikaivelan et al. 2007; Thanikaivelan et al. 2005; Thanikaivelan et al. 2006; Wang et al. 2009). The move to enzymatic depilation would achieve significant improvements. Briefly, enzyme depilation has the following prospective positive aspects (Gehring et al. 2002; He et al. 2006; Passman 1997; Taylor et al. 1987; Thanikaivelan et al. 2000):

- The process can be extremely specific depending on the enzyme used
- It would eliminate sulphide contamination of wool

- It would provide safer and more pleasant work environment
- It would eliminates the risk of hydrogen sulphide gas production
- It would reduce air emissions
- It would provide an effluent nominally free of sulphide

Previous work has suggested that the removal of specific proteins from the surface of the skin are the cause of damage that often occurs to enzymatically depilated skins (Allsop et al. 2007) and removal of collagen VI in particular is implicated (Edmonds et al. 2008). The use of highly specific proteolytic enzymes which did not damage these proteins would therefore be prudent.

Significant research has been carried out over a number of years examining bulk reagents in gram quantities on their ability to depilate with many proving to cause damage or to incompletely depilate. The main reason that enzymes are available in bulk supply is because they have been found to be successful in for some non leather industrial application and so are prepared in industrial quantities. There are however many more proteolytic enzymes that are only available in extremely small quantities, or at a very high price. To date they have been mostly overlooked due to the fact that purchasing enough of these enzymes to carry out a trial is prohibitive.

The approach taken in the development of this method was that the solution to this problem is to develop a test for depilation and possible damage to skin on a scale that could be applied to the microgram quantities of untried enzyme that were available. Very limited quantities of pure enzymes reported to not have collagen VI activity were available, so a method was required to determine depilation efficacy on a microscopic scale.

A method was developed using the SACPIC stain (Nixon 1993; Yates 1968). The ability of an enzyme solution to depilate lambskin was assessed by applying it to a single 20um thick section on a microscope slide for 16 hours at 20°C. The section was then stained with the SACPIC stain. Methods for immunohistology have been previously used to investigate morphological characteristics in skin (Fukunaga, 2006). In this work an immunohistological technique developed for collagen VI (Edmonds et al, 2008) was also extended to investigate small quantities of potential depilatory enzymes.

Materials and methods

Sections for depilation were prepared by taking samples of frozen raw skin 10mm long by 3mm wide, cutting 20um thick sections at right angles to the skins long axis, and air fixing (24 hours, 20°C, 65% relative humidity) to glass slides. The sections were then held at -20°C until required.

A known effective depilatory enzyme (Purafect 4000L, Zymus Inc. (Edmonds et al. 2008)) was used as a comparison to ensure the new test adequately represented effects noted in bulk enzyme depilation trials. Depilation could be achieved on whole skins by the application of

2% w/w enzyme within a carbonate buffer system (pH 10.5). We evaluated the micro method for the assessment of depilation on a single section by applying a range of concentrations of Purafect 4000L in the 0.5M buffer to the air dried raw skin section. Ultimately, a 0.6%v/v solution of enzyme in the micro test was found to generate equivalent results to 2% enzyme solution in bulk trials.

Due to the fragility of skin sections the enzyme depilatory test solution (and all subsequent solutions) were applied slowly in 200uL amounts using a Hamilton syringe over 10 seconds. Test solutions were held in a bubble over the test section by the use of a wax (PAP) pen.

Figure 1: Slide with a single section of skin surrounded by the wax of a PAP pen and flooded with 200 uL test solution

After exposure to the enzyme the test solution was removed using a Hamilton syringe and stained using the SACPIC stain (appendix 1). It was determined that the sections after enzyme treatment were too fragile to be treated as is with the stain in the conventional manner, so the sections were first treated with a 70% ethanol solution. This effectively toughened the section enough for staining to be completed.

