

REPORT

# Effect of Immobilization on Solubility of Soleus and Gastrocnemius Muscle Collagen

## —Biochemical Studies on Collagen from Soleus and Gastrocnemius Muscles of Rat—

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**Abstract.** We have been studying mainly the changes in collagen fiber solubility with respect to its influence on immobilization of the soleus muscle and Achilles tendon of rats. We decided to investigate also the change of the collagen fiber solubility in the gastrocnemius muscle which, like the soleus muscle, is assumed to influence the range of motion of the ankle joints. The purpose of this study, therefore, was to investigate the effects of a 7-week immobilization on the solubility of the gastrocnemius muscle and soleus muscle collagen fiber of rats. The results were that in a 7-week immobilization period, hydroxyproline concentration in tissue was increased and salt and pepsin soluble collagen was decreased in both the soleus and the gastrocnemius muscles. The results suggest an increase in the collagen concentration in tissue and an increase in the intermolecular cross-link with a stronger molecular structure. As far as the amount of collagen and solubility were concerned, the immobilization had a similar influence on the collagen fiber in both muscles.

**Key words:** immobilization, collagen solubility, soleus and gastrocnemius muscles

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Joint contractures after a long period of immobilization are a common problem for physical therapists. As for research on joint contracture, mainly histological and biochemical studies have been performed. In the biochemical study of joint contracture, it is considered that changes in the collagen fiber in the soft tissue around the joint are related to joint contracture and that an increase in collagen in the tissue and changes in the cross-link formed in the collagen fiber lead to a decrease in tissue flexibility<sup>1,2</sup>.

This cross-link contributes as an important factor to collagen fiber solubility. It is generally recognized that the collagen fiber which is solubilized by neutral salt contains a small quantity of cross-link, and that the collagen fiber which contains an intramolecular cross-link is primarily solubilized by acid. Also, the collagen fiber which contains an

intermolecular cross-link becomes insoluble collagen which is not solubilized by either salt or acid solution.

We have been mainly studying the changes in collagen fiber solubility with regard to its effect on immobilization of the soleus muscle and Achilles tendon of rats<sup>3-5</sup>. We reported<sup>3</sup> that the rate of collagen in tissue was increased significantly by a 3-week immobilization, and that salt soluble collagen and the pepsin solubilization rate of insoluble collagen was decreased significantly by a 3-week immobilization. In view of this, we suggested that there was an increase in the amount of collagen in tissue, abnormal changes in cross-link, and the possibility of a cross-link having developed with a stronger molecular structure<sup>3,5</sup>.

On the other hand, with regard to the Achilles tendon, we reported<sup>4</sup> that the amount of collagen in tissue had not changed significantly after a 7-week immobilization, and the change in collagen solubility was less than that in the soleus muscle after a 7-week immobilization. We suggested that a 7-week immobilization had little effect on the Achilles tendon collagen fiber, as compared with the soleus muscle

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collagen fiber.

Then we further investigated the change in the collagen fiber solubility in gastrocnemius muscle which, like the soleus muscle, is assumed to influence the range of motion of ankle joints. The purpose of this study was to investigate the effects of a 7-week immobilization on the solubility of the gastrocnemius muscle and soleus muscle collagen fiber of rats.

## Materials and Methods

### 1. Materials

Six male Wistar rats (weight  $221.0 \pm 6.0$  g), aged eight weeks, were used for this study. We immobilized the hind limb joints for 7-weeks. After anesthetizing the rats with sodium pentobarbital, we immobilized the left ankle joint in a neutral position with a cast, exposing the distal foot in order to observe the existence of edema. Next, we put the trunk of each rat in a cast enhancing the immobilization of the leg joints. The right hind limb was left free to serve as a non-immobilized group (Fig. 1). After the casting, the

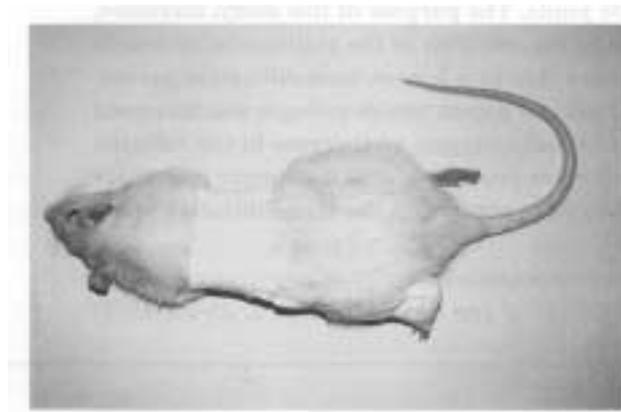


Fig. 1 Immobilization of rat hind limb

rats were able to move in the cage by using both front limbs and the right hind limb, to drink water and eat food freely. When a loose cast or edema on the fixed limb was observed, the cast was reapplied.

