The Microstructure of Raw Hides of *Crocodylus niloticus*

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Abstract

Crocodile leather with its unique pattern and scarcity is regarded as a leader in the high-grade leather products industry. In order to further improve the quality of crocodile leather products, based on the *Crocodylus niloticus* raw hides, the trichrome-udan IV staining method, aldehyde fuchshin staining method, scanning electron microscope and polarising microscope were used to study the histology characteristics and fibre weaving of the raw hides, which provides a theoretical basis for formulating tanning scientifically and reasonably.

摘要：鳄鱼皮制品作为高档真皮制品界的标志性奢侈品之一，以其独特的花纹和稀有性，受到各方面的追捧。为了进一步提高鳄鱼皮制品的内在质量，本文以尼罗鳄盐湿皮为原料，采用三色苏丹IV染色法、醛品红染色法及扫描电子显微镜、偏光显微镜等现代技术手段，对尼罗鳄原料皮的组织结构特点以及纤维编织情况进行了研究，为科学、合理地制定制革工艺方案提供了理论依据。关键词：尼罗鳄，原料皮，微观组织结构

1 INTRODUCTION

Histological and technological researches of various skins have been undertaken since the 1970s, including the skins of pigs, buffalo, goats, sheep and snakes[1-11]. The histology of the raw hides and the composition and characteristics these different raw hides were analysed, provide a practical and effective reference more efficient and sustainable.

In recent years, modern tanning technology has developed rapidly driving the development of the special leather tanning industry. However, there are very few domestic large-scale tanneries processing crocodile leather left. Thus there is a necessity to study the histology of crocodiles systematically. At present, the microstructure of crocodile skins is confined to the ordinary microscope and naked eye observation, there are few studies on the structure, identification and microscopic morphology of each part of the skin there being. As a result, it brings a great inconvenience for studying crocodile leather.

In this study, based on the *Crocodylus niloticus* raw hides, the trichrome-Sudan IV staining method, aldehyde-fuchsin staining method, scanning electron microscope and polarising microscope were used to study the structural characteristics and fibre network of the *Crocodylus niloticus* raw hides.

2 EXPERIMENTAL PROCEDURES

2.1 Materials and Instruments

Materials: *Crocodylus niloticus* wet salted skins, were provided by Guangzhou Longze Leather Co., Ltd. Acid Fuchsin, AR, was supplied by Beijing Procurement and Supply Station of China Pharmaceutical Company; Orange G, AR, was obtained by Sinopharm Chemical Reagent Co. Ltd.; molybdophosphoric Acid, AR, was supplied by Shanghai Chemical Reagent Company, Chinese Academy of Medical Sciences; Aniline Blue, AR, was provided by Shanghai Specimen Model Factory; absolute alcohol, AR, glacial acetic acid, AR, and acetone, AR, were supplied by Tianjin Fu Yu Fine Chemical Co. Ltd.; Sudan IV, AR, was obtained by Beijing 57601 Organic Chemical Factory; arabia gum, AR, was supplied by Tianjin Tianli Chemical Reagent Co. Ltd.; Basic Fuchsin, AR, was obtained by Shanghai Reagent No. 3 Factory; concentrated hydrochloric acid, AR, was supplied by Kaifeng Dongda Chemical Co. Ltd.; potassium permanganate, AR, was provided by Wuqiao Xinghua Pharmaceutical Co. Ltd.; oxalic acid, AR, was supplied by Tianjin Hengxing Chemical Reagent Co. Ltd.

Instruments: electronic balance, LT1001, Metter Toledo group; Fast freezing and paraffin section machine, HH-3658, Jinhua City, Hua Hai Teaching Instruments Factory; microscope, UB100i, Chongqing Ao Pu Photoelectric Technology Co. Ltd.; upright microscope, DM2500M, Leica micro system (Shanghai) Trading Co. Ltd.; scanning electron microscope, S-4500, Japan Hitachi company; iransmitted polarised microscope, XP400D, Shanghai Wan Heng Precision Instrument Co. Ltd.

2.2 Sampling

Examination of the hide revealed that the middle of the belly is the most smooth and clear of residues of fat, ground meat waste and fibre membrane present

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to the smallest extent and there exists no clusters of fat layers in the reticular layer, thus reducing the difficulties of the tanning process. The most difficult part of the processing is the tail because the thickness of the skin is greatest, up to 6-8mm, and there are fat layers under the reticular layers of the scutella [shield-like structure]. Therefore, both sides of the belly midline/tail were chosen as the sampling site for the histology. As shown in Figure 1, the sample size is 5cm x 5cm, which is then frozen to conserve it after labelling and after finishing all processes and sampling.

