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Quantification of Total Collagen in Rabbit Tendon by the Sirius Red Method

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Introduction

Collagen, as a fibrous protein constituting approximately one third of the total protein in the body is synthesized by fibroblasts and is a major component of extracellular matrix in many tissues such as vessels, heart, kidney, liver, skin, tendons, bone and cartilage. The collagen molecule consists of three polypeptide chains which have three main amino acids: glycine, proline and hydroxyproline (OHPr). (1, 2, 3).

The common method of collagen determination is based on the quantitation of OHPr which accounts for approximately 10% of the collagen molecule. According to this method, the oxidation of OHPr to pyrrole and pyrrole 2 carboxylic acid and a chromophore formation with a p-dimethylaminobenzaldehyde (DMBA) reaction is measured spectrophotometrically at 550 nm (4, 5, 6).

There are some altrenative methods such as radioactive labelling of proline and enzymeimmunoassays using antibodies specific to each collagen type. Although these methods are very specific, they are expensive and many sample handling steps are required (7).

Sirius red F3BA, a strong anionic dye, stains collagen by reacting, via its sulphonic acid groups, with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fibre in such a way that their long axes are parallel. Therefore, sirius red F3BA in picric acid solutions has been used for quantitation of collagen in tissue sections for many years (8, 9).

In this study, our purpose was to measure the total collagen content of tendon species, as a good source for collagen type I, using sirius red F3BA dye and to compare the results with those measured by conventional hydroxyproline assay.

Materials and Methods

Animals: Thirteen normal male rabbits, 12-14 months old, were obtained from Ege University Animal Care Center and sacrified according to the rules of the ethical committee. After death, the patellar tendons were dissected and removed completely.

Tissue preparation: Each tendon was weighed to obtaion a wet weight and then lyophilized for 8 hours to obtain the dry weight. Lyophilized samples were divided into two groups and the collagen of all tissues was measured using both the sirius red F3BA and the DMBA assay.

Sirius red assay: Dry samples were continuously stirred in a solution of 4 M guanidine-HCl in 0.05 M sodium acetate (pH 5.8) at 4°C to remove the proteoglycans (11). After centrifugation for 30 minutes at 30000g the residue was collected and washed three times using 0.5 M acetic acid. The collagen residue was added to a solution of 1mg/ml pepsin in 0.5 M acetic acid at 4°C for 3 days (11, 12). Undigested material was removed by centrifugation at 30000g for 20 minutes. The supernatant was diluted with distilled water first and then 0.2 ml of diluted sample was mixed with 1.8 ml of siriues red in acetic acid (0.5 M) and incubated at room temperature for 20 minutes. After the incubation period, the samples were centrifuged at 2500 g for 10 minutes and the absorbance of the supernatant was read at 540 nm against acetic acid (0.5 M) as blank (13). The assay was calibrated using Type I collagen purified from rat tail. The method sensitivity was found to be in the range of 5 to 40 μg/ml of collagen. The linearity between the concentration of sirius red and optical density was assessed prior to the study and 0.5 μM of dye concentration was found to be suitable. To test the effect of pepsin on dye binding, 1mg/ml of pepsin was added to

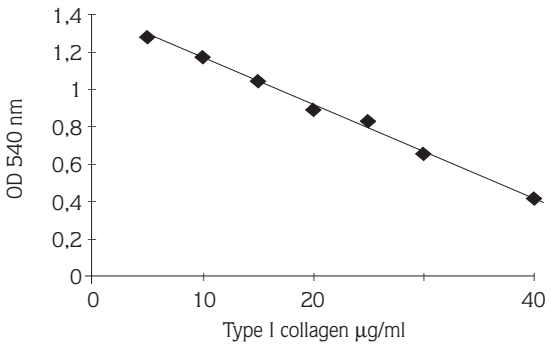

Figure 1. Standard curve showing absorbance of sirius red at 540 nm versus amount of type I collagen. Each point is the mean of triplicate samples and the regression coefficient for this line is 0.997, ($p<0.001$).

sirius red solution and a decrease in optical density of 0.010 was detected.

