Study on molecular-level collagen structure changes caused by enzymatic depilation process using X-Ray Scattering

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1 Introduction

The leather industry, specifically tanning and its associated operations can be a source of considerable environmental impact [1]. A crucial beamhouse process – the conventional chemical depilation, uses sulfides to break down disulphide (S-S) bonds in the cysteine (Cys) residue of the keratin [2]. The use of sulfides in increases the total dissolved solids as well as chemical and biochemical oxygen demand (COD and BOD) in the effluent [3,4]. The chemicals used for depilation can also leached into the environment. Moreover, most solid tannery wastes, including sheepskin pelts, are currently landfilled. These factors contribute to the environmental impact of the leather industry which are under increasingly stringent regulatory requirements [5].

Recently, enzymes has been gaining attention as a sustainable alternative to the conventional depilation process [6,7]. Use of enzymatic depilation reduces the pollution load in the process effluent and improves working conditions since the use of sulphide is reduced drastically [8].

The depilation process removes components such as keratin and epidermis as well as alter the collagen fibre structure [9,10]. These changes can affect the grain surface and the quality of the final leather produced. The changes in the structure of collagen fibres at the molecular level can be studied using X-ray scattering technique [11], as type I collagen are aligned in a quarter stagger structure with repeating gap/overlap regions within the fibril [12]. However, studies focused on structural features of collagen caused by unhairing are limited. Our group has previously established synchrotron-based small-angle X-ray scattering (SAXS) as a tool for studying structural features of collagen with each step of beamhouse operation [13,14], as well as tanning using various tanning agents [15]. Similarly, SAXS has also been used to describe molecular level packing of keratin by Rafik et al. [16].

Ideally, unhairing enzymes for industrial application should have specificity towards keratin and epidermis with minimal activity towards collagen. The present study investigates the efficacy of the protease produced by *A. hydrophila* in the depilation of calf skins. The process effluents were analysed to assess the effect of depilation process on its environmental impact and tanning agent uptake. SAXS was also used to provide insight into the molecular-level structural changes of collagen and other skin components and its relationship with physical properties of the leather produced.

2 Methods and Materials

2.1 Enzyme preparation

Aeromonas hydrophila previously isolated from sweated sheepskins [17] was used in this study. *A. hydrophila* cryo-preserved with glycerol at -80 °C was activated by inoculation in 5 mL LB broth at a ratio of 1:1000 and cultured overnight at 25 °C on a shaking incubator. The activated culture was used to inoculate 3 L of freshly prepared fermentation media which was then cultured under the same conditions for a further 48 h. The crude enzyme extract was collected by centrifuging the resultant culture at 8000x g RCF for 20 min at 4 °C. Ammonium sulphate precipitation was carried out at 90% saturation to separate the total proteins from the crude protease extract, followed by centrifugation at 20,000 g for 30 min at 4 °C on Thermo Scientific™ Sorvall™ LYNX 6000 superspeed centrifuge. The partially purified protease was rehydrated using 40 mL 1x phosphate-buffered saline (PBS) and frozen down with 20% glycerol at -20 °C before subsequent experiments.

2.2 Protease activity

Protease activity was determined against the standard curve generated with trypsin, using Azo dye impregnated collagen (Azocoll) as a substrate. Briefly, 100 µl diluted enzyme solution was mixed with 900 µl 5 mg ml-1 Azocoll in 100 mM Potassium Phosphate Buffer, pH 7.8. After incubation at 37 °C for 15 min, the mixture was centrifuged and the absorbance of supernatant at 520 nm was measured on a Varian Cary® 50 UV-Vis spectrophotometer. Protease activity was expressed as the amount of equivalent trypsin per mg proteins.

The optimum temperature for enzymatic activity was determined by performing the enzyme assays in the temperature range of 15 to 60 °C (pH 7.8). The optimum pH for enzymatic activity was determined by performing the enzyme assays in the pH range of 7 to 12 at 37 °C, with the substrate being suspended in buffers with different pH values.

2.3 Depilation and tanning process

Two calf skins were split along their backbone, the left half was depilated using conventional chemical depilation, followed by standard liming, deliming and pickling procedure. The right half of the calf skin was depilated with enzymatic depilation, using depilatory paint prepared by mixing

the enzyme solution with 25g/L of Solvitose as the thickener, then wrapped in plastic sheet and stored at 37 °C for 24 h. The depilated skins from both processes were then processed into crust leather with liming, deliming and pickling procedure followed by standard leather processing including chrome tanning, re-tanning and fat liquoring process. The entire process for the enzymatically depilated half is referred to as “enzymatic process” while the chemically depilated half is referred to as “chemical process”.