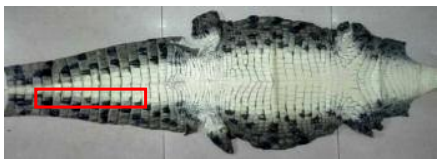

Figure 1. Sampling site of histology.

2.3 Fixing

The *preparation of 2,4,6- trinitrophenol* (picric acid) saturated aqueous solution: 500g water was added to a beaker, and the temperature was raised to 90°C. Then 12g picric acid weighed and added to the water, the solution was stirred and heated for 30 minutes. Finally, the supernatant was filtered,saved and cooled to room temperature.

The *preparation of Bouin fixative* (Boeing): 150ml formaldehyde solution (40%) and 30g glacial acetic acid were added to the 450ml of supernatant picric acid prepared above. Later the mixture was sealed and stored after being mixed evenly.

The cryo-preserved samples were cross-cut into small pieces of 5mm x 10mm, and 3-5 pieces were put into 100mL sealed sample bottles and fixed for 24 hours in 20mL Bouin fixative and then thoroughly washed.

2.4 Sectioning

The CO_2 freezing and paraffin wax microtome was used.The samples (x1)were attached to the freezing microtome using elbow forceps at -25°C, then the samples were flattened by tweezers and surrounded by a little water, thus freezing the samples to form a circular truncated cone. Sectioning was carried out at below -38°C.

The sample was fixed to the specimen holder at 0-1° blade adapter angle and 20-25μm section thickness. The sliding tool apron was adjusted to the appropriate distance and sectioning started at a knife speed of 0.5cm/s or so. The samples were gently put into a culture dish with distilled water.

2.5 Trichrome-Sudan IV staining method

Preparation of 0.5% dye liquor: 1.25g portions each of Acid Fuchsin, Orange G, Molybdophosphoric Acid and Aniline blue were weighed, dissolved respectively in 250mL distilled water and left aside until completely dissolved afterwards being filtered.

Preparation of Sultan IV dye liquor: 1.0g Sultan IV dye were dissolved in 100mL acetone, then 100mL 70% alcohol were added. After being completely dissolved, it was filtered after 24 hours and set aside.

Preparation of 10% Arabia gum: 50g Arabia gum were dissolved in 450mL hot water, then filtered, dyed and degassed by centrifugation before sealing.

70%, 50%, 30% alcohol solutions were prepared according to the volume fraction.

The whole staining process was finished on a watch glass, using 1-3 samples. 1-3 pieces of microtome sections were used. They were rinsed with water and residual moisture was absorbed with a pipette, which is similar to other staining methods.

The staining method is as follows:

Section washing→acid fuchsin for 30s→Orange G for 30s→molybdophosphoric acid differentiation for 60s→aniline blue for 120s→rinsing with distilled water→quick washing with 30% alcohol→quick washing with 50% alcohol→70% alcohol for 60s→Sultan IV for 45s→quick washing with 50% alcohol→quick washing with 30% alcohol→washing with distilled water→Arabia gum sealing.

After sealing, the samples were observed under an ordinary microscope to choose the appropriate sections, photographed and saved.

2.6 Aldehyde-fuchsin staining method

Preparation of aldehyde-fuchsin dye: 1g magenta was dissolved in alcohol solution with a volume fraction of 70%. After being dissolved entirely, 2mL concentrated hydrochloric acid and 2mL trioxymethylene were added, then kept for 24h and preserved in the refrigerator/freezer after filtration.

Preparation of 50% acid alcohol: 2 drops of hydrochloric acid were dropped into 100mL 50% alcohol.

Staining operation was similar to Trichrome-Sudan IV staining method, the whole staining process was finished on a watch glass. The specific staining steps are as follows:

Section washing→5g/L potassium permanganate aqueous solution for 1.5min→washing with distilled water 3 times→10g/L oxalate aqueous solution for 1min→washing with distilled water for 1min→50% alcohol for 1-2min→aldehyde-fuchsin liquor for 10min at 37°C→50% acid alcohol for colour separation 2min→quick washing with 30% alcohol→washing→Arabia gum sealing.

2.7 Upright microscope observation

After staining and sealing, one drop of Arabia gum, a cover glass was placed on the sample before placing on the microscope observation platform, lenses with 4, 10, 20, 40, 100 powers were used.