Results and discussion

The enzymes chosen for examination using the single section depilation investigation were as follows:

- Purafect - used as a control because analysis of the exposure of lambskins had already been analysed using SACPIC staining, proteomic techniques, and immunohistochemistry. The results with different enzymes will therefore be able to be compared with those using Purafect.
- Dispase (Sigma, D4818 -2MG) - chosen because claims had been made that this enzyme could unhair without damage to the enamel layer of the skin (Paul et al. 2001).
- Human MMP-11 (Sigma, M7692-10 uG)– chosen because it is described as having specific proteolytic activities against extracellular proteins including laminin, and fibronectin, but not against collagens (Kreis and Vale 1999). This is an important distinction because if a protease was found with no activity against any collagen but was found to be highly active against the remainder of the extracellular proteins there would be a real possibility to release the wool fibre from the extracellular matrix without the concomitant damage of any of the collagens that go into the final product

Purafect was made up in 0.5M 10.5 pH carbonate/bicarbonate buffer. Dispase was applied in a neutral buffer containing 50mM calcium chloride set to pH 7.4 using NaOH. MMP-11 was applied in the solution as received in its active form at 5 ug/100 uL (pH 7.0) diluted 1:1 with a solution containing 100mM calcium chloride, 100mM zinc chloride adjusted to pH 7.0. The results for each in comparison to control sections treated with buffer only are shown in Figure 2.

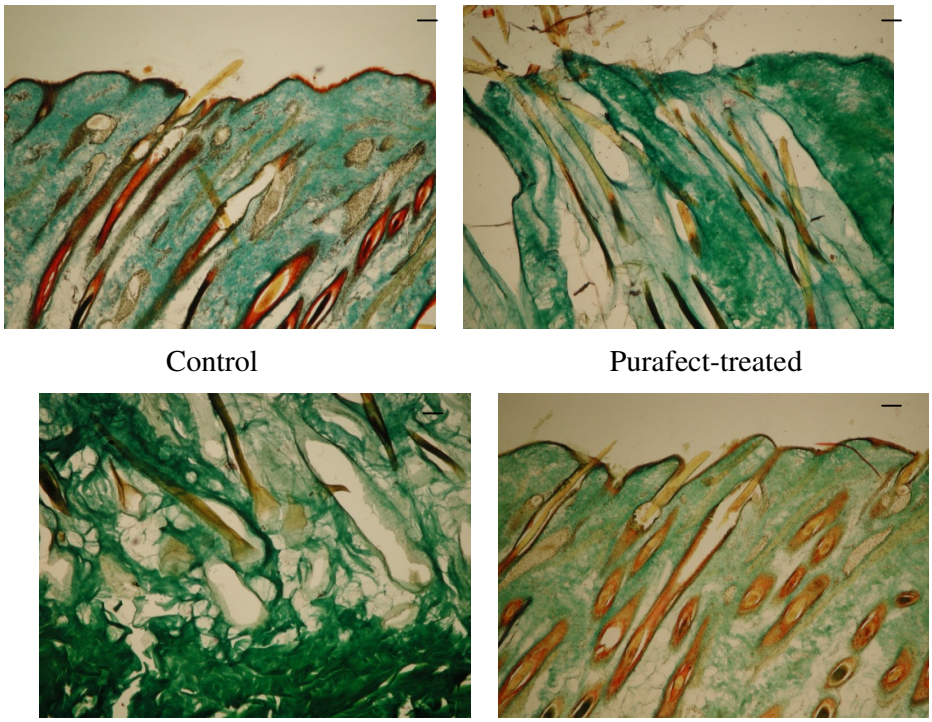

Figure 2: SACPIC stains of single section depilation micro assay: Note evidence of the processes of depilation in sections treated with Purafect or Dispase. All bars are 50 um

The untreated control section shows fully intact structures around the wool root and at the skin surface as expected.

Examples of the single section of skin that had been treated with Purafect showed the same form of destruction of the wool root sheath that was observed on sections cut from whole skins that had been similarly treated. Dispase also caused some destruction of the wool root confirming previous reports of its activity in skins. The Dispase treated section, however, had significantly more intact wool bulb in comparison to skins treated with Purafect indicating that it was not attacking the proteins in those structures as required. Although Dispase may be useful in removing cells of the epidermis as previously described it would appear that the removal of wool by this enzyme may be a more difficult task. These results, therefore, may explain the findings of (Paul et al. 2001) who showed that while Dispase acted as a depilatory agent it was not a complete depilatory in that some fine hairs were left behind.

Sections treated with MMP-11 were not significantly different from the controls which were treated with buffer only. Specifically the structures of the wool root sheath remained predominantly intact indicating that the use of MMP-11 under these conditions would not result in successful depilation.

