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Changes in Hydrophobic Surface of Collagen by Chondroitin Sulfate : Fluorescence Intensity Measurements with Bis-ANS as the Probe

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Abstract

The important components of extracellular matrix (ECM) are collagen and chondroitin sulfate. The hydrophobic surface of collagen is one of the determining factors of diameter of collagen fiber and also is closely related to the aging phenomena. The controlling mechanism of the diameter of collagen fiber influenced by the interaction with chondroitin sulfate was evaluated using bis-ANS as a hydrophobic probe. Hydrophobic surface area of collagen molecule shielded by chondroitin sulfate was evaluated. Relative fluorescence intensity of collagen in the presence of chondroitin sulfate was measured using bis-ANS as a hydrophobic probe. The fluorescence intensity decreased with the increase in chondroitin sulfate up to 3.8 chondroitin sulfate / collagen (mole / mole). Further increase in the ratio of chondroitin sulfate to collagen did not change the fluorescence intensity. Similar changes in the relative fluorescence intensity were observed for both rat tail and lathyritic rat skin collagen. The fluorescence intensity indicated by the binding between bis-ANS and hydrophobic sites of collagen was pH dependent, and the shielding effect of collagen-chondroitin sulfate interaction could not be detected at pH above 6.0. This is probably due to the charge repulsions caused by negatively charged collagen molecules at higher pH.

Key words : collagen, chondroitin sulfate, hydrophobicity

INTRODUCTION

It has been recognized that the hydrophobic interactions plays an important role in the aggregation or fibrillation of collagen (1). Type I collagen molecules contain two $\alpha 1$ chains and one $\alpha 2$ chain. According to the primary sequence, there are 38 hydrophobic residues in $\alpha 1$ chain and 71 in $\alpha 2$ chain. Therefore, Type I collagen has 147 hydrophobic residues among total about 3000 amino acids. The fibrillation of the collagen is a self-assembly process, regulated by the amino acid sequence of the collagen molecule. The regulatory function is attributed predominantly to the charged polar amino acid residues. However, the hydrophobic amino acids such as valine, leucine, isoleucine, methionine, phenylalanine, and tyrosine still may play a role in the process of aggregation of the molecules. Collagen molecules in neutral salt solutions are soluble at the cold temperature ; however, heating to 37°C results in aggregation of the molecules into fibril (1). While electrostatic forces are not sufficient to cause aggregation, the increase in hydrophobic interactions may lead to fibrillation. The primary sequence and staining of segment-long-spacing crystallites (2) indicated that the cluster of hydrophobic amino acid residues of different collagen chains are distributed in a close proximity to each other. For the fibril formation, the distribution increases the size of hydrophobic pocket so that it is larger than that of a single chain.

The involvement of hydrophobic interaction in aggregation and fibrillation is suspected, if not recognized. Therefore, it is the objective of this study to evaluate the change in surface hydrophobicity of collagen in the extracellular matrix. Understanding the change in the surface hydrophobicity of collagen fundamental to understand the change in mechanical and physicochemical properties of tissues.

There are many significant age-related changes in the extracellular matrix. One of these changes is the increases in the rigidity and brittleness of the tissue with aging probably caused by the loss of glycosaminoglycan in connective tissue (3,4). These changes are caused mainly by the change in size of collagen fib-

ril. The brittleness and rigidity of the tissue, which increased by age, altered the mechanical properties of tendons and cartilage and also decreased the transparency in the cornea of eye. The loss of glycosaminoglycan, especially hyaluronic acids and chondroitin sulfate, may decrease tissue hydration and increase tissue surface hydrophobicity, thus causing an increase in the diameter of collagen fibril (4,5).

The age related changes in extracellular matrix (6) and the functions (3) of the glycosaminoglycan (GAG) relating to collagen or collagen fibril are the subjects of extreme interest. Therefore, a component of GAG, chondroitin sulfate, was used to study the effect of GAG on the surface hydrophobicity of collagen. Bis-Anilino-Naphthalene-Sulfonate (bis-ANS) was used to determine the surface hydrophobicity and the shielding effect of chondroitin sulfate on collagen. The shielding effect of chondroitin sulfate on collagen was evaluated in terms of hydrophobicity and electrostatic interaction.