On completion of the immobilization period, the rats were anesthetized with sodium pentobarbital again and the soleus and gastrocnemius muscles on the immobilized and non-immobilized sides were extracted. At that time, the musculotendinous junction was excluded as much as possible, preventing mixture with the collagen fiber of the tendon tissue.

For experimental purposes the muscles extracted from the immobilized side were classified as the immobilized group, and muscles extracted from the non-immobilized side were classified as the non-immobilized group.

### 2. The Solubilization of the Collagen Fiber (Fig. 2)

By using the method of Fujii *et al.*<sup>6)</sup>, we first isolated and prepared collagen from the soleus and gastrocnemius muscle tissue to obtain the muscle collagen fiber. The collagen fiber was then solubilized. To summarize the details of the method: first, 24-hour stirring and extraction were done twice with 1.0M NaCl in 0.05M Tris-HCl buffer pH 7.4, and the extracted solution was isolated by centrifugation. The supernate was collected as neutral salt soluble collagen. Next, 0.1M acetic acid was added to the sediment and the same stirring and extraction procedure was performed. After extraction and centrifugation, the supernate was collected as the acid soluble collagen and the sediment as the insoluble collagen.

### 3. Pepsination of the Insoluble Collagen (Fig. 2)

The insoluble collagen, which was not solubilized by salt and acid solution, was pepsinated in 0.5M acetic acid. Pepsin digestion was carried out by stirring for 48 hours with a magnetic stirrer. This solution was isolated by

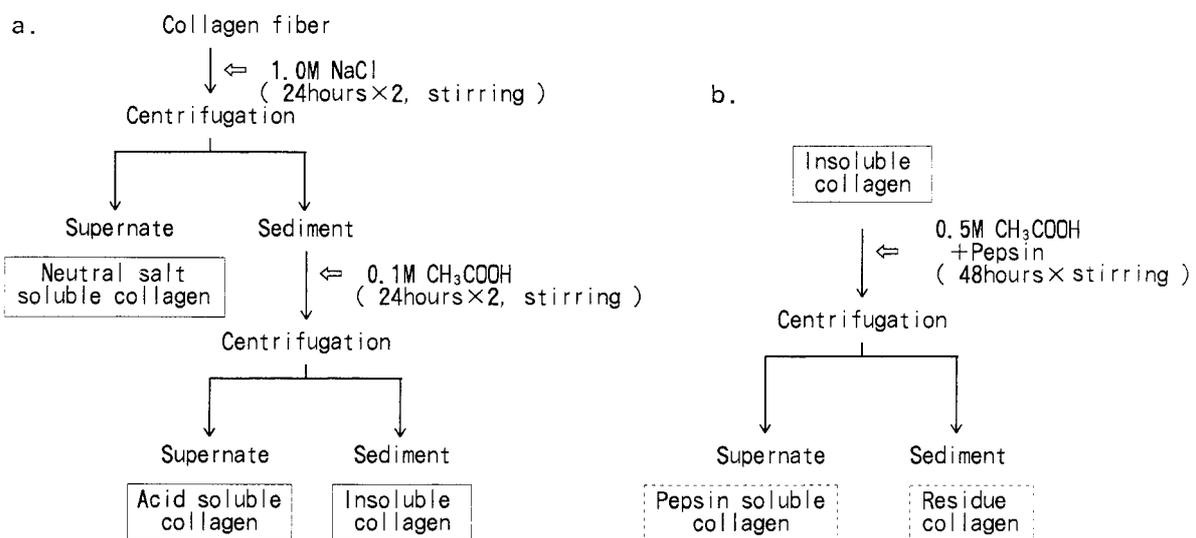


Fig. 2 The solubilization of collagen fiber (a) and the pepsination of the insoluble collagen (b)

**Table 1** Muscle wet weight

	Soleus		Gastrocnemius	
	Non-immobilization	Immobilization	Non-immobilization	Immobilization
Muscle wet weight (mg)	119 ± 7	64 ± 16	1672 ± 73	1171 ± 73

Values are means ± SD. \*: p<0.05.

**Table 2** Amount of hydroxyproline

	Soleus		Gastrocnemius	
	Non-immobilization	Immobilization	Non-immobilization	Immobilization
Amount of hydroxyproline (µg)	138 ± 27	151 ± 25	3127 ± 149	2877 ± 284
Amount of hydroxyproline per muscle wet weight (%)	0.12 ± 0.03	0.24 ± 0.04	0.19 ± 0.01	0.25 ± 0.03

Values are means ± SD. \*: p<0.05.

centrifugation and the supernate was collected as the pepsin soluble collagen, and the sediment as the residue collagen.

In order to prevent degeneration of the collagen, the above experiments were carried out at a temperature of 4°C or lower.