DMBA assay: Freeze-dried ligament samples were hydrolyzed in 4 ml of 6N HCl for 10 hours at 100°C. Aliquots of this hydrolysate were processed for quantification of hydroxyproline as an index of collagen content (4, 14). Collagen and hydroproline concentrations were expressed on a dry weight basis for each sample.

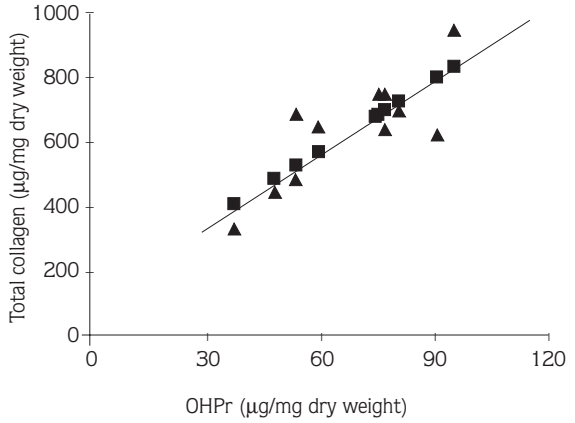

Figure 2. Relationship between total collagen and hydroxyproline assay. Each value is the mean of three measurements and the regression coefficient is 0.185, ($p<0.001$, $n=13$).

Results

Fig. 1 shows the calibration curve of Type I collagen. The decrease of the absorbance of the dye solution reveals a linearity with the amounts of the collagen. The wet and dry weights were found to be 113.06±45.09 and 31.24±8.84 mg (SD), respectively. The total collagen and hydroxyproline contents were found to be 626.94±152.96 and 67.81±17.36 µg/mg dry weight (SG), respectively ($n=13$, Table). The ratio between OHPr and total collagen was calculated and found to be 0.108±0.016 (SD). Linear regression on the data of the two methods showed a good correlation between the total collagen and hydroxyproline ($r=0.815$, $p<0.001$, Fig. 2).

Discussion

The collagen content in tissue sections has been measured using both histophotometry and colorimetric analysis. According to this method the sirius red dye is eluted in alkali solution and measured spectrophotometrically. These findings have been found to be correlated with quantification of the hydroxyproline content (8, 9, 10, 15). In the present study, the total collagen content of tendon species was determined spectrophotometrically after the digestion with pepsin without any histologic preparation. The ratio between OHPr and total collagen was calculated and found to be 0.108. Therefore, the results were in fairly good agreement when the total collagen content was estimated on the basis of a 10% correction factor, as suggested by Bornstein and Sage (16).

Wet weight (mg)	Dry weight(mg)	Total collagen	OHPr
118.90	40.47	327.34	37.08
111.12	31.94	561.79	59.54
95.97	26.93	440.89	47.86
74.82	27.71	639.65	59.44
109.45	31.87	738.72	75.44
105.19	22.38	935.96	95.43
80.15	31.45	478.24	53.33
74.05	15.85	611.41	90.84
148.82	42.01	631.68	77.10
93.04	24.30	679.66	53.68
141.46	27.48	688.65	80.62
88.62	26.68	738.72	76.92
118.36	33.99	677.60	74.36

Table 1. Wet weight, dry weight, collagen and hydroxyproline contents of ligaments. Total collagen and hydroxyproline levels were expressed as µg/mg dry weight.

Pierard has shown that the sirius red polarization method is useful for visualizing the organization of connective tissues but not for identifying the molecular nature of their fibrous polymers (17).

Walsh et al. have reported that sirius red was specific for many collagen types (Types I, III, IV, V) and gave similar calibration curves for each collagen type. In the present study, we used only type I collagen for the calibration of the assay because 80-95% of tendon collagen is composed of type I collagen (3, 18).

Previous studies have demonstrated that the addition of various noncollagenous proteins did nto cause any significant effect on the assay (13, 19). We tested pepsin solution (1mg/ml) and observed a decrease in the optical density of the sirius red solution after the addition of pepsin but this interaction was not found to be significant.

In conclusion, sirius red is a reliable, easy and inexpensive method requiring low cost reagents and equipment and might be used for quantitation of the total collagen content in tendons and other tissues in clinical and experimental studies.

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