MATERIALS AND METHODS

Collagen

Two samples of collagen were used. Rat tail collagen (Type I, cat. #40236, Collaborative Research Associates, Bedford, MA) was purchased as a solution in 0.02N acetic acid. The collagen solution was diluted to 1.2mg/ml with deionized water as a stock solution and kept at 4° C. Lathyritic rat skin collagen (Type I, cat. # 1063812, Boehringer Mannheim, Germany) was obtained as lyophilized powder. The collagen powder was dissolved in 0.005N acetic acid and 1.2mg/ml solution was prepared as a stock solution.

Chondroitin sulfate

Chondroitin sulfate of 70% Type A and 30% Type B (cat. # C-8529, Sigma Chemical Co., St. Louis, MO) were prepared to make two stock solutions of concentration 1.2mg/ml and 0.12mg/ml.

Hydrophobic probe

In preliminary experiments, several fluorescent probes such as bis-ANS (1,1′-bi[4-anilino] naphthalene-5,5′- disulfonic acid, dipotassium salt), ANS (1-anilinonaphthalene-8-sulphonate), DPH (1,6-diphenyl-1,3,5-hexatriene), and TNS (2-p-toluidinylnaphthalene-6-sulfonate) were tested and the sensitivities of probes were compared to the results of Fonscca *et al.* (7). Bis-ANS was considered the most suitable in terms of sensitivity and effectiveness and therefore was selected for this experiment. Bis-ANS (cat. # 4524-2, Molecular probes, Inc., Eugene, OR) showed a highest binding affinity and highest quantum yield. The concentration of stock solution was adjusted to 100μM.

Another chemical

Bovine serum albumin (BSA, cat. # A-7906, Sigma Chemical Company, St. Louis, MO) was used as a reference for evaluating the binding ability of bis-ANS in two different pH solutions.

Fluorescence intensity measurement

The intensity of fluorescence was measured at room temperature using an LS-50 Luminescence Spectrophotometer (Perkin-Elmer, England). All readings were made with a 1% attenuator at 400nm excitation and 500nm emission, and slit widths of 10nm.

The time of reading was determined by time drive under same conditions. The reaction mixture showed a stable fluorescence after 45 to 55 minutes of standing at room temperature. Therefore, the fluorescence intensity of the mixture of collagen and bis-ANS with or without chondroitin sulfate was measured after 1 hours at room temperature. Data were normalized by subtracting the intensity value of the mixture without probe and collagen.

Preparation of samples

All solutions were equilibrated to room temperature. Collagen alone or collagen with chondroitin sulfate was mixed with bis-ANS.

In order to determine the effect of chondoitin sulfate on the hydrophobic surface of collagen, fluorescence intensities were observed under various chondroitin sulfate/collagen ratio (0.0 to 12.5mole/mole). For evaluation of the effect of pH, 0.005N acetic acid (pH 3.5) and phosphate buffered saline (PBS, pH 7.4) were

used to adjust the pH. Phosphate buffered saline was prepared by dissolving 0.2g of potassium chloride, 0.2g of potassium phosphate monobasic (anhydrous), 8.0g of sodium chloride and 1.15g of sodium phosphate dibasic (anhydrous) in 1000ml of deionized water.

RESULTS

Determination of optimum concentrations of bis-ANS and collagen

The concentration of bis-ANS which gave a maximum fluorescence intensity was determined with a specific collagen concentration (0.24μM). The maximum fluorescence intensity was observed when 20 μM of bis-ANS was used (Fig. 1). Further increase of concentration of bis-ANS decreases the fluorescence intensity. Therefore, bis-ANS concentration of 20μM was used throughout the experiments.

Fig. 2 shows that the fluorescence intensity increased with the increase in concentration of collagen. The intensity showed a linear increase up to 0.24μM; however, further increase of collagen concentration showed a slow increase of intensity and finally reached a plateau when the concentration was higher than 0.6μM. Therefore, the concentration of collagen, 0.24 μM, which gives the highest intensity in the initial linear increase, was selected for the experiments.

