

PERSISTENCE OF MINORITY MACROMOLECULES OF HIDE THROUGH THE BEAMHOUSE. II. REMOVAL OF COLLAGEN TYPE XIV*†

by

PAUL L. KRONICK AND SUSAN IANDOLA

U. S. Department of Agriculture

Agricultural Research Service, Eastern Regional Research Center

600 EAST MERMAID LANE

WYNDMOOR, PA 19038-8598

ABSTRACT

Collagen type XIV, which is located on the surfaces of the collagen fibrils of hide and skin, is a minority collagen that might play a role in leather drying. We show by immunohistological techniques that most of the non-helical portion of collagen type XIV is removed by lime. Unlike collagen type VI, it does not persist in the grain enamel. The triple-helical segments, however, might still persist, since these are not expected to react with the antibody stains used here. How type XIV collagen is removed might be an important feature of hide and grain quality in leather.

INTRODUCTION

Due to the pressing need for more environmentally sound tannery processes, much research has been devoted to developing a reliable enzymatic unhairing that would reduce sulfides in tannery effluent.^{1,2,3} Since it would be more costly than traditional chemical liming, efforts have in some instances been directed toward an enzymatic process that would be a hair-save as well, leaving tanneries with a valuable by-product.^{4,5} Investigators have delved into animal proteases, such as trypsin and pepsin, plant proteases, such as pectinase, and most recently microbial proteases, particularly those obtained from bacteria.^{2,5,6,7,8}

Reports on all of these methods have concluded that physical test results are comparable to those achieved with lime-sulfide unhairing.^{3,4,6,9} Other kinds of results, though,

vary widely. Grain is said to be greatly improved in some cases, while in others grain irregularities have been noted. These irregularities include a loss in fullness and smoothness of the grain,¹⁰ and an irregular "buffing effect" resulting in uneven dyeing later.³ The time required by each enzyme system also varies widely, from as little as 6 hours to over 24 hours.^{2,4,9}

It is no wonder that many tanneries have been cautious about adopting an enzymatic unhairing procedure. Questions naturally arise regarding the efficacy of transposing a procedure reported to work well on goat skin to bovine or porcine hide. Will the value-added by adopting a more drawn-out hair-save procedure outweigh the savings achieved by going for a shortened liming time without hair recovery? In the end though, nothing is worth the investment in money and time if it does not result in leather of better quality. The proliferation of available enzymes and the resulting questions raised have created a need for more precise methods of chemically analyzing the effects of enzyme treatments upon a hide.

Further, retans and fatliquors are added to leather to control the adhesions among its fibers, but it is not clear why fibrils should adhere to each other, nor whether they actually would if their surfaces comprised only type-I collagen. Besides collagen, the fibrils of hide contain proteoglycans and non-fibrillar collagens. Although present in small amounts, they are concentrated on the surfaces of the fibrils and probably enhance the tendency of the fibers and fibrils of leather to clump when it is dried. It is therefore necessary to determine their role if fiber adhesion during drying is to be understood.

The type XIV collagen molecule is twice as large as that of type I¹¹ but does not form fibrils by itself, because most of the molecule is not triple helical (Figure 1). Unlike the fibrous collagens its triple helix is interrupted by a nonhelical segment (NC2) and is attached to a globule joining three ramified fingers visible in the electron microscope, comprising the unraveled ends of the three polypeptide chains. This nonhelical portion (NC3) is very large, with a molecular weight of 190 kDa.¹¹ The protein associates tightly with the surface of fibrils of type I collagen.^{12,17} In these characteristics, type XIV resembles collagen type XII, on which we reported earlier,¹⁴ with which it is 61% homologous in its amino-acid sequence. Both minority collagens are classified as FACIT's (fiber-associated collagens with interrupted triple helices),¹⁵ which are often found in tissues that comprise type-I collagen fibrils.

In this work we analyze the removal of collagen XIV in the beamhouse by staining treated tissue with antibodies that bind specifically to that protein. Because few antibodies bind to the helical parts of the molecule, our observations apply strictly to only the non-helical domains.

MATERIALS AND METHODS

Rawstock

The hide from a fetal dairy calf of 6 months was procured fresh and immediately packed in dry ice for transport. A portion of the fetal hide was pulverized under liquid nitrogen in a freezer mill (SPEX Industries, Princeton, NJ) and stored at -80°C for immunoscreening work. The remaining hide was held at -80°C, unfixed, for immunohistochemical staining. The fresh hide from a 21-month-old Hereford was procured for immunohistology/tanning experiments. The Hereford hide was held at the slaughterhouse at 4°C for 24 hours before being transported to the ERRC pilot tannery. It was trimmed and sided prior to fleshing with a Wilmington automatic roller fleshing machine (Wilmington, DE). The two sides were then cut in half crosswise to provide four distinct areas for sampling (Figure 2).