2.8 Scanning electron microscope observation

The samples were dried after mounting at 50°C and the sputtering time was 30 seconds, then the *Crocodylus niloticus* raw hides and chrome tanned samples were examined by field emission scanning electron microscope.

2.9 Polarising microscope observation

Chamfering is a way of sampling with 30~45° angle between cutting direction and the pelt. The sections of cross sections, cut both chamfered and flat, were sealed by Arabia gum, then placed on the rotating observation platform of the polarising microscope and observed with 4x, 10x, 20x and 40x magnification.

3 RESULTS AND DISCUSSION

3.1 Histology characteristics of the *Crocodylus niloticus* raw hides

As shown in Figure 2, The trichrome-sudan IV and aldehyde fuchshin staining methods were used to observe the histological of the raw hides. Figures 2a and b display the result of staining *Crocodylus niloticus* tail skin with trichrome-Sudan IV .The yellow fixative shows the dense lamellar keratin tissue attached to the surface of the skin, this is called the scale layer. In leather processing, if the scale layer is not removed it will seriously hinder the penetration of chemicals. The thickness of the scale layer is not uniform, the groove parts between the scutella are the thinnest and the middle parts of the scutella are the thickest. The epidermal layer is a brownish-black fibre layer connected with the scale layer and plays the most important part in highlighting the surface characteristics of the resultant leather, therefore the epidermal layer should be protected to prevent epidermal disruption which would result in the decline of the value of the resultant leather. The thickness of the bottom of the epidermal layer is comparable to that of the scale layer. The grain layer is a fibrous layer woven by finer fibre bundles and is also an important part of highlighting the softness and elasticity of leather. As in the case of cattle skins and sheepskins, the main part of the *Crocodylus niloticus* skin is known as the reticular layer, but the reticular layer of *niloticus* is thicker, the bundles are stronger and the fibre weave is more uniform. The diameter of the fibre bundles in the reticular layer is about 50-200μm and, the closer to the grain surface, the smaller the diameter of the fibre bundle is. As shown in the red part of Figure 2a-b, in the belly/tail of these skins under the reticular layer is a layer of fat, which is not uniform in thickness and is composed of globules of fat. In the tanning process, due to the effect of degreasing agents, bating enzymes, acid and salt, the adipose layer will be dispersed into the reticular layer, and even into the grain layer which will have a serious effect on tanning, so the adipose layer should be removed before tanning. Subcutaneous tissue is under the adipose layer (the subcutaneous tissue is directly connected with the reticular layer when there is no adipose layer), the main components of the subcutaneous tissue are still the collagen fibre bundles. However, the array of the fibre bundles is obviously different from that of the reticular layer but presents a uniform direction perpendicular to the line of the belly midline.

Figure 2c shows the histology of the *Crocodylus niloticus* tail skins when stained by aldehyde fuchsin. As shown in the Figure 2c, the scale layer is chequered in black and white, the epidermal layer is dark purple, the background colour is light purple and the elastic fibres around the fibre bundle are deep purple. The scale layer will absorb some aldehyde fuchsin dyes in the staining process which cannot be completely washed off in the washing process, therefore, partial dye permeation shows bright purple and the part where there has been no dye permeation still shows the yellow of the fixative solution. The epidermis layer shows dark purple, which indicates that a large amount of elastic fibres are contained in the epidermis layer. In the tanning process, particular attention to the control of processing should be paid when destroying the elastic fibres to a extent,as these can keep the epidermis layer from excessive loss of collagen fibres and avoid giving rise to a rough surface and blistering. The array of the fibre bundles in the grain layer, reticular layer and subcutaneous tissues can be better displayed by the method of aldehyde fuchsin staining. The fibre bundles in the grain layer are finer and the weaving is messy [tangled], the fibre weaving in order in the upper half of the reticular layer and the lower half of the reticular layer belongs to the transition state of the reticular layer and the subcutaneous tissue, which has strong fibres and a certain degree of weaving.

It can be seen from the analysis of Figure 2 that there was a big difference between the *Crocodylus niloticus* skins and the traditional leathers such as cattle, sheep, pig skins and so on. *Crocodylus niloticus* skins lack pores, hair, hair follicles and the sweat glands, these are consistent with the situation that *Crocodylus niloticus* can only rely on water temperature and sunlight for temperature regulation.[12]

Through histological section staining, although the different features can be stained by various dyes, the observation of the histology of *Crocodylus niloticus* can only be obtained under the light microscope when the magnification is low and does not meet the requirements of research into collagen fibre structure. Therefore, in this study, the scanning electron microscope was used to observe the cross sections of the belly of the *Crocodylus niloticus* including collagen fibre bundles and fibres. The sampling site was near the belly midline, which was close to the sampling location of the tissue section. The cross section and SEM results for raw hides are shown in Figure 3.