The shielding effect of chondroitin sulfate at acidic pH

Rat tail collagen can be dissolved in the acidic solution at pH around 3 to 4. Therefore, rat tail collagen in pH 3.5 solution was tested first in order to avoid the complication due to fibril formation. Chondroitin sulfate decreased the fluorescence intensity of collagen. As shown in Fig. 3, this decrease probably indicates the shielding effect of chondroitin sulfate on the hydrophobic surface of collagen. Two molecules, chondroitin sulfates and collagen, interact by their electrostatic attractions, and their binding shields the hydrophobic surface area of collagen. This interaction results in the decrease of bis-ANS binding to collagen and thereby in the decrease of fluorescence intensity. With the increase in the ratio of chondroitin sulfate to collagen from 0 up to 3.75mole/mole, the intensity decreased. However, further increase of chondroitin sulfate to collagen did not change the fluorescence intensity. Thus, the ratio of 3.75mole/mole of chondroitin sulfate to collagen indicates the maximum amount of chondroitin sulfate bound to collagen. This value agrees with the amount of chondroitin sulfate binding to the collagen reported by Örink and Sundelöf (8).

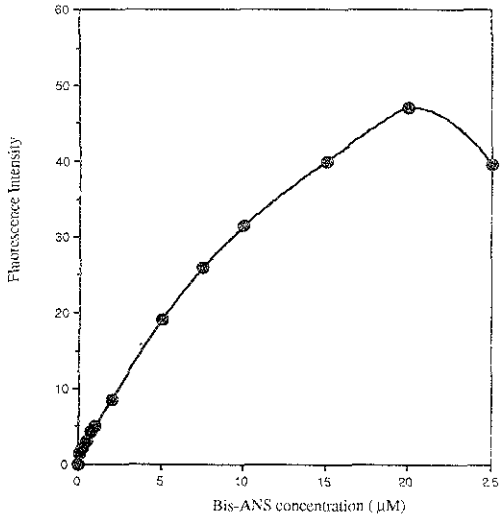

Fig. 1. Fluorescence intensity of collagen (60μg/ml) indicated by various concentration of bis-ANS.

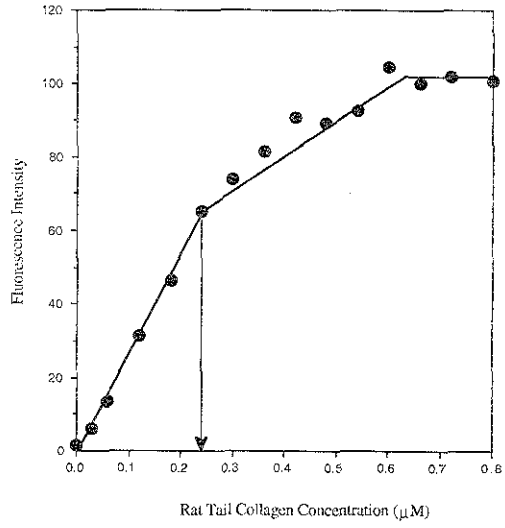

Fig. 2. Optimum concentration of collagen for fluorescence intensity measurements.

The Effect of chondroitin sulfate at pH higher than 4

The effects of chondroitin sulfate on the hydrophobic surface of collagen at higher pH and at physiological pH were evaluated. Chondroitin sulfate shields the hydrophobic surface area of collagen at pH 5.0 or lower as shown in Fig. 4. However, the shielding effect of hydrophobic sites by chondroitin sulfate diminished as the pH increased, and no effect could be observed at pH higher than 6.0. It was not clear whether chondroitin sulfate interacted with the collagen molecule or not at this pH value because the intensity value was similarly low in the presence as the absence of chondroitin sulfate. On the other hand, in the views of charge repulsions, there may have been no interactions between the chondroitin sulfate and collagen. The overall charge indicates that pH 5.0 is the isoelectric point of collagen molecule. Therefore, the collagen takes net negative charge at pH higher than 5.0 (Table 1). This may lead to the charge repulsions between the chondroitin sulfate and collagen, therefore no interaction and no resultant shielding. Another possible explanation is fibril formation of collagen with or without chondroitin sulfate at higher pH during 1 hour reaction. The lag time for the fibril formation was 10 to 20 minutes. After the lag time, the fibril formed very rapidly and reached equilibrium. Upon the fibrillation, most of the hydrophobic sites of collagen might be buried inside the fibrils, thus the numbers of hydrophobic sites of collagen may decrease. This might result the low intensity of fluorescence from the bis-ANS and collagen mixture with or without chondroitin sulfate at pH higher than 4.