Tanning

The standard USDA chrome process¹⁶ was followed, involving a traditional 18-hour unhairing. Lime and bate processing took place in a 36-gal Canbar drum. The hide quarters were removed to a 36-gal Dosemat for pickling and tanning. Consecutive samples were excised from all four areas when the hide was at each of the following stages: fresh, lime, bate, pickle and wet-blue. Specimens were preserved at 4°C in 4% paraformaldehyde buffered with 200 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) at pH 7.4. Hair was razor-shaved from fresh specimens.

Immunohistochemistry

Sample blocks 3 x 5 x 4 mm were removed from the preserved specimens and prepared for sectioning by first reversing the fixation with an 18-hour wash in PBS- 4% sucrose at 4°C. The frozen fetal calf skin was washed in the same manner for consistency, although it was not fixed. The sample blocks were then surrounded by cryostat embedding media and snap-frozen at liquid nitrogen temperature. Full cross sections of hide were cut 8-10 µm thick at -28°C with a freezing microtome. The sections were mounted on polylysine-coated glass slides. The immunostaining was carried out following the procedures established by Amenta et al.¹¹ Bovine serum albumin (BSA) was used at 5% to block nonspecific binding of antibodies by competing with them, and at 1% as a diluant. Appropriate antibody concentrations were determined by testing serial dilutions on fetal calf skin, as this is known to contain observable quantities of types III and XIV collagen. The antibody to type III collagen was used as a positive control, and the antibody diluant (1% BSA) as a negative control.

Antibody Specifications

The two primary antibodies used were affinity-purified rabbit anti-collagen type III (human and bovine), supplied by Rockland, Inc., Gilbertsville, PA and polyvalent rabbit serum against human undulin (Heyltex Corporation, Houston, TX) cross-reacting with bovine undulin, a majority fragment of the collagen type XIV molecule, comprising most of the NC3 domain but not the collagenous domains.¹¹ Goat anti-rabbit immunoglobulin (Boehringer Mannheim Corporation, Indianapolis, IN) and rabbit peroxidase anti-peroxidase (PAP) (Cappel Laboratories, Durham, NC) were used as the secondary and tertiary antibodies, respectively. Immunohistological detection was accomplished using Sigma Co. (St. Louis, MO) Fast DAB Tablet sets. In this PAP amplification method, an (primary) antibody against the antigen to be detected (collagen XIV) is first applied to the sample. After excess is washed away, a second antibody is applied that is directed against the primary one and also against a third. The third is directed against horse-radish peroxidase (HRP) and forms a complex with it containing 3 molecules of HRP. In this system, a single molecule of antigen binds single molecules of primary antibody, but this binds multiple secondary antibodies that in turn bind clusters of the third antibody, giving amplification at this stage of 37.2 HRP's to antigen.¹⁸ Each molecule of the enzyme HRP, in turn, catalyzes the oxidation of many molecules of diaminobenzidine (DAB) by hydrogen peroxide, staining the area of the sample brown. For example, purified HRP from Sigma Chemical Co., St. Louis, MO can generate 80 x 10⁴ mmol diaminobenzidine color product/

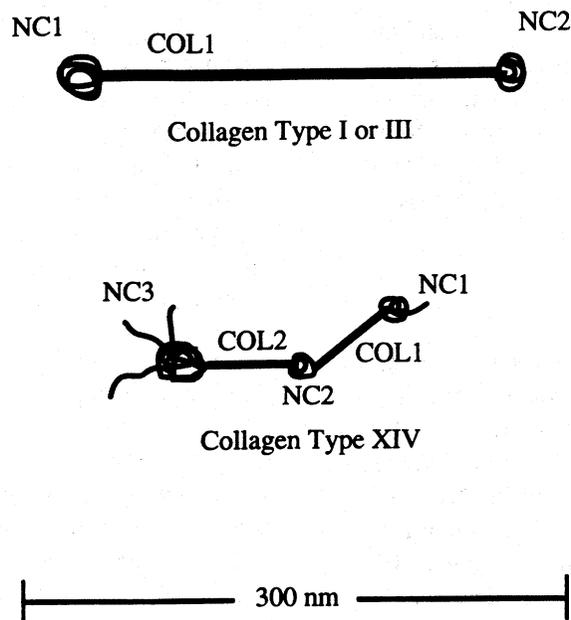


FIGURE 1. — Comparison of sizes and conformations of molecules of collagens type I and XIV. Segments COLx are triple helical; NCx, non-helical. The non-helical segments of collagen type XIV prevent the molecules from packing into fibrils; those of type I are small enough to fit into the lattice of the fibril.