Additional sections that had been exposed to prospective enzyme depilatory reagents were assessed for persistence of collagen VI. The results are illustrated in Figure 3 below:

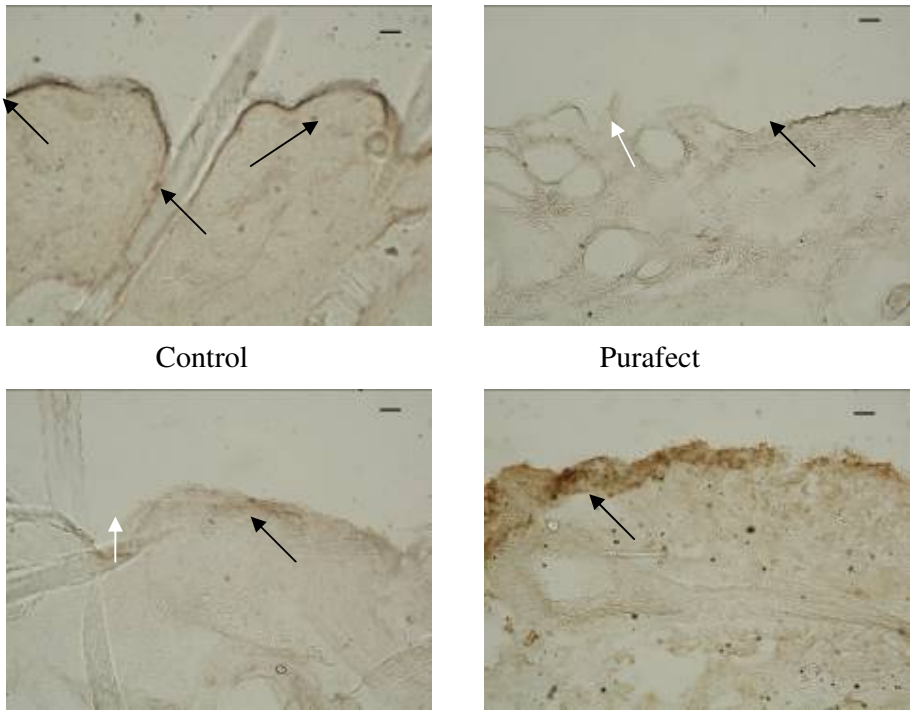

Figure 3: Collagen VI immunohistology of single section depilation micro assay (Collagen VI stained dark brown/black: Note some collagen VI in each section (black arrows) with evidence of the removal of stained material within the grain enamel of sections treated with either Dispase or Purafect (white arrows). All scale bars are 25 um

The collagen VI immunohistological stain again shows intact collagen VI at the surface of the control section showing that as expected collagen VI remained in the grain enamel layer in the presence of buffer (Controls using only water were the same – results not shown).

When whole skins were treated with Purafect there was reduced staining in the grain enamel consistent with the loss of collagen VI from this area of the skin. In particular, the regions in which the staining was absent corresponded to those regions of the skin surface that were most damaged. Specifically the surface fibres appeared frayed and did not present a smooth surface. The same results occurred as illustrated above for the section treated with purafect

The section treated with Dispase showed some damage to the collagen VI and some disruption of the grain surface in those regions. This indicates that dispase causes some damage to collagen VI, which could potentially result in a reduction in the quality of the grain surface in the finished product.

It was hoped that the MMP-11 would result in a significant degree of destruction to the structures of the extracellular matrix but without damage to collagen VI. The results show that while there was still significant immunohistological staining at the skin surface indicating that a significant amount of collagen VI remained intact, the SACPIC staining indicated that there was hardly any change to the proteins in the wool root shaft. This indicated that MMP-11 would not cause depilation. It is possible that the use of a human (MMP-11), which is supposedly specific to human extracellular proteins, meant that it was not active against the ovine proteins. Alternatively because MMP-11 is characterised as being relatively less active in comparison to other endogenous proteases, it failed to degrade enough of the proteins under the conditions used to remove the proteins around the wool root. It is still possible that ovine MMP-11 might achieve effective depilation without affecting collagen VI. This would require the isolation of ovine MMP-11 which is outside the scope of this work. However, the most likely explanation for the inability of MMP-11 to degrade the proteins around the wool root is that it simply does not have a broad enough spectrum of activity.

It may be worthwhile in the future to use the techniques developed here to examine other pure, and highly specific proteases to check their activity against collagen VI and the different proteins around the wool shaft.