Figure 3b displays the scale layer on the surface of the raw skins. This was very dense and there were many small layers in the dry state, the epidermal layer under the scale layer was not obvious and the thickness of the grain layer was quite similar to that of the scale layer, moreover, there was obvious layering in the grain layer, scale layer and reticular collagen fibre layer. The collagen fibre bundles and their array were not observed in Figure 3b. It can be seen from Figure 3c that the fibres of C raw hides with a tight weave was surrounded by inter-fibrillary substance.

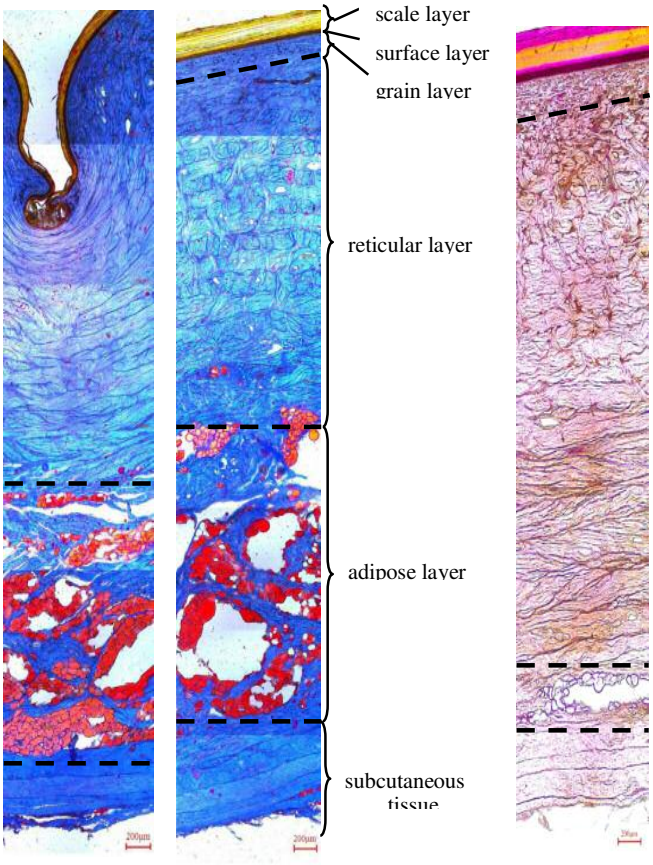

Figure 2. Cross section of *Crocodylus niloticus* raw hides histology (×10) **a**, **b**;trichrome-sudan IV staining method **c**; aldehyde-fuchsin staining method.

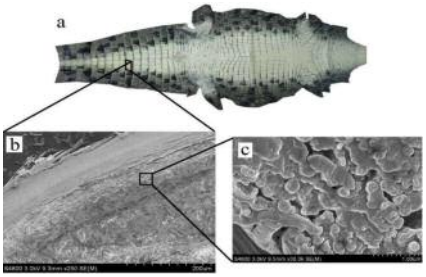

Figure 3. Cross section and fibre scanning electron micrograph of the *Crocodylus niloticus* raw hides (**b**; ×250, **c**; ×30000).

The tight degree of the fibre weaving of the skin directly influences the permeation of chemical materials in the tanning process. The bating enzymes, tannins, neutralising agents, vegetable tanning extracts, retanning and fatliquoring agents all of which have larger molecular weights, permeate with difficulty into the fibres, so materials with small molecular weights should be given preference during tanning. In addition, the intensity of bating and degreasing needs to be strengthened in the tanning process owing to the higher content of interfibrillary substance and fat.

3.2 Fibre weave of *Crocodylus niloticus*

Figure 4 shows the cross-section, chamfering and flat cutting micrographs obtained under a polarizing microscope at x10 magnification. It can be seen from

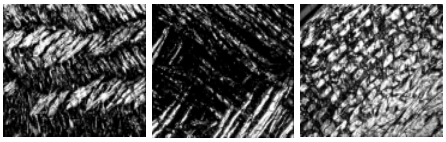

Figure 4. Fibre weaving polarizing micrograph of *Crocodylus niloticus* raw hides (×4) **a**; cross section, **b**; chamfering, **c**; flat cutting.