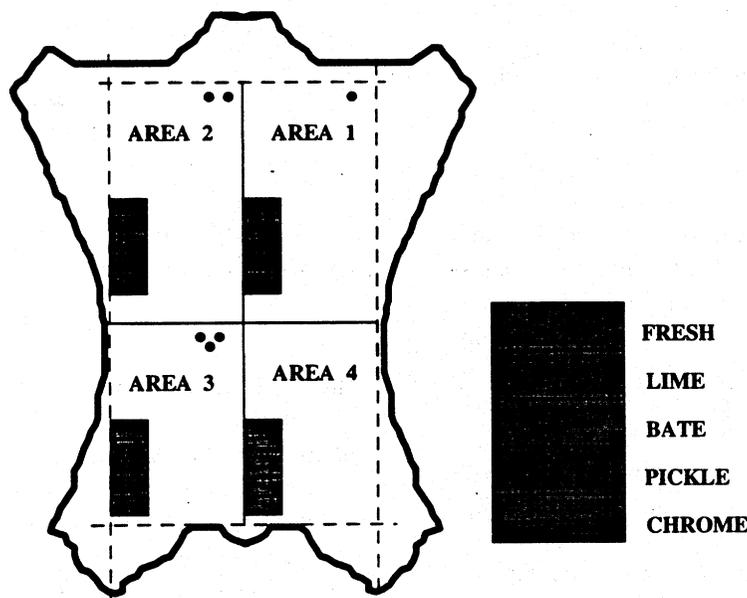


FIGURE 2. — Locations of sampling areas on hide for microscopy.

mmol HRP in our 20 min incubation time, giving a molar amplification of 3×10^8 . To be clearly observable, the optical density of the oxidized DAB should be 0.5 or greater; with a molar absorption coefficient as low as 500, we would need to generate 4 M DAB to see it clearly in the microscope, still requiring only $4/(3 \times 10^8) = 1.3 \times 10^{-8}$ M collagen XIV.

Immunoscreening

Bovine collagen type XIV was extracted from fetal calfskin following Auerbet-Fauchet et al.¹⁹ Fractions containing collagen type XIV as judged by gel electrophoresis²⁰ (SDS-PAGE with 5% gels and Tris-glycine buffer) under reducing and denaturing conditions were blotted from the electrophoresis gels to cellulose nitrate film along with type I

collagen (Vitrogen X100, Collagen Corp., Palo Alto, CA). The film was immunostained as described above to affirm the crossreactivity of the bovine collagen XIV spots with rabbit anti-human undulin and lack of such reactivity of collagen type I. These Western blots²¹ were performed from Mini-Protean II Ready Gels (Bio-Rad Inc., Rockland, MD) onto 0.45- μ m nitrocellulose membranes, using the Bio-Rad Mini Trans-Blot electrophoretic transfer cell.

RESULTS

The reliability of our immunohistochemical labeling technique depends on a primary antibody binding selectively to a component of hide and then being made visible by an amplifying developing system that, by itself, would generate no colored product. To confirm the selectivity of the developing system, we compare staining of fresh, limed, and wet-blue hide by antibodies against collagen III (Fig. 3b-d) with staining by the PAP development system with no primary antibody in Figure 3a. There is no positive artifact in the fibers shown in Figure 3a, even though the development system also depends on antibody binding (the secondary antibody to a primary), proving that the secondary antibody of the developer does not bind at all to our tissues. There is, however, a positive artifact in the hairs and fibroblast cells, probably due to endogenous peroxidase, which causes the oxidation of the diaminobenzidine in the developer to its colored product.

