# A Study of Implantable EGCG Formulated Collagen Bioprosthetic Material

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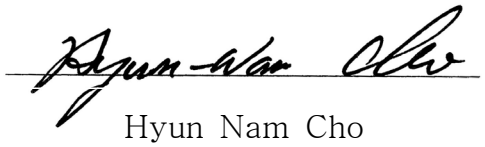

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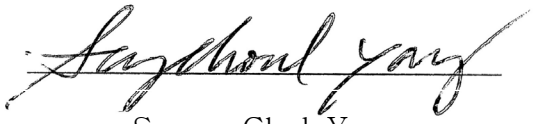

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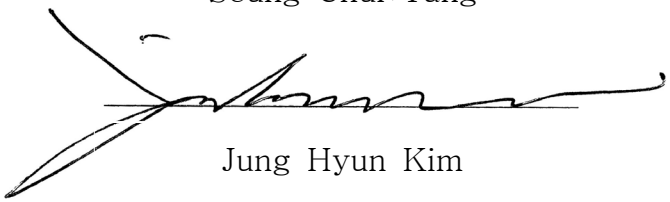

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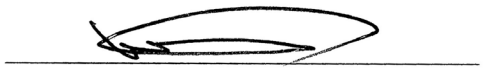

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# Abbreviations

Col : collagen

dCol : 37 ℃ incubated collagen

dCol-RGD : RGDS treated fibroblasts culture on 37 ℃ incubated collagen

Col+RGD : RGDS treated fibroblasts culture on collagen

E+col : EGCG treated collagen

dE+Col : 37 ℃ incubated EGCG treated collagen

dE+Col-RGD : RGDS treated fibroblasts culture on 37 ℃ incubated EGCG treated collagen

E+Col-RGD : RGDS treated fibroblasts culture on EGCG treated collagen

S-Col : succinylated collagen

E+S-Col : EGCG treated succinylated collagen

EGCG : (-)-epigallocatechin-3-gallate

C : (+)-catechin

EC : (-)-epicatechin

GC : (+)-gallocatechin

EGC : (-)-epigallocatechin

ECG : (-)-epicatechin-3-gallate

GCG : (-)-gallocatechin-3-gallate

HA : hyaluronic acid

ECM : extracellular matrix

DMEM : Dulbecco's modified Eagle's medium

FBS : fetal bovine serum

PBS : phosphate buffered saline

EDTA : ethylenediaminetetraacetic acid

MMP : matrix metalloproteinase

SDS : Sodium dodecyl sulphate

CD : circular dichroism

ELISA : enzyme-linked immunosorbent assay

GAPDH : glyceraldehydes-3-phosphate dehydrogenase

# Abstract

## A Study of Implantable EGCG Formulated Collagen Bioprosthetic material

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Collagen is regarded as one of the most useful biomaterials. The excellent biocompatibility and safety due to its biological characteristics, such as biodegradability and weak antigenicity, made collagen the primary source in biomedical application. In addition to its biological function, because collagen has the ability to persisting the body without developing a foreign body response that could lead to premature rejection, it has been extensively investigated as a biomaterial for artificial skin, tendons, blood vessels, cartilage, and bones.

Collagen has been widely used in the crosslinked form to extend the durability of collagen for the implantable bioprosthesis. The chemical treatment influences the structural integrity of collagen molecule resulting in the loss of triple helical characteristic. The structural characteristic of collagen is importantly related to it's biological function for the interaction with cell.

In this study, structural stability of collagen was enhanced through EGCG treatment, that resulting in high resistance against degradation by bacterial collagenase and mammalian collagenase MMP-1, which was confirmed by collagen zymography. The triple helical structure of EGCG

treated collagen could be maintained at 37 ℃ of physiological temperature in comparison with collagen, which confirmed by CD spectra analysis, and EGCG treated collagen demonstrated a high free radical scavenging activity. Also, in the fibroblasts-culture on EGCG treated collagen, its structural stability of EGCG treated collagen provided a favorable support for cell functions in cell adhesion, actin filament expression, and cell proliferation. EGCG treated collagen was less susceptible to macrophage adhesion than collagen, and there was no significant difference in macrophage adhesion between EGCG treated collagen and collagenase treated EGCG treated collagen. In addition, the toxicity test, such as intradermal test, sensitization test, genetic toxicity test, and subacute toxicity test, of EGCG treated collagen bioprosthesis using animal model did not show toxicity. From the intradermal test, skin response such as erythema and edema was not visualized after the subcutaneous injection of EGCG treated collagen bioprosthesis from the naked eye observation, and EGCG treated collagen bioprosthesis did not exhibit specific histological indications such as inflammatory cell infiltration and fibrosis, comparison with the negative control. In case of the sensitization test, EGCG treated collagen bioprosthesis and negative control, both the fraction response (FR) (positive number of animals/total number of animals) and the mean response (MR) (summation of score/total number of animals) after the challenge phase was zero, which indicates no sensitization. The Ames test of genetic toxicity test showed that the EGCG treated collagen bioprosthesis did not induce mutagenicity, in spite of false positive reaction owing to native character of collagen. And, there was no subacute toxicity on EGCG treated collagen bioprosthesis.

These observations underscore the needs of native, triple helical collagen conformation as a prerequisite for integrin mediated cell adhesion

and functions. According to this experiment, EGCG treated collagen assumes to provide a practical benefit to resist the degradation by collagenase retaining its structural characteristic, and could be an attractive biomaterial, especially for soft tissue augmentation.