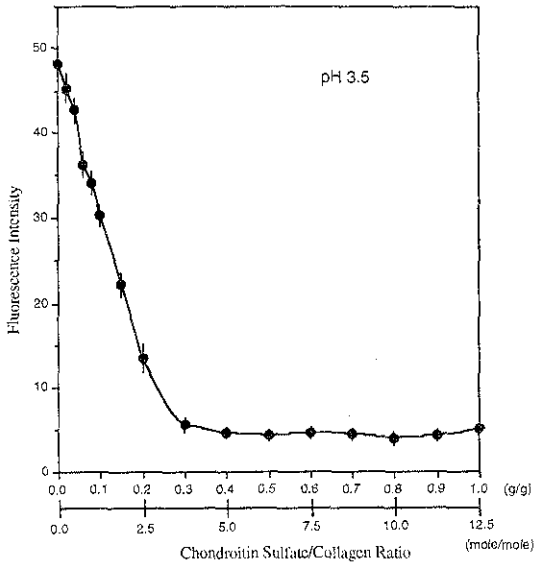

Fig. 3. Shielding of the hydrophobic sites of rat tail collagen by chondroitin sulfate.

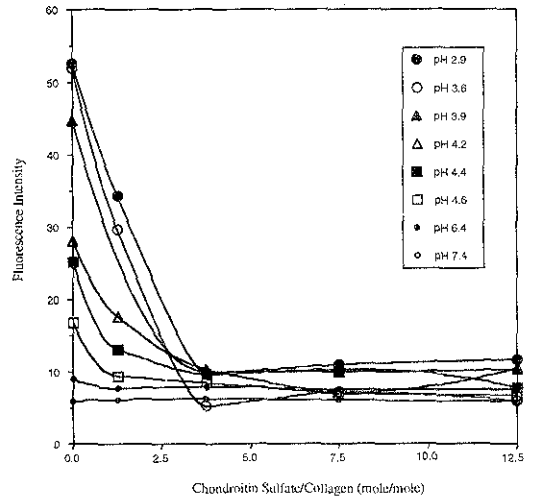

Fig. 4. Shielding of the hydrophobic sites of rat tail collagen by chondroitin sulfate at various pH.

The amount of bis-ANS binding to collagen at various pH

The amount of bis-ANS binding to collagen decreased with the increase in pH as shown in the Fig. 5. This indicates that the binding of bis-ANS to collagen is not solely due to hydrophobic interactions but also influenced by the electrostatic interactions. As the pH becomes higher, the total charge on collagen molecule becomes negative (Table 1), which may inhibit the binding of negatively charged bis-ANS to

Table 1. Calculated net charges of collagen at different pH using amino acids composition of collagen (9) by Henderson-Hasselbach equation

pH	Net charges	pH	Net charges	pH	Net charges
3.50	+242	4.98	0	6.50	−88
3.75	+220	5.25	−36	6.75	−89
4.00	+188	5.50	−58	7.00	−9[illegible]
4.25	+144	5.75	−72	7.25	−92
4.50	+ 92	6.00	−80	7.50	−93
4.75	+ 41	6.25	−85		

collagen. As mentioned earlier at higher pH, it is not clear whether less binding of bis-ANS to collagen is due to the fibril formation of collagen or not.

