## Original Article

# Increased Crystallinity but Unchanged Crystallographic Fiber Period of Collagen Fiber with Aging

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**Abstract:** The x-ray small angle scattering method disclosed that the crystallographic fiber period of gerbil tail tendon collagen did not change with aging. Two kinds of scattering profiles were recognized ; crystalline diffraction spots and the amorphous diffuse scattering halo. The crystalline diffraction spots were broad in a younger tendon. However, they became sharper with aging. The amorphous diffuse halo that is marked in young tendon diminishes with aging. These results indicate that 1 ; the collagen fiber of a young tendon contains many crystal defects, 2 ; the content of proteoglycan in an younger tendon is higher than that in an older tendon. The x-ray diffraction profile becomes clear with aging.

**Key words:** collagen, fiber period, x-ray small angle scattering, crystallinity, proteoglycan

## INTRODUCTION

Many biomaterials change their concentrations *in vivo* responding to any change of their environment. However, the reaction time is usually short. On the other hand, connective tissues sometimes show a long reaction time. Variation in any components of connective tissues with aging and embryo developmental stage has been recognized since the 1960's. In some examples, the ratio of chondroitin sulfate A to sulfate C changes with aging and embryo developmental stages ; Keratan sulfate content increases with aging in costal cartilage[1].

However, in tendons are there such changes with aging ? The diameter of tendon collagen fibril increases with aging[2-5]. Scott et al. obtained the following interesting results about aging of rat tail tendon. They stained a proteoglycan in rat tail tendon by caproic acid and observed it using an electron microscope[6),7]. The proteoglycan could be seen as like a fiber which is coiled around collagen fibril perpendicular to the axis. The interval of the proteoglycan increased with aging, and the content of the proteoglycan decreased with aging by the biochemical analyses[6),7]. These results are self-consistent, because the increase in the period results in decreased content.

It is of interest that the composition and the contents of the mucopolysaccharides, and the diameter of collagen fibril change with aging. A question could be raised whether the crystallographic fiber period of collagen fiber itself changes with aging. (In this paper, collagen fiber is recognized as a crystal in a broad sense.) We may expect that the amino acid sequence of tropocollagen could not be changed with aging, because its gene structure would not change with aging. Therefore, the crystallographic fiber period would not change with aging. However, this is not self-evident. This must be confirmed experimentally. This question could be clarified by the x-ray small angle scattering method (XSAS). We define here the crystallographic fiber period as a spacing which can be determined by x-ray small angle scattering of the first order reflection. It is usually about 64 nm.

The present report deals with the fiber period, the sharpness of the diffraction spots and the diffuseness of the amorphous halo.

The diameter of the collagen fibril or microfibril is not to be dealt with in this paper, because the resolution power of our XSAS apparatus is too low to resolve these issues.

## MATERIALS AND METHODS

The experimental animals used were mongolian gerbils (*meriones urguiculateus*), aged 1, 3, 13, and 29 months old. The animals were sacrificed and their tails were excised, and cut to about 1 cm in length. The skins were cut longitudinally. The tendons were gently taken out by forceps in order not to cause any mechanical effects on the fiber period. The tendons were washed with phosphate-buffered saline (PBS) containing protease inhibitors (0. 1 M 6-amino-hexanoic acid, 5 mM benzamidine-HCl and 1 mM phenylmethane-sulphonyl-fluoride) and 0. 02 % sodium azide with ice-cooling to remove any blood and fat components, then stained with 0. 05 % phosphotungstic acid in 0. 15 M NaCl for 20 min according to the method of Fraser and MacRae[8], and

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washed with 0.15 M NaCl and water. They were gently straightened on a clean glass slide and air-dried. The dried tendons were bundled and used as the x-ray scattering samples.

The optics of the x-ray small angle scattering apparatus was a pinhole-system ($\phi$=0.5 mm), and its camera length was 411 mm. Incident x-ray was Ni-filtered CuK$\alpha$. The exposure time lasted about 3 h at 40 kV×43 mA. An imaging plate was used.

## RESULTS AND DISCUSSION

Figure 1 shows the x-ray small angle diffraction photographs of the mongolian gerbil tail tendons of the designated ages in months. Two kinds of scattering profiles were recognized ; the crystalline diffraction spots from collagen fibers on the equatorial line and the amorphous diffuse scattering halo which may attribute to amouphous proteoglycans. Increased crystallinity of collagen fiber makes the diffraction spots sharper. When the content of proteoglycan decreases, the halo may diminish. The positions of the diffraction spots from the center of the photograph determine the fiber periods. Table 1 is a list of the fiber periods *vs.* the age in months, where the fiber period is consistent with the repeating length along the fiber axis of collagen crystallites. In Table 1, it appears that the fiber period does not change with aging. Thus I statistically tested this point. The regression line between the period and age could be written as

$$\text{period} = 64.8 - 0.0 \times \text{age (nm)}.$$

A hypothesis considering the slope as zero can be adopted within 95 % confidence limits[9]. Therefore, it is reasonable to regard that the period does not change with aging.

As can be seen in Fig. 1, the diffraction spots become sharper with aging. This means that the crystallinity increases with maturation of the animal.