The beamhouse cannot remove collagen type III. With our techniques, staining of type-III collagen is always observed, not depending on the treatments, showing that any absence of staining of collagen type XIV in the presence of its antibodies would be due to the removal of antigenic protein, not to our technique. Figure 3c shows an effect of opening up of the fibers by lime (cf. Figure 3b), with heavier labeling of collagen III due to the deeper penetration of the large molecules of the antibody into them. The steps of neutralization, bating, pickling, and chrome tanning do not cause any diminution of labeling (Figure 3d). The tanning process, as drastic as it is, apparently leaves the antigenic sites on collagen type III apparently intact, even though these are located on the nonhelical telopeptides, where they are more vulnerable than in the triple helix. The distribution of type III, although it was even throughout each area, appeared to be greater in the corium than in the grain. The corium was labeled consistently more darkly than the corium minor layer over all four areas through the beamhouse. Uneven distribution of type III collagen throughout the thickness of the skin when observed by immunohistochemical staining was also reported by Viidik et al.²²

Figure 4a shows the labeling of collagen type XIV at the upper surface of the corium minor. It is absent from the epidermis, hair follicles, and blood vessels, which contain no collagen XIV, but is associated with fiber bundles throughout the dermis. Close examination of the darkly stained "dots" shows them to be fibroblasts, which synthesize the protein. A narrow continuous band just underneath the epidermis is also labeled; this layer might later become the grain enamel. The corium minor was labeled slightly more heavily than the corium major (not shown).

In contrast to type III collagen, which lies within the hide fibers and is integral to them, the epitopes of collagen type XIV, to which the antibodies bind, are largely removed by lime (cf. Figure 4a with 4b), along with the epidermis. These epitopes are limited to the NC3 domain of collagen type XIV and cannot include the triple-helical parts of the molecule, because the indulin against which the antibody was raised does not include them. (see **Materials and Methods, Antibody Specifications**); we have no information about them from this experiment. No labeling is evident throughout most of the fibers; the staining observed appears to be the peroxide artifact seen in Figure 4a.

Figures 4c, taken after the bate, and 4d, after chrome tanning, confirm that the NC3 domain of collagen type XIV is removed in the tannery to a level below our level of detection. Given the concentration of type I collagen in fibrils, about 5×10^{-3} M, and a requirement of the collagen XIV to be at least 10^{-8} M to be discernable (q.v. **Materials and Methods, Antibody Specifications**), the ratio of collagen XIV:collagen I remaining intact must be less than 2×10^{-6} .

DISCUSSION

The fibrils of hide are composed substantially of three types of fibrogenic collagen: I, III, and V. It is known that they all coexist in the same fibrils, which are therefore described as "heterofibrils." We confirm (Figure 3) that the type III collagen resists lime, bate, and pickle as does the type I collagen, and is present in the fibers of chrome-tanned leather. The opening-up effect of liming and neutralization, however, does make it more accessible to the large antibody molecules, as we observed in the enhanced staining. It is more than likely that this increased accessibility due to liming would also pertain to enzyme molecules that might be introduced into the leather making process for purposes of chemical modification.

When we entered this research, we considered collagen XIV, or at least its NC3 fragment, to be a likely interfibrillar adhesive. This seemed to be an especially attractive



FIGURE 3. — Corium minor layer of hide immunostained for collagen type III. (a) sample from area 3 of Figure 2, developed without primary antibody, showing lack of development artifact; (b) sample from area 3, fresh hide with full development system; (c) sample from area 1, after lime, full development system; (d) sample from area 1, after chrome tanning, full development system. Bar in (a) = 100 μ m; same for (b) - (d).