The effect of chondroitin sulfate on lathyritic rat skin collagen

In order to carry out the experiment in the absence of the fibril formation, lathyritic collagen was used to determine the shielding effect of chondroitin sulfate on the collagen molecules (Fig. 6). The trend in binding of chondroitin sulfate and the decrease in surface hydrophobicity observed in the lathyritic rat skin collagen were similar to those of rat tail collagen at pH 3.5 as shown in Fig. 3. The shielding of hydrophobic sites on lathyritic collagen by chondroitin sulfate could be observed at pH 3.5. Again, the chondroitin sulfate and collagen ratio for the maximum binding was 3.75mole/mole. However, no effect of chondroitin sulfate on shielding hydrophobic site of collagen could be observed at pH 7.4. If it is assumed that there is no fibril formation at pH 7.4 by the lathyritic collagen with chondroitin sulfate, chondroitin sulfate could not bind to collagen probably due to the charge repulsions between chondroitin sulfate and collagen at pH 7.4.

In order to evaluate the binding capacity of bis-ANS as influenced by pH, bovine serum albumin (BSA) was used to measure the fluorescence intensity at pH 3.5 and 7.4 (Fig. 7). There was nearly no effect of pH. The hydrophobicities of BSA or the fluorescence intensities indicated by bis-ANS were similar for both pH 3.5 and 7.5. This implies that the change in the hydrophobicity of collagen measured with bis-ANS is not due to the inability of bis-ANS to bind to protein but can be attributed to particular nature of the collagen molecule.

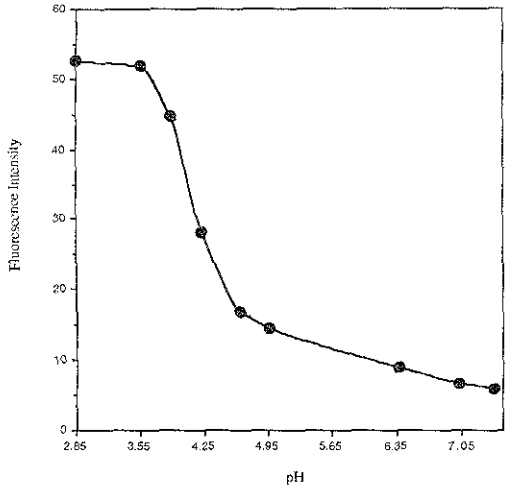

Fig. 5. Binding of bis-ANS to collagen.

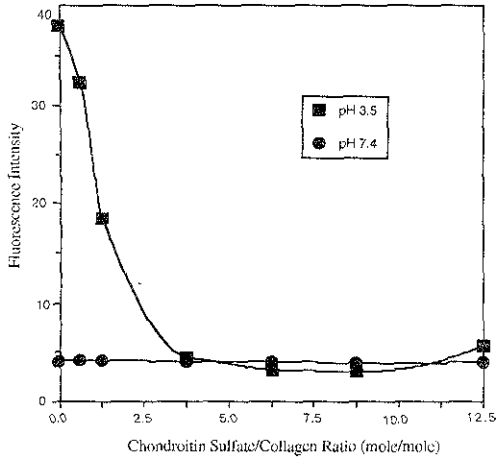

Fig. 6. Shielding of hydrophobic sites of lathyratic rat skin collagen by chondroitin sulfate.

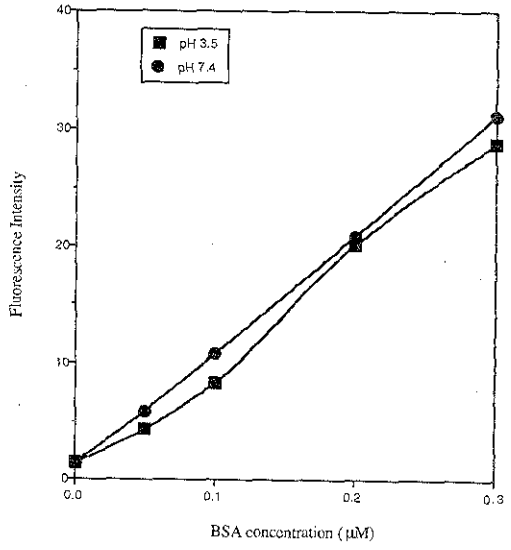

Fig. 7. Effect of pH on fluorescence intensity of bovine serum albumin.