2.4 Physical properties of crust leather

To evaluate the physical properties of the dried crust leather. Leather from the chemical process and enzymatic process were conditioned at 20 °C/65% relative humidity for 48 h prior to physical properties testing according to IULTCS/IUP6 standard procedures for tear strength, tensile strength, elongation at break, and distension and strength of surface [18–20].

2.5 Analysis of physico-chemical parameters of the process effluent

To evaluate the effect of the depilation process on the pollution load of the process effluents, spent liquor were collected at the pre-tanning, tanning, and post-tanning steps. The effluent were then analysed by an accredited third party laboratory using standard methods for common tannery effluent parameters including biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), total suspended solids (TSS), calcium, chloride, sulphate, sulphide, and chromium [21].

2.6 Thermal characterisation

To evaluate the thermal characteristics of the leather, shrinkage temperature was determined using a standard method as per ISO 3380 [22]. Differential scanning calorimetry (DSC) measurements were also carried out on DSC Q2000, TA Instruments. Ten 50 µm cross-sections of samples were obtained using a freezing microtome (Leica CM1850 UV, Germany), rehydrated with 0.2 M sodium acetate, and then sealed in Tzero aluminium pans for 2 hours. The rehydrated samples were then analysed under heating rate of 5 °C/min from 30 °C to 120 °C under a nitrogen gas purge of 50 ml/min. The onset temperature of the endothermic peaks on heat flow curves were taken as the denaturation temperature of the sample.

2.7 Structural changes of skin with SAXS

Hide samples were cut into squares of size 0.3 cm× 0.3 cm×200 μm (L × W × H) using a microtome from leather cross-section. The sections were then air-dried at room temperature prior to SAXS measurement. SAXS experiments were conducted on beamline I22 at Diamond Light Source. The measurements were carried out at 12.4 keV X-rays and a 9.7 m (q = 0.021–1.7 nm−1) sample-to-detector distance.

Peak fittings were then performed using protocol according to previous studies using SAXSFit and Fityk [23]. Briefly, the scattering intensity I(q) is presented as a function of scattering vector, q, where $q = 4\pi \sin(\theta/2)/\lambda$, and where θ is the angle between incident and scattered radiation. Relative diffraction peak intensity is calculated as $R_n/m = A_n/A_m$, where A_n and A_m stands for the area of peak order n and m. Peak positions were recorded and converted to real-space distance $d = 2\pi/q$.

3 Results and Discussion

3.1 Protease activity of enzymes from *A. hydrophila*

Proteins produced by *A. hydrophila* were precipitated using ammonium sulphate, followed by dialysis and cryopreservation with glycerol. The total protein concentration was 1.33 mg/ml and 1 mg of partially purified *A. hydrophila* enzymes exhibited protease activity equivalent to 14.13 mg trypsin.

Enzymes from *A. hydrophila* exhibited maximum protease activity at 35 °C, while 90% of the activity was still detected at 50 °C. Protease activity remained above 40% in the temperature range of 30 °C to 60 °C. The optimum pH for protease activity in *A. hydrophila* was found to be 8 and 60% of activity was still present when pH was at 7. The protease activity dropped drastically once the pH increased to over 9. The results confirm the enzyme from *A. hydrophila* as a promising candidate with high depilation efficiency, similar to other studies using enzymes with high proteolytic activity [10,24].

3.2 Physical Properties and visual assessment of leathers

Physical properties of crust leather produced from enzyme depilation were comparable with those made from chemical process, with higher tear strength in parallel direction and lower tensile strength in the perpendicular direction. These results shown in Table 1 suggest differences in collagen fibre arrangement, which might had been altered by enzymatic depilation process.

Table 1. Physical properties of leather including tear strength, tensile strength, elongation at break and grain crack strength produced using the chemical process and

Method	**Tear strength (N mm^{-1})**		**Tensile strength (N mm^{-2})**		**Elongation at break (%)**		**Grain crack strength**	
	Parallel	Perpendicular	Parallel	Perpendicular	Parallel	Perpendicular	Distension (mm)	Load (kg)
Chemical Process	50.8 ± 3.64	46.8 ± 2.06	32.7 ± 3.58	24.1 ± 2.56	41.2 ± 0.74	63.6 ± 5.52	9.7 ± 0.21	52.0 ± 8.62
Enzymatic Process	64.4^{a} ± 8.24^{a}	49.8 ± 3.65	30.3 ± 2.58	17.8^{b} ± 2.67^{b}	41.4 ± 4.46	70.4 ± 5.64	10.6 ± 0.69	61.5 ± 12.08

* ^{a} and ^{b} are statistically different values

Visual inspection of the leather revealed that complete hair removal was accomplished with both chemical and enzymatic process. The samples were free from hair/short hair/scud, as well as epidermal layer (a white surface on the grain side), achieving comparable extent of depilation with studies using enzymatic depilation process [25].