Conclusions

A method was developed such that low quantities of a potential depilatory enzyme could be evaluated for its ability to destroy the structures surrounding the wool fibre. The method involved taking a thin cross section of the material to be depilated, exposing that cross section to the depilatory system and staining the resulting section with a tri colour stain known to highlight the structures adjacent to the wool fibre. The method was found to produce stained sections similar to those made from sections of skin cut from whole skins to which an enzyme depilatory had been applied. The method was then extended to include immunohistology so that specific proteins could be investigated in this case specifically collagen VI. Potential depilatory enzymes were subsequently selected based on reported activities likely to be useful for a successful depilatory and the enzyme Dispase was found to depilate with a reduced level

of damage in comparison to the broad spectrum protease Purafect 6L. It is likely that this method would be useful in the future for assessment of depilatory activity of enzymes Particularly during the discovery and developmental phases of enzyme unhairing research due to the limited supply of enzymes at these stages.

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Appendix

SACPIC stain reagents

Celestin blue

10g iron alum (Ammonium ferric sulfate dodecahydrate, NH4Fe(SO4)2.12H2O) (BDH chemicals Ltd, England) was dissolved in 200mL water and mixed overnight. To this 1.0g Celestin blue powder (Fluka, Switzerland) was added and heated slowly. The solution was then boiled for 3 min and then cooled. The stain was then filtered and 6.8mL glycerol (#100-GLEP.2500, Pure science, New Zealand) added and shaken well.

Scott's tap water

2 g/L Sodium bicarbonate, 20 g/L magnesium sulphate

Safranine solution

The following ingredients were mixed for one hour: 2g safranine (BDH Chemical Ltd, England), 49mL absolute ethanol, 49mL RO water.

Picric acid in ethanol

0.5mL picric acid (Saturated aqueous solution) and 50mL absolute alcohol were mixed

Picro-indigo-carmine

0.125g indigo carmine powder (Sigma, USA), and 50mL picric acid (saturated aqueous solution) were mixed.

SACPIC Staining protocol

All solutions were carefully applied and removed using a Hamilton syringe. 200uL of solution was used for each step of the procedure (unless otherwise stated). The steps taken were as follows:

Rinse section with water, apply Celestin blue solution for 5 min, rinse with water, apply haematoxylin (Mayers Hamalaun solution, Applichem, Germany) for 5 min, rinse with water, apply Scott's "tap water" for 2 min, rinse with water rinse, apply Safranine solution for 5 min, apply 70% ethanol for 2 min, apply absolute ethanol for 2 min, apply picric acid in ethanol for 2 min, apply 95% ethanol for 2 min, apply 70% ethanol for 2 min, rinse with water for 2 min, apply Picro-Indigo-Carmine for 1 min, rinse with water. The section was then mounted in DPX and coverslipped for assessment under the microscope.

Staining protocol for Immunolocation of collagen VI

The biotin-streptavidin detection system was used as follows: Sections were equilibrated in phosphate buffered saline (PBS) (0.01M Phosphate NaCl 9.0g/L), pH 7.2 for 1 min. The effect of endogenous peroxidase was mitigated by exposure to 3% hydrogen peroxide in PBS for 30 m then equilibrating again in PBS for 1 min. The section was encircled with a PAP pen (Sigma) to create a fluid barrier to hold the reagents. Non-specific binding sites were blocked by covering the section with 1% bovine serum albumin (BSA Sigma) and allowing the slide to stand for 5min, before incubating it in a humidity chamber for 60 min. The sections were then drained and washed for 1 min in each of three changes of PBS. Anti-collagen VI antibodies (Chemicon AB7822 diluted 1:40 in BSA) were then added to the sections (20uL) and again incubated in a humidity chamber for 60 min after which they were drained and washed in three separate changes of PBS. Biotinylated IgG (~100 uL) (Amersham anti-rabbit from sheep (RPN1004) diluted 1:200 in BSA) was added and the sections were incubated in a humidity chamber for 30 min. They were then drained and washed in three changes of PBS followed by a preformed biotin-streptavidin-peroxidase complex (Amersham Streptavidin biotinylated horseradish peroxidase RPN1051 diluted 1:200 in BSA). Sections were then drained and washed in three changes of PBS before being reacted in 3,3 diaminobenzidine (DAB) for approximately 3 min. The reaction was halted by immersion in PBS. The sections were rinsed in tap water before being counter stained with Mayer's haemalum for 1min, after which they were rinsed in tap water, dehydrated, cleared and mounted in DPX. The sections were examined under a light microscope at 40x magnification where collagen VI was visualised as a brown/black stain

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