DISCUSSION

In the soluble state of collagen (acidic pH), the shielding effect of chondroitin sulfate could be detected. As a consequence of the interaction of collagen with GAG materials, the hydrophobic sites of collagen are shielded. The consequent change in exposed hydrophobic surface area of collagen can be detected by the relative fluorecence intensity. The hydrophobic probe used in this experiment is bis-ANS as the hydrophobic probe. The fluorescence intensity decreased with the increase in the ratio of chondroitin sulfate to collagen upto 3.75mole/mole. At the chondroitin sulfate to collagen ratio higher than to 3.75 mole-/mole, the fluorescence intensity does not decrease further indicating perhaps saturation of the binding sites in the collagen. The shielding effect of chondroitin sulfate on hydrophobic site is not apparent at higher pH. The relative fluorescence intensity of collagen alone decreases with an increase in pH to the level as low as or lower than that of collagen completely shielded by chondroitin sulfate at pH 6.4. The addition of chondroitin sulfate does not lower the relative fluorescence intensity. Fibrillation occurs at high pH and that may deter the binding and determination of shielding effect of chondroitin sulfate. In order to eliminate the interference by fibrillation, the lathyritic collagen which has the tendency not to form fibers was used. The result with the lathyritic collagen also showed that there was no change in fluorescence intensity, therefore probably there is no hydrophobic site shielding by chondroitin sulfate nor the binding of the bis-ANS to collagen at higher pH. From these result, it can be concluded that bindings of chondroitin sulfate and/or bis-ANS to collagen involve electrostatic interactions. Bis-ANS is used as a hydrophobic probe since it binds to hydrophobic sites and gives fluorescence in conjunction with hydrophobic residues. However, it also contains two sulfonic groups which are negatively charged at the most of pH ranges. The amount of bis-ANS which bind to collagen decreases with the increase in pH due to the fact that the overall charge of collagen is negative at pH above 5.25 (Table 1) and charge repulsion prevents binding of bis-ANS to collagen (Fig. 8). Although there was some fluorescence intensity level observed in colla-

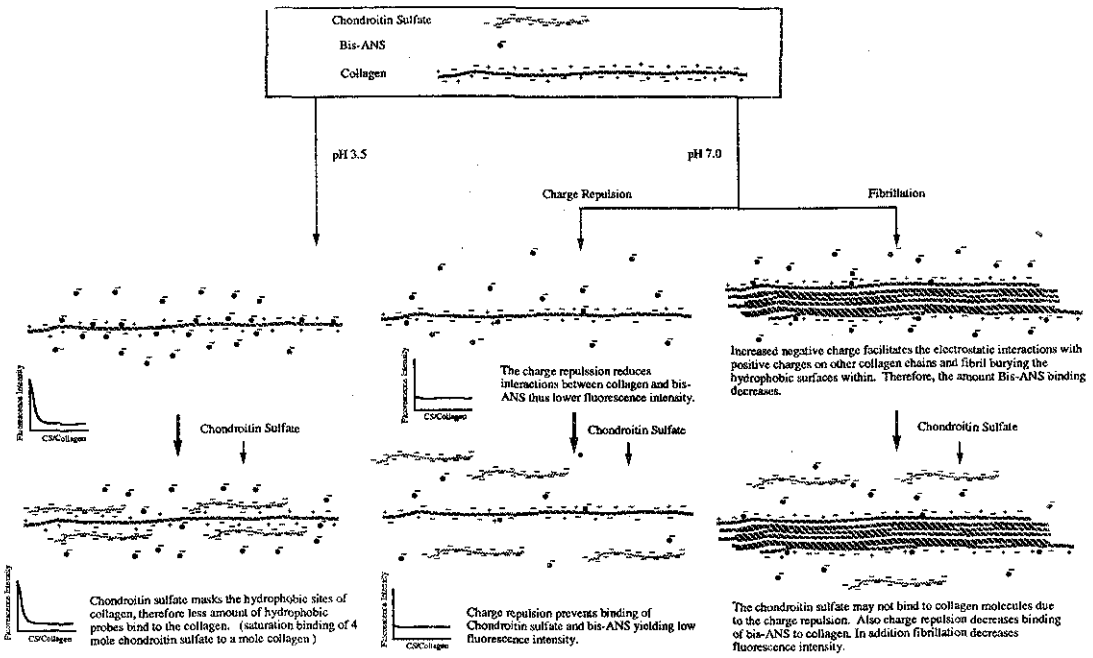

Fig. 8. The fluorescence intensity resulting from the interaction between collagen, chondroitin sulfate and/or bis-ANS.