3.3 Enzymatic process reduces pollutant load in the process effluents.

The chemical depilation step uses large amounts of lime and sulphide, while enzymatic depilation step involves a relatively simple application of the depilatory paint followed by washing. As a result, water consumption of the pre-tanning process was reduced by 75.7%, with most effluent parameters showing a significant decrease (See Table 2). At the tanning and post-tanning steps, a significant reduction of chromium load was also observed for the enzymatic

process, with a reduction of 63.7% and -35.2 % for tanning and post-tanning step respectively. This is attributed to the enhanced uptake of the tanning agent [25]. Since beamhouse operation commonly accounts for 70–80% of BOD and COD loading in tannery wastewater, the drastic reduction of pollutants at this step is expected to have a significant effect on the overall environmental impact of the leather production process [26].

Table 2. Difference in total load (%) of the enzymatic process with respect to the chemical process of key effluent parameters for the pre-tanning, tanning and post tanning process

Parameter	Difference in total load[*a](%)		
	Pre-tanning	Tanning	Post tanning
BOD	-77.4	+6.3	+12.1
COD	-77.8	-15.1	+4.3
TDS	-68.3	-26.2	+2.3
TSS	-83.9	-42.3	-33.9
Calcium	-81.5	-53.7	-
Chloride	+23.6	-26.6	-11.3
Sulphate	-97.1	-22.2	+2.2
Sulphide	-73.7	+28.6	-
Chromium	-	-63.7	-35.2
Effluent volume	-75.7	+2.6	+25.6

[*a] indicates the percentage difference of the total load (concentration * effluent volume) of the enzymatic process with respect to the chemical process.

3.4 Thermal properties

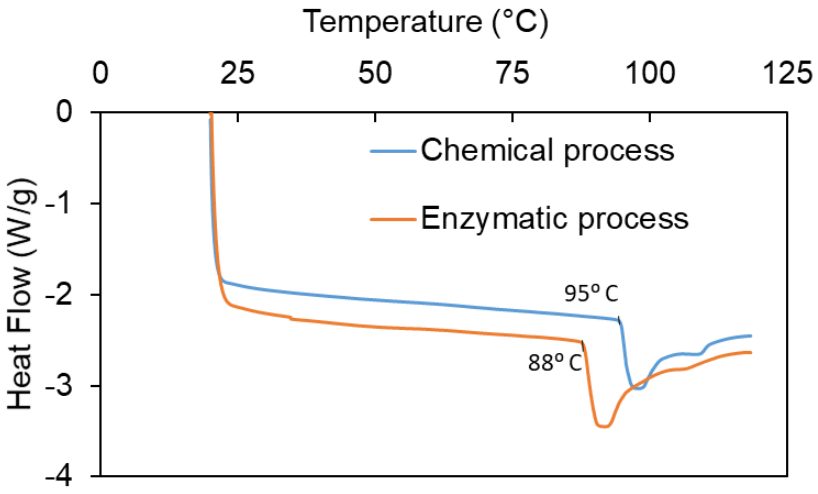

Figure 1. DSC results of crust leather produced using the chemical and the enzymatic process

The denaturation temperature of leather produced via the chemical process was found to be higher than leather produced via enzymatic process. The results were confirmed using shrinkage temperature test, with leather from chemical process showing a shrinkage temperature of 97 º C, while leather from enzymatic process showing a shrinkage temperature of 92 º C. The reduction of thermal stability of leather from enzymatic process combined with an increase in chromium uptake suggest an increase in carbonyl (-COO) groups, the primary functional group available for chrome crosslinking [27]. This indicates some enzymatic degradation to the collagen fibre structure. Despite the reduction in thermal stability, the physical properties outlined in Section 3.2 indicate that the enzymatic process can produce leather of comparable quality from calf skins.