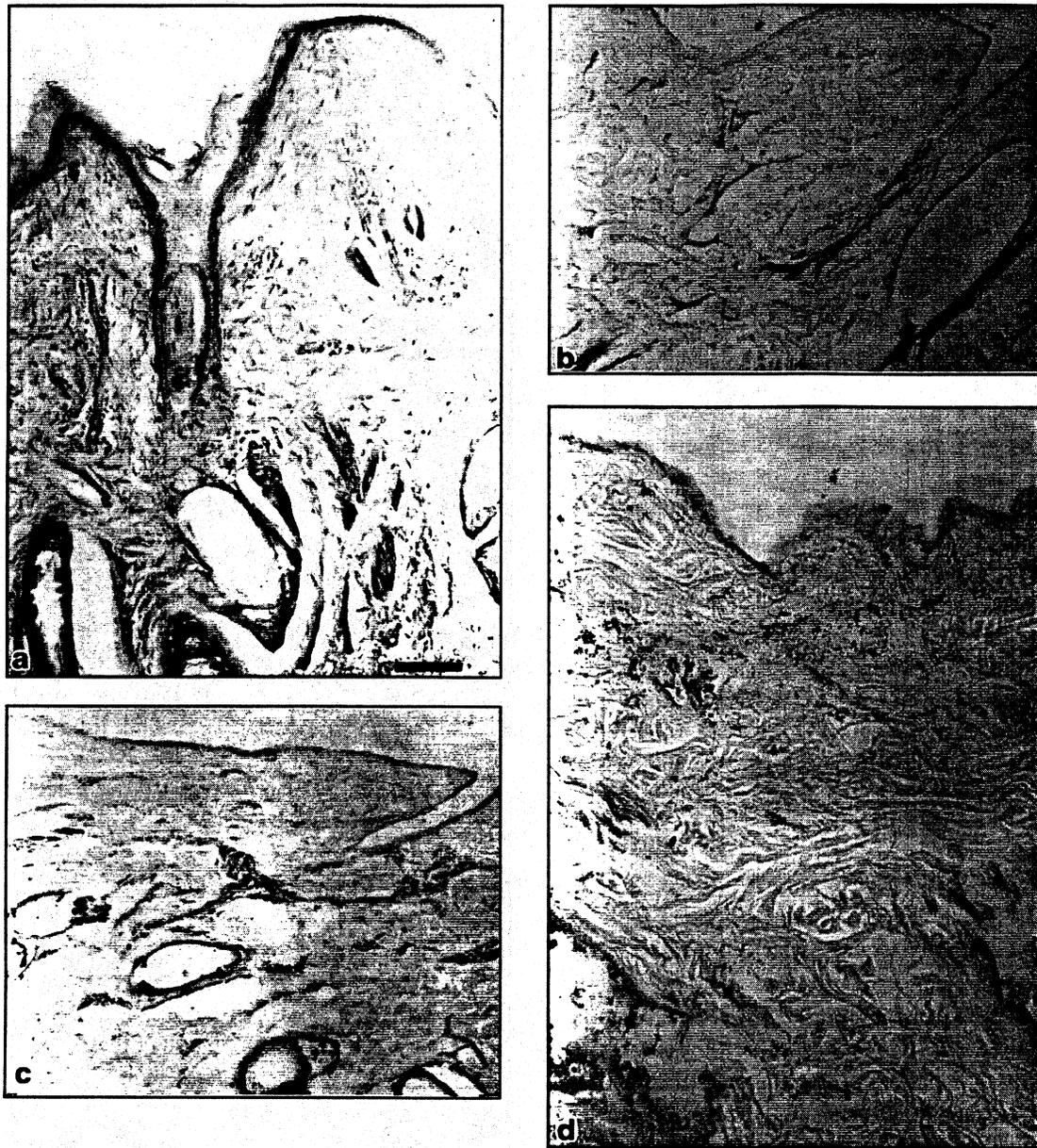


FIGURE 4. — Corium minor layer of hide immunostained for collagen type XIV. (a) sample from area 3 of Figure 2, fresh hide with full development system; (b) sample from area 3, after lime, full development system; (c) sample from area 3, after bate, full development system; (d) sample from area 3, after chrome tanning, showing absence of collagen type XIV from the fibers. Bar in (a) = 100 μ m; same for (b) - (d).

hypothesis, because type XIV binds to the surfaces of the heterotypic fibrils as well as to the proteoglycan decorin, which also is attached firmly to fibril surfaces. In fact it binds to the dermatan sulfate side chain of decorin,¹⁹ but ELISA assays have shown no evidence of binding of collagen type XIV directly to monomeric forms of collagens I, III, or V (23). Our observation of the removal of the NC3 domain of this collagen by lime is therefore consistent with the Font et al.¹² hypothesis that the glycosaminoglycan of decorin is a necessary intermediate for the binding of type

XIV to the heterofibrils of hide,¹² since the two are removed together when lime cleaves the decorin side chain in the tannery.

An important observation is the large concentration of collagen XIV in the "proto-enamel," just below the epidermis. Unlike the edge artifact along the side edges of the section, this staining projects into the corium minor layer, signifying that collagen XIV is a component of the layer in raw hide that will become enamel in the leather. Because of its

location at the surface of corium minor splits or full leather, it is for esthetic qualities the most valuable layer. Little else is known about its composition; collagens IV²⁴ and VI¹⁴ have been reported as other components.

We found in this work that a major part of the collagen type XIV molecule is removed by lime (Figure 4b), including from the enamel. This is the first identification of a component of enamel that is removed by liming.