The other observation made when we look at Fig. 1

**Table 1 Variations of the crystallographic fiber period with aging.**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | one month old | | | | | | |
| spacing (nm) | 15.9 | 10.9 | 7.3 | | | | |
| order | 6 | 8 | 9 | | | | |
| spacing×order | 63.6 | 65.4 | 65.7 | | | | |
| average | 64.9 | | | | | | |
| | 3 months old | | | | | | |
| spacing (nm) | 30.6 | 21.5 | 16.5 | 13.0 | 10.6 | 7.1 | |
| order | 2 | 3 | 4 | 5 | 6 | 9 | |
| spacing×order | 61.2 | 64.5 | 66.0 | 65.0 | 63.6 | 63.9 | |
| average | 64.0 | | | | | | |
| | 13 months old | | | | | | |
| spacing (nm) | 33.1 | 21.5 | 16.3 | 12.6 | 10.3 | 7.5 | 6.9 |
| order | 2 | 3 | 4 | 5 | 6 | 9 | 10 |
| spacing×order | 61.2 | 64.5 | 66.0 | 65.0 | 63.6 | 63.9 | 69.0 |
| average | 65.4 | | | | | | |
| | 29 months old | | | | | | |
| spacing (nm) | 31.8 | 21.5 | 15.9 | 12.8 | 10.6 | 7.9 | 7.1 |
| order | 2 | 3 | 4 | 5 | 6 | 9 | 10 |
| spacing×order | 63.6 | 64.5 | 63.6 | 64.1 | 63.6 | 63.2 | 63.9 |
| average | 63.9 | | | | | | |
| total average | 64.4±1.6* | | | | | | |

*: standard deviation.

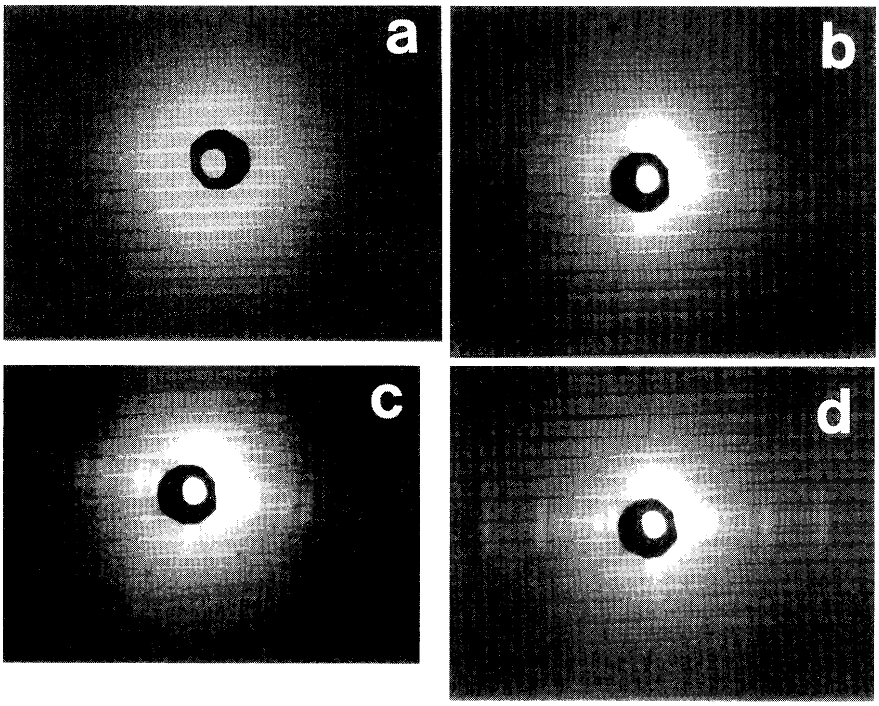


**Fig. 1. The X-ray small angle scattering profiles of the collagen fibers of mongolian gerbil tail tendons at one month old (a), 3 months old (b), 13 months old (c) and 29 months old (d), respectively.**
There are two kinds of the scattering profiles. One is the crystalline diffraction spots arranged horizontally, the other is a diffuse halo spread out from the center (an incident x-ray stopper) to the surroundings. The axis of the tendon is set horizontally.

is the diffuse halo, which can be especially seen in a young tail tendon but which diminishes with age, though it is still recognized at even 29 months old. The halo is remarkable, especially in a one month old gerbil. Scott et al. reported that the proteoglycan content of rat tail tendon decreased rapidly within 1-3 months of age. Their results agree with our experimental results using x-ray diffraction in which the halo is marked in a younger gerbil but diminishes with maturation. Therefore, it is rational that the halo could be attributed to the higher content of proteoglycans in the tendon. When we touched the tendons with tweezers, we observed that the tendons of 1-3 month old gerbils were very soft, but they were harder for the older gerbil tendons. The hardness may result from decreased body fluids as well. The environment around the collagen fibers changes dramatically within a couple of months after birth.

The sharpness of the crystalline diffraction spots and the diminution of the diffuse halo happened at same time, phenomenally. I speculate that the mechanism for the improvement of the crystallinity of collagen fibers may well be related to decreased content of proteoglycans. However, it has not been obvious yet whether the two phenomena are substantially correlated each other.

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