Figure 4a that there were two different areas showing light and dark in the cross section of the raw hides, and different distributions appeared in each layer. In the grain layer, the bright region was small and the distribution was not obvious. In the upper half of the reticular layer, the bright region was larger, the bright layer and the dark layer appeared alternately and the layering was obvious. In the lower half of the reticular layer, the bright region continued to increase and the dark region appeared as a thin line. Looking at the chamfering and flat cutting micrographs of Figures 4b and c, the fibre of the reticular layer has a distinct layering, the adjacent two layers of fibres showed different directions and the angle between the fibre bundles was close to 90°. The results show that there was no obvious regularity of the fibre weaving in the grain layer of the raw hides and the reticular layer shows histology which is arrayed tightly and regularly, the layering of the fibrous layer was also regular.

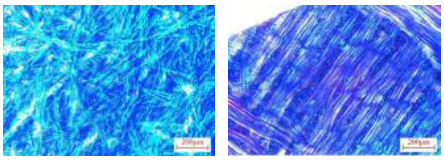

Figure 5. Fibre weaving of *Crocodylus niloticus* pelt (trichrome-Sudan Sudan IV staining method, flat cutting ×10) **a**; grain layer, **b**; reticular layer.

In this experiment, the experimental study on the *Crocodylus niloticus* pelt was carried out by polarising microscope, it is found that the polarizing microscope showed a significant effect in observing the distribution of fibres, the direction of the fibre bundles and the fibre weaving, this use of a polarising microscope could also be promoted in the synthetic leather and textile industry. In order to verify the conclusions obtained using a polarising microscope, trichrome-sudan staining and scanning electron microscopy were used to observe the pelt. Figure 5 displayed the microstructure between the grain layer and the reticular layer of *Crocodylus niloticus* raw hides under the microscope at a magnification of x10. It can be seen from Figure 5a that the diameter of the collagen fibre bundles was less than 50μm and their weaving was messy [untidy], moreover, the fibre bundles had no distinct direction. Figure 5b showed that the diameter of the collagen fibre bundles was 50-100μm, which was stronger than that of the fibre bundles, and of the fibre bundles in the reticular layer which were arrayed in a straight line in the same layer. The bright blue dots

between the collagen fibre bundles were bundles perpendicular to the flat cutting side, these bundles were relatively thin and closely distributed around the thicker bundles. Based on the observation of the samples prepared using trichrome-udan IV staining method, the laminar distribution of the reticular layer fibres in the pelt was further clarified.

4 CONCLUSIONS

The sections of *Crocodylus niloticus* raw hides were stained by trichrome-sudan IV and aldehyde-fuchsin staining methods, and the histology was observed under light microscope, scanning electron microscope and polarizing microscope to study the fibre weaving of the pelt, the conclusions are as follows.

There existed some fat layers with different thicknesses in the pelt, only a few supporting fibres were present in the fat which were easy to break up thus, direct freezing sectioning should be adopted, the appropriate thickness was 20μm.

(2) The histology of *Crocodylus niloticus* differs from cowhide, sheepskin and pigskin, it can be divided into scale layer, surface layer, grain, reticular, adipose and subcutis layers [deepest layer of the dermis mainly fat and connective tissues], while the reticular layer can be subdivided into the reticular upper layer and lower layer according to the different characteristics of the fibre weaving. Therefore, in the tanning process, the adipose layer and subcutis layer should be removed by fleshing after soaking and degreasing. The scale layer was removed in liming and the lower reticular layer was removed after tanning, finally shaving the pelt to the required thickness before finishing.

(3) Scanning electron microscopy was used to observe the histology of raw hides. It was found that, the structure of the scale layer was dense, the bundles between the grain and reticular layers were woven closely and the fibres were tightly surrounded by interfibrillary proteins. So it was necessary to remove the scale layer and interfibrillary proteins so the intensity of enzyme bating and degreasing should be strengthened, moreover, chemicals with good permeation should be given preference during tanning.

It can be seen from the analysis of pelt fibre weaving that the fibre bundles in the grain layer were finer and the weaving was messy, the bundles in the reticular layer were stronger and the weave was more ordered. The reticular layer can be divided into a plurality of fibre layers, and the direction of the bundles was exactly identical in any particular layer, also the angle between the bundles and the adjacent fibre layer was about 90°. Through the interweaving connection of fine and bending fibre bundles, two adjacent fibre layers formed a network system which was regular with a tight weave.

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