#### 4. Determination of Amount of Collagen

Hydroxyproline, an amino acid which is a characteristic component of collagen, was determined by the method of Inayama *et al.*<sup>7)</sup> for the estimation of the collagen content. To summarize the method of Inayama: after oxidation of hydroxyproline to pyrrole by chloramine-T, pyrrole was condensed with Ehrlich's reagent, thus producing colors. Using a spectrophotometer, the absorbance at 560 nm of colored solution was measured, and the content of hydroxyproline in the sample solution was estimated from the absorbance.

#### 5. The Amount of Total Hydroxyproline

The amount of total hydroxyproline in the obtained tissues consisted of hydroxyproline of neutral salt soluble collagen, acid soluble collagen, pepsin soluble collagen, and residue collagen. It was also calculated as a percentage of the muscle wet weight.

#### 6. The Ratios of Soluble and Insoluble Collagen as Percentages of Total Collagen

The percentages of neutral salt soluble, acid soluble, and insoluble collagen in relation to total collagen were indicated by the hydroxyproline of each sample in relation to the total hydroxyproline. The amount of hydroxyproline in insoluble collagen was total hydroxyproline in the pepsin soluble collagen and the residue collagen.

#### 7. Pepsin Solubilization

Pepsin solubilization ratios of insoluble collagen were

indicated by the percentages of the amount of hydroxyproline in the pepsin soluble collagen relative to the amount of hydroxyproline in the insoluble collagen.

#### 8. Data Analysis

The data were analyzed by independent sample *t*-test. In all statistical analyses, a level of p<0.05 was required for significance.

## Results

#### 1. Muscle Wet Weight (Table 1)

The soleus muscle wet weight was 119 ± 7 mg in the non-immobilized group and 64 ± 16 mg in the immobilized group. The gastrocnemius muscle wet weight was 1672 ± 73 mg in the non-immobilized group and 1171 ± 73 mg in the immobilized group. The soleus muscle wet weight and gastrocnemius muscle wet weight of the immobilized group was decreased significantly by the 7-week immobilization.

#### 2. The Amount of Total Hydroxyproline in Tissue (Table 2)

In both the soleus muscle and the gastrocnemius muscle, there was no significant difference between the non-immobilized group and the immobilized group in the amount of total hydroxyproline. The hydroxyproline concentration in the soleus muscle was 0.12 ± 0.03% in the non-immobilized group and 0.24 ± 0.04% in the immobilized group, and in the gastrocnemius was 0.19 ± 0.01% in the non-immobilized group and 0.25 ± 0.03% in the immobilized group. In both the soleus muscle and the gastrocnemius muscle, the hydroxyproline concentration was increased significantly by the 7-week immobilization.

#### 3. Ratios of Neutral Salt Soluble Collagen in Relation to Total Collagen (Table 3)

**Table 3** Solubility of collagen fiber

	Soleus		Gastrocnemius	
	Non-immobilization	Immobilization	Non-immobilization	Immobilization
Relation to the total collagen (%)				
Salt soluble collagen	1.6 ± 0.7	0.8 ± 0.4	5.9 ± 0.6	4.3 ± 0.8
Acid soluble collagen	18.9 ± 2.9	16.9 ± 2.2	14.0 ± 3.3	12.7 ± 1.7
Insoluble collagen	79.5 ± 3.1	82.3 ± 2.4	80.1 ± 3.8	82.9 ± 2.0

Values are means ± SD. \*:p<0.05.

In both the soleus muscle and the gastrocnemius muscle, the neutral salt soluble collagen of the immobilized group was decreased significantly by the 7-week immobilization. In the soleus muscle, the percentage of neutral salt soluble collagen was 1.6 ± 0.7% in the non-immobilized group and 0.8 ± 0.4% in the immobilized group. In the gastrocnemius muscle, it was 5.9 ± 0.6% in the non-immobilized group and 4.3 ± 0.8% in the immobilized group.

#### 4. Ratios of Acid Soluble Collagen in Relation to Total Collagen (Table 3)

In the soleus muscles, the percentage of acid soluble collagen in the non-immobilized group was not significantly different from that in the immobilized group.

In the gastrocnemius muscle, there was no significant difference between the non-immobilized and immobilized groups.

#### 5. Ratios of Insoluble Collagen in Relation to Total Collagen (Table 3)

In the soleus muscle, the percentage of the insoluble collagen was not significantly different in the non-immobilized and immobilized groups.

In the gastrocnemius muscle, there was no significant difference between the non-immobilized and immobilized groups.

#### 6. Pepsin Solubilization (Table 4)

In both the soleus muscle and the gastrocnemius muscle, the percentage of solubilized collagen fiber, when the insoluble collagen was pepsinated, was decreased significantly by a 7-week immobilization. In the soleus muscle, the pepsin solubilization was 76.1 ± 5.1% of insoluble collagen in the non-immobilized group and 61.0 ± 4.2% in the immobilized group. In the gastrocnemius muscle, it was 47.6 ± 2.4% of insoluble collagen in the non-immobilized group and 40.5 ± 6.9% in the immobilized group.