3.5 Collagen structural changes: SAXS analysis

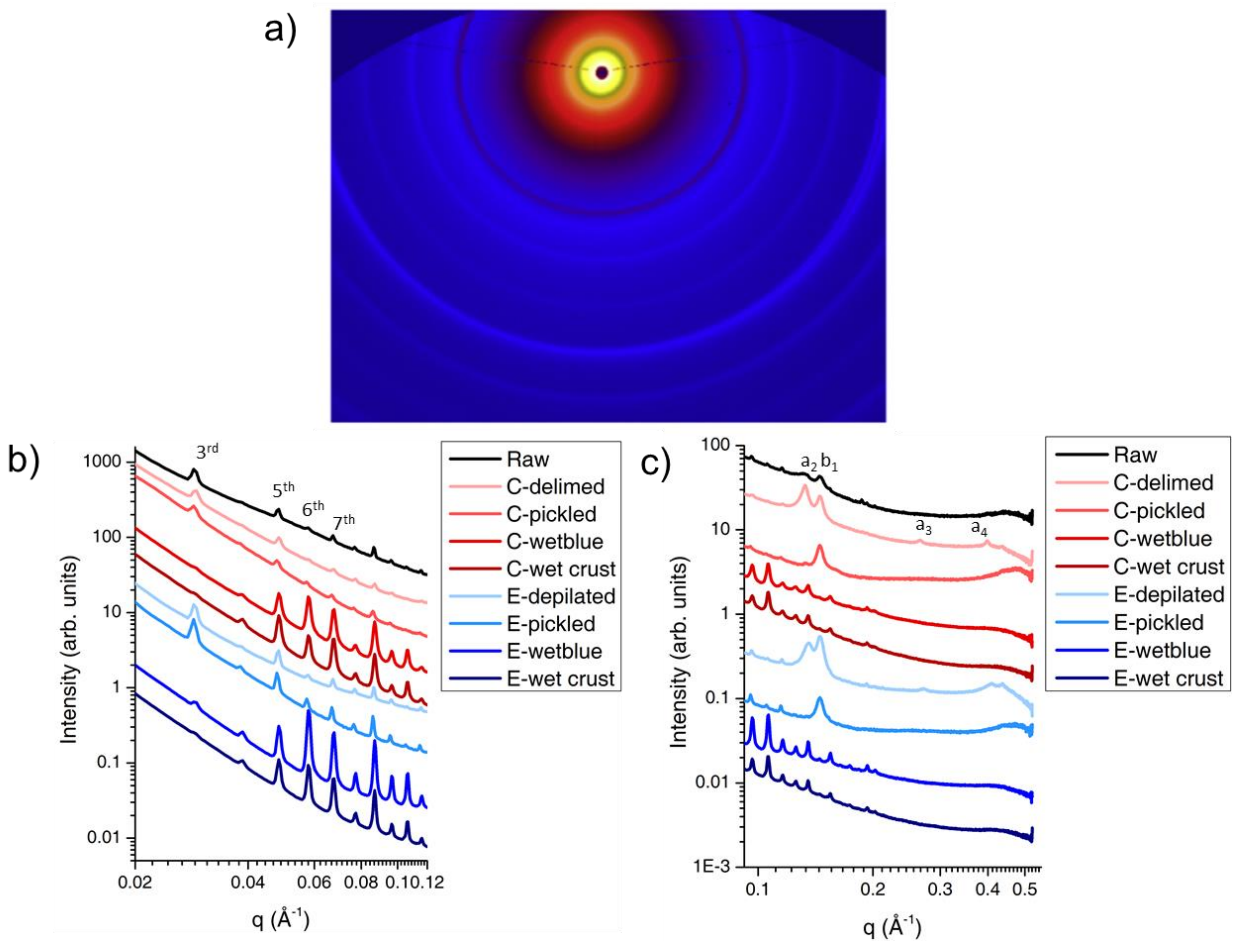

Figure 2. (a) 2D SAXS pattern of raw calf skin showing characteristic diffraction rings caused by the long-range ordered collagen structure. (b): Integrated SAXS data in the q region of 0.02–0.12 A^{-1} showing changes of overall peak intensities during the process from raw to wet crust. C): Integrated SAXS data in high q region of 0.1 – 0.52 A^{-1} showing specific peaks indicating the keratin intermediate filament ($a_{2, 3, 4}$) and fat (b_1). C = chemical process, E = enzymatic process.