gen in the absence of chondroitin sulfate at higher pH (higher than 6.0), hydrophobic sites shielding by chondroitin sulfate could not be demonstrated. The fluorescence intensity remained same regardless of the amount of chondroitin sulfate present (Fig. 3). Therefore, there may be no binding between chondroitin sulfate and collagen at higher pH (Fig. 8), and that is most likely due to the charge repulsion. Another possibility is that the fibrillation of collagen occurs at high pH. The fibrillation buries the hydrophobic sites of collagen and prevents the access of bis-ANS to the collagen molecules (Fig. 8). This might cause the decrease in fluorescence intensity of collagen regardless of the interaction with chondroitin sulfates (Fig. 4).

It was difficult to evaluate the hydrophobic sites shielding effect of GAG on collagen at higher pH or physiological pH due to the electrostatic properties. However, there was a clear indication of shielding of hydrophobic sites by GAG when collagen was at a soluble state.

Further experiments should be conducted with a neutral hydrophobic probe such as nile red for the evaluation of charge shielding effect of GAG material on collagen.

CONCLUSIONS

From the results of the determination of fluorescence intensity upon interaction of collagen with chondroitin sulfate using bis-ANS as hydrophobic probe, following conclusions could be made.

Hydrophobic sites of collagen was shielded by the interaction with chondroitin sulfate with collagen in a ratio ranging from 3.75mole/mole at acidic pH. Increasing the ratio of chondroitin sulfate to collagen to over 3.75mole/mole did not change the fluorescence intensity. The maximum binding ratio is 4 molecules of chondroitin sulfate per each collagen molecule.

Low fluorescence intensity at higher pH may be due to the charge repulsion between bis-ANS and collagen. The shielding effect of chondroitin sulfate on hydrophobic sites of collagen was not evident at pH above 6. This lack of effect is probably due to the charge repulsion between chondroitin sulfate and collagen.

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콘드로이친 설페이트에 의한 콜라젠 소수성표면의 변화 : Bis-ANS를 발색단으로 이용한 형광도 측정

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요 약

세포외 구조물질 (Extracellular Matrix, ECM)을 구성하고 있는 성분 중 가장 중요한 성분으로 콜라젠과 콘드로이친 설페이트를 들 수 있다. 콜라젠 분자의 소수성 아미노산 잔기에 의해서 나타내는 소수성 표면이 콜라젠 섬유의 굵기를 결정하는 요인으로 작용되고 있으며 아울러서 노화(aging)와 밀접한 관계를 가지고 있다. 세포외 구조물질의 구성 성분인 콘드로이친 설페이트와 콜라젠의 상호 작용에 의해 콜라젠 섬유의 굵기를 조절하는 기작을 소수성 발색단인 bis-ANS를 이용하여 측정하였다. 콘드로이친 설페이트에 의해서 가리워지는 콜라젠의 소수성의 표면적을 소수성 발색단으로 bis-ANS를 사용한 상대 형광도 측정으로 분석할 수 있었다. 콘드로이친 설페이트 첨가시 콜라젠의 상대 형광도는 콘드로이친 설페이트/콜라젠의 비가 3.8이 될 때 까지 감소하였다. 이 이상의 콘도로이친 설페이트의 농도 증가는 콜라젠의 상대 형광도에 아무런 영향도 미치지 못하였다. Rat tail 및 lathyritic rat skin 콜라젠에서도 비슷한 결과를 얻을 수 있었다. 콜라젠의 소수성 표면과 bis-ANS의 결합에 의해서 나타내어지는 상대 형광도는 pH에 의존적이었다. pH 6 이상의 콜라젠 용액에서 콘드로이친 설페이트에 의한 콜라젠 분자의 소수성 표면 shielding 효과를 확인할 수 없었다. 이는 높은 pH에서 콜라젠 분자의 음전하에 의한 반발력에 기인하는 것으로 추측할 수 있다.