## Discussion

Our results indicated that the amount of hydroxyproline in the soleus and gastrocnemius muscle in the immobilized

**Table 4** Pepsin solubilization rates (%)

	Non-immobilization	Immobilization	
Soleus	76.1 ± 5.1	61.0 ± 4.2	p<0.05
Gastrocnemius	47.6 ± 2.4	40.5 ± 6.9	p<0.05

Values are means ± SD.

and non-immobilized groups did not change significantly over a 7-week period. Converting this to the amount of hydroxyproline per wet weight of muscle, the soleus and the gastrocnemius muscles had a significant increase in the immobilized group. These results suggest an increase in the collagen concentration in tissue. We considered that the increase in the collagen concentration in muscle tissue was caused by decreased wet weight of muscle, because there was no significant change in the amount of hydroxyproline in the soleus and gastrocnemius muscle, while the muscle wet weight decreased significantly.

With regard to the relationship between the elasticity of soft tissue and the collagen fiber, Woo *et al.*<sup>8)</sup> pointed out that a cross-link formed in the collagen fiber decreased the flexibility of the tissue. This cross-link is an important factor in collagen fiber solubility. It is generally recognized that the collagen fiber which is solubilized by neutral salt has a small quantity of cross-link, and that the collagen fiber which has an intramolecular cross-link is primarily solubilized by acid, and that the collagen fiber which has an intermolecular cross-link becomes insoluble collagen<sup>9)</sup>. Considering the above, we estimated changes in collagen fiber solubility for the purpose of estimating the cross-link change. The results showed that the salt soluble collagen in soleus and gastrocnemius muscles was decreased significantly by a 7-week immobilization. These results indicate that the collagen fiber with few cross-links had decreased while the collagen fiber with a larger number of cross-links had increased after the 7-week immobilization.

With regard to the physical stability of the collagen fiber, it is considered that the intermolecular cross-link has a closer relationship to physical stability than the intramolecular cross-link. It has become clear that a cross-link in organisms tends to promote the development of a stronger and more stable molecular structure<sup>10)</sup>. Also, as a more stable cross-

link is formed, the resistance to enzymes will increase, and the collagen fiber solubilized by pepsin digestion decreases<sup>11)</sup>. In view of this we carried out pepsin digestion of the insoluble collagen for the purpose of estimating the strength of the intermolecular cross-link. The pepsin solubilization rate in the immobilized group was found to be decreased significantly, in both the soleus and gastrocnemius muscles. It was therefore presumed that an intermolecular cross-link with a stronger molecular structure had begun to form in the collagen fiber in both soleus and gastrocnemius muscles after the 7-week immobilization.

Comparing the gastrocnemius muscle to the soleus muscle, the soleus muscle has more Type I fiber and the gastrocnemius muscle has more Type II fiber<sup>12)</sup>. In the case of disused muscle atrophy with joints immobilized, Herbinson *et al.*<sup>13)</sup> reported that the degree of muscle atrophy depended on the muscle fiber type.

With regard to the change in collagen with immobilization, Savolainen *et al.*<sup>14)</sup> reported that a much greater specific activity of prolyl 4-hydroxylase was observed in the soleus muscle than in the gastrocnemius muscle, and greater prolyl 4-hydroxylase activity relative to collagen suggests a greater fractional turnover rate for collagen. Actually, their results indicated a much greater decrease in prolyl 4-hydroxylase activity in the soleus muscle than in the gastrocnemius muscle after immobilization. Our results indicated that there was no difference between the soleus muscle and the gastrocnemius muscle in terms of changes in the amount of collagen and the collagen fiber solubility with joints immobilized. But this study only investigated the change in the amount of hydroxyproline and collagen solubility in the soleus and gastrocnemius muscles, therefore we could not determine exactly how much the rate of collagen synthesis and degradation had changed. As Savolainen *et al.*<sup>14)</sup> reported that the rate of collagen synthesis and degradation is changed by immobilization, we need to consider the synthesis and degradation of collagen fiber as well as the amount of collagen and collagen solubility in future.

In conclusion, in a 7-week immobilization period, the collagen concentration in tissue was increased and salt soluble and pepsin soluble collagen was decreased, in both the soleus and gastrocnemius muscles. As far as the amount of collagen and solubility was concerned, the immobilization had a similar influence on the collagen fiber in both types of muscle.

It is considered that the change in collagen fiber is one of the most important factors influencing the flexibility of tissue, and that these basic experiments will be helpful in the study of physical therapy and its influence on joint contracture caused by immobilization.

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