The SAXS diffraction pattern in Figure 2 (a) demonstrated a group of well-resolved diffraction rings within the q region of 0.01–0.3 A^{-1} confirming the long-range ordered packing of collagen molecules within the collagen fibrils of raw calf skin. SAXS data obtained for enzymatic process and chemical process process in different steps are shown in Figure 2 (b) and Figure 2 (c), the structural changes observed from the four steps from raw to wet crust were consistent with the previous SAXS/WAXS results, indicating a comparable structure to standard leathers. [14,28]. In the low q region (0.02–0.12 A^{-1}), the peak intensity decreased from raw to pickling step, likely caused by the removal of non-collagenous proteins. During chrome tanning, the 3rd order peak

decreased while the other peaks increased significantly. From Figure 2(b), C-wet blue compared to E-wet blue, a slightly higher 6th order peaks of E-wet blue, suggesting a relatively higher uptake of chromium in the tanning step for enzymatic process.

In the q region of 0.1 – 0.52 A^{-1}, we observed a pair of broad peaks, a_2 and b_1, at around q = 0.15 $Å^{-1}$. According to previous studies, peak a_2, a_3 and a_4 can be attributed to the regular packing of keratin intermediate filaments [16,29]. The size of packing distance was calculated to be around 90 Å. The peak b_1 - assigned to the scattering that originates from the layered structure of fat or triglyceride molecules confirms the typical bilayer distance of 45 Å [30]. The intensity of peak a_2-a_4 reduces significantly from the deliming to pickling step for both the enzymatic process and the chemical process. At the unhairing step, some keratin molecules remain in the follicle in the grain of skin, but these were completely removed by the high salt concentration at the pickled step. The decrease in intensity of peak b_1 highlights the removal of fat (lipid) during the degreasing step after pickling and before chrome tanning. The structural changes observed in keratin and lipid were similar to our previous study [13].

4 Conclusion

The enzymatic depilation process from a novel bacterium - *A. hydrophila* was found to produce calf skin leather of comparable physical and thermal properties, with a significant reduction in pollutant load in process effluents compared to a conventional chemical process. The specificity of the enzymatic activity remains a challenge with a decrease of thermal stability observed. Further characterisation of the protease activity and the optimisation of depilatory paint application procedure can improve the depilation applicability of *A. hydrophila*. The use of SAXS demonstrated the technique's ability to detect structural changes of lipid, collagen, and keratin through different leather processes. The findings of this study can guide future developments of sustainable manufacturing methods for the leather industry.

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6 References

1. Milã i Canals, L.; Domènech, X.; Rieradevall, J.; Puig, R.; Fullana, P. Use of Life Cycle Assessment in the procedure for the establishment of environmental criteria in the Catalan eco-label of leather. *Int. J. Life Cycle Assess.* **2002**, *7*, 39–46.
2. Covington, A.D.; Covington, T. *Tanning Chemistry: The Science of Leather*; 2009; ISBN 0854041702.
3. Thanikaivelan, P.; Raghava Rao, J.; Unni Nair, B. Development of a leather processing method in narrow pH profile: Part 1. Standardisation of dehairing process. *J. Soc. Leather Technol. Chem.* **2000**, *84*, 276–284.
4. Marsal, A.; Cot, J.; Boza, E.G.; Celma, P.J.; Manich, A.M. *Oxidizing unhairing process with hair recovery. Part I. Experiments on the prior hair immunization*; 1999; Vol. 83;.
5. Dixit, S.; Yadav, A.; Dwivedi, P.D.; Das, M. Toxic hazards of leather industry and technologies to combat threat: A review. *J. Clean. Prod.* 2015, *87*, 39–49.
6. George, N.; Sondhi, S.; Soni, S.K.; Gupta, N. Lime and Sulphide-Free Dehairing of Animal Skin Using Collagenase-Free Alkaline Protease from Vibrio metschnikovii NG155. *Indian J. Microbiol.* **2014**, *54*, 139–142.
7. Vijay Kumar, E.; Srijana, M.; Kiran Kumar, K.; Harikrishna, N.; Reddy, G. A novel serine alkaline protease from Bacillus altitudinis GVC11 and its application as a dehairing agent. *Bioprocess Biosyst. Eng.* **2011**, *34*, 403–409.
8. Khambhaty, Y. Applications of enzymes in leather processing. *Environ. Chem. Lett.* **2020**, *18*, 747–769, doi:10.1007/s10311-020-00971-5.
9. Sivasubramanian, S.; Manohar, B.M.; Puvanakrishnan, R. Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. *Chemosphere* **2008**, *70*, 1025–1034.
10. Riffel, A.; Ortolan, S.; Brandelli, A. De-hairing activity of extracellular proteases produced by keratinolytic bacteria. *J. Chem. Technol. Biotechnol.* **2003**, *78*, 855–859.
11. Maxwell, C.A.; Smiechowski, K.; Zarlok, J.; Sionkowska, A.; Wess, T.J. X-ray studies of a collagen material for leather production treated with chromium salt. *J. Am. Leather Chem. Assoc.* **2006**, *115*, 373–379.
12. PETRUSKA, J.A.; HODGE, A.J. A Subunit Model for the Tropocollagen Macromolecule. *Proc. Natl. Acad. Sci. United States* **1964**, *51*, 871–876.
13. Zhang, Y.; Mansel, B.W.; Buchanan, J.K.; Su, J.; Zhang, Z.; Holmes, G.; Prabakar, S. Studies on Collagen Structure using X-Ray Scattering on a Closed-Loop Leather Process. *J. Am. Leather Chem. Assoc.* **2020**, *115*, 373–379.
14. Buchanan, J.K.; Zhang, Y.; Holmes, G.; Covington, A.D.; Prabakar, S. Role of X-ray Scattering Techniques in Understanding the Collagen Structure of Leather. *ChemistrySelect* **2019**, *4*, 14091–14102.
15. Zhang, Y.; Ingham, B.; Cheong, S.; Ariotti, N.; Tilley, R.D.; Naffa, R.; Holmes, G.; Clarke, D.J.; Prabakar, S. Real-Time Synchrotron Small-Angle X-ray Scattering Studies of Collagen Structure during Leather Processing. *Ind. Eng. Chem. Res.* **2018**, *57*, 63–69.
16. Rafik, M.E.; Briki, F.; Burghammer, M.; Doucet, J. In vivo formation steps of the hard α-keratin intermediate filament along a hair follicle: Evidence for structural polymorphism. *J. Struct. Biol.* **2006**, *154*, 79–88.
17. Mehta, M.; Liu, Y.; Waterland, M.; Holmes, G. Characterization of the Degradation of Sheepskin by Monitoring Cytochrome c of Bacteria by Raman Spectroscopy. *Anal. Lett.* **2020**, *54*, 1–18.
18. IULTCS/IUP6 Leather — Physical and mechanical tests — Determination of tensile