Removal of collagen type XIV by liming differs from what we observed earlier for collagen type XII in fetal chick skin, which we labeled with a monoclonal antibody.¹⁴ In that case the part of the type XII molecule that the antibody labeled, at the end opposite to the NC3 region, was observed to persist until our simulated bating step (treatment with pure trypsin). The difference in persistence between collagens XII and XIV might only be apparent; the NC3 domain might have been removed in both cases, but while the antibodies against that domain, used for collagen XIV, would detect this removal, those against NC1 of collagen XII would not. Therefore, the collagenous domains, NC1, and NC2 of the two collagens might still persist even through the bate. Since the collagenous molecular fragments physically are not unlike the helical ones of type I collagen, however, they might not add to the adhesion among fibrils.

CONCLUSION

Besides the well-known effects of liming, opening-up and cleaving of glycosaminoglycans, we find that it also removes at least the major non-collagenous portion of collagen type XIV and possibly of type XII (14). Evidently the evaluation of the effectiveness of a liming process in a tannery should involve more than swelling and pH profiles. Removal of minority macromolecules can affect the properties of completed leather. Knowledge of the persistence of collagen type XIV might be important in predicting the degree of fiber adhesion in the dried leather. The effects on other minority macromolecules, such as the protein part of decorin, are also being explored.

REFERENCES

1. Taylor, M. M., Bailey, D. G., and Fairheller, S. H.; *JALCA* **82**, 153, 1987.
2. Raju, A. A., Chandrababu, N. K., Samivelu, N., Rose, C. and Rao, N. M.; *JALCA* **91**, 115, 1996.
3. Cantera, C. S., Angelinetti, A. R., Altobelli, G. and Gaita, G.; *J. Soc. Leather Tech. and Chem.* **80**, 83, 1996.
4. Dhar, S. C.; *Leather Sci.* **24**, 199, 1977.
5. Puvanakrishnan, R. and Dhar, S. C.; *Leather Sci.* **33**, 177, 1986.
6. Campbell, D. W. and Donovan, R. G.; *JALCA* **68**, 96, 1973.
7. Puvanakrishnan, R., Bose, S. M. and Dhar, S. C.; *Leather Sci.* **28**, 32, 1981.
8. Deselnicu, M., Bratulescu, V., Siegler, M. and Anghel, A.; *JALCA* **89**, 352, 1994.
9. Alexander, K. T. W., Haines, B. M. and Walker, M. P.; *JALCA* **81**, 85, 1986.
10. Siva Parvathi, M. and Nandy, S. C.; *Leather Sci.* **27**, 327, 1980.
11. Amenta, P. S. and Martinez-Hernandez, A.; *Meth. Enc.* **145**, 133, 1987.
12. Font, B., Aubert-Foucher, E., Goldschmidt, D., Eichenberger, D. and van der Rest, M.; *J. Biol. Chem.* **268**, 25015, 1993.
13. Gordon, M. K., Gerecke, D. R., Nishimura, I., Ninomiya, Y. K. and Olsen, B. R.; *Connective Tissue Res.* **20**, 179-186, 1989.
14. Kronick, P. L., Maleeff, B. E., and Dahms, M. P.; *JALCA* **86**, 209-224, 1991.
15. Gordon, M. K., Gerecke, D. R., Dublet, B., van der Rest, M., and Olsen, B. R.; *J. Biol. Chem.* **264**, 19772-19778, 1989.
16. Taylor, M. M., Diefendort, E. J., Hannigan, M. V., Artymyshym, B., Phillips, J. G., Fairheller, S. H. and Bailey, D. G.; *JALCA* **81**, 43, 1986.
17. Trueb, J. and Trueb, B.; *Eur. J. Biochem.* **107**, 549-557, 1992.
18. Sternberger, L. A., Hardy, P. H., Cuculis, J. J., and Meyer, H. G.; *J. Histochem. Cytochem.* **18**, 315-333, 1970.
19. Auberet-Foucher, E., Font, B., Eichenberger, D., Goldschmidt, D., Lethias, C. and van der Rest, M.; *J. Biol. Chem.* **267**, 15759, 1992.
20. Laemmli, U. K.; *Nature* **227**, 680, 1970.
21. Towbin, H., Staehelin, T. and Gordon, J.; *Proc. Natl. Acad. Sci.* **76**, 4350, 1979.
22. Viidik, A., Danielsen, C. C. and Oxlund, H.; *Biorheology* **19**, 437, 1982.
23. Brown, J. C., Mann, K., Wiedemann, H., and Timpl, R.; *J. Cell Biol.* **120**, 557-567, 1993.
24. Loutis, H., Stirtz, T., Fietzik, P. P., and Heidemann, E.; *Das Leder* **35**, 75-77, 1984.