strength and percentage extension. 2020.
19. IULTCS/IUP6 Leather — Physical and mechanical tests — Determination of tear load — Part 2: Double edge tear. 2016.
20. IULTCS/IUP6 Leather — Determination of distension and strength of surface (Ball burst method). 2015.
21. Baird, R.; Rice, E.; Eaton, A. Standard methods for the examination of water and wastewater. In *American Journal of Public Health and the Nations Health*; 2017.
22. IULTCS/IUP6 Leather — Physical and mechanical tests — Determination of shrinkage temperature up to 100 °C 2015.
23. Ingham, B.; Li, H.; Allen, E.L.; Toney, M.F. *SAXSFit: A program for fitting small-angle x-ray and neutron scattering data*; 2009.
24. Yates, J.R. Studies in depilation. Part X: The mechanism of the enzyme depilation process 1972.
25. Ranjithkumar, A.; Durga, J.; Ramesh, R.; Rose, C.; Muralidharan, C. Cleaner processing: a sulphide—free approach for depilation of skins. *Environ. Sci. Pollut. Res.* **2017**, *24*, 180–188.
26. Bhavan, S.; Rao, J.R.; Nair, B.U. A potential new commercial method for processing leather to reduce environmental impact. *Environ. Sci. Pollut. Res.* **2008**, *15*, 293–295.
27. Zhang, Y.; Mehta, M.; Mansel, B.W.; Ng, H.W.; Liu, Y.; Holmes, G.; Le Ru, E.C.; Prabakar, S. Anion-regulated binding selectivity of Cr(III) in collagen. *Biopolymers* **2020**, *111*
28. Zhang, Y.; Snow, T.; Smith, A.J.; Holmes, G.; Prabakar, S. A guide to high-efficiency chromium (III)-collagen cross-linking: Synchrotron SAXS and DSC study. *Int. J. Biol. Macromol.* **2018**, *126*, 123–129
29. Rafik, M.E.; Doucet, J.; Briki, F. The Intermediate Filament Architecture as Determined by X-Ray Diffraction Modeling of Hard α-Keratin. *Biophys. J.* **2004**, *86*, 3893–3904.
30. Fraser, R.D.B.; MacRae, T.P.; Rogers, G.E.; Filshie, B.K. Lipids in keratinized tissues. *J. Mol. Biol.* 1963, *7*, 90–91.