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Key words: collagen; EGCG treated collagen; bioprosthesis; collagenase resistance; free radical scavenging; triple helix structure

# 1. Introduction

## 1.1. Structure of Skin

In the view of surface area and weight, the skin is the largest organ in the mammalian body. In adult, it ranges in area about from 2 to 2.5 $m^2$ and in weights from 4.5 to 5 kg, about 16% of total body weight. It covers an thickness from 0.5 (on the eyelids) to 4.0 mm (on the heels). However, and is 1~2 mm thick over most of the body.

Skin consists of different tissues that are joined to perform specific functions. Two parts, epidermis and dermis, are principal in consisting skin, and deep to the dermis is the subcutaneous layer, or hypodermis which is not part of the skin as shown in figure 1-1.

Epidermis, the superficial and thinner part of skin, consists of keratinized stratified squamous epithelium that contains four types of cells such keratinocytes, melanocytes, Langerhans cells, and Merkel's cells. Keratinocytes, about 90 % of epidermal cells, produce not only keratin protein that helps protect the skin and underlying tissues from heat, microbes, and chemicals but also lamella granules, which release a waterproofing sealant. Melanocytes, about 8 % of the epidermal cells, produce the pigment melanin that contribute skin color and absorbs damaging ultraviolet (UV) light. Langerhans cells, small portion of the epidermal cells, participate in immune responses mounted against microbes invaded skin. Merkel cells, the least numerous epidermal cells, function in sensing of touch with sensory neuron.

Several distinct layers of keratinocytes are in various stages of

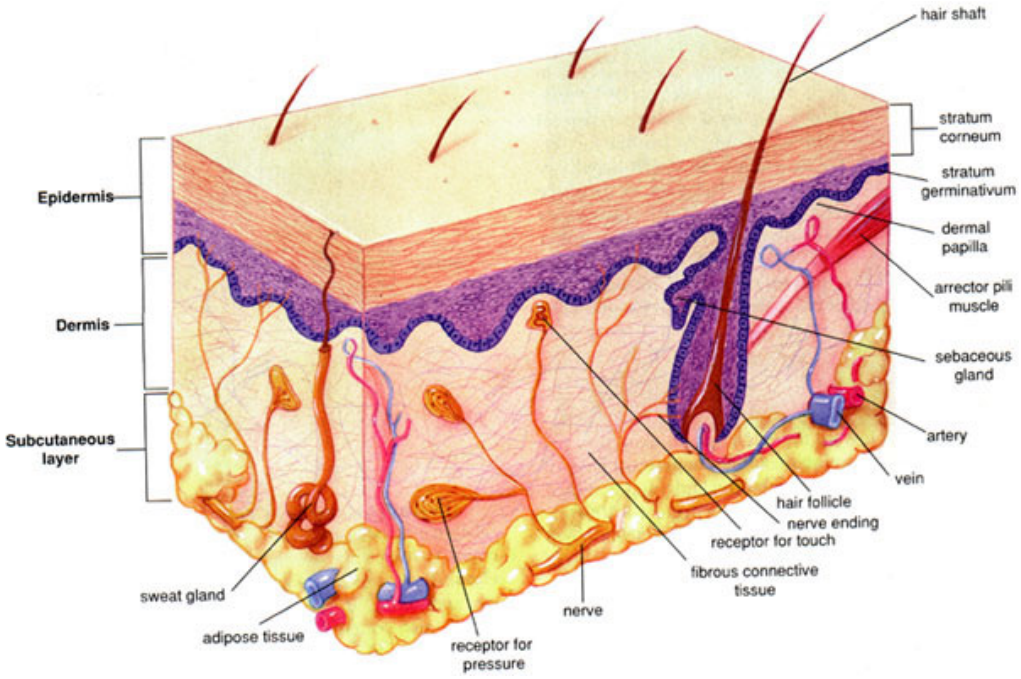



Figure 1-1. The structure of skin. The skin consists of athin superficial epidermis and a deep, thicker dermis. Deep to the skin is the subcutaneous layer, which attaches the dermis to underlying organs and tissue.

development form the epidermis. In most regions of the body the epidermis has five strata (layers) of stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and a thin stratum corneum. The description of epidermal strata is shown in table 1-1.

Dermis, the second deeper part of skin, is composed mainly of connective tissue containing collagen and elastic fibers. The few cells present in the dermis include fibroblasts, macrophages, and some adipocytes. Blood vessels, nerves, glands, and hair follicles are embedded in dermal tissue. Based on its tissue structure, the dermis can be divided into a superficial papillary region and a deeper reticular region. The description of dermal regions is shown in table 1-2.

## 1.2. Collagen

Collagen is the major structural component of connective tissues. In vertebrates, it represents about one-third of their total protein contents [1, 2]. About one half of the total body collagen is in the skin and about 70 % of the material other than water present in dermis of skin and tendon is collagen. The structure and function of collagen have been studied by many researchers [3-8].

Collagen occurs in a variety of macroscopic forms in tissues as diverse as skin, tendon, blood vessels, cartilage, bone, cornea, vitreous humor, and basement membrane. And it presents in the framework and interstices of all other tissues and organs with the exception of blood, lymph, and the keratinous tissues [9]. In most cases the prime role of the collagen is to provide the tissue with its structural integrity [10, 11]. This property is due to its unique molecular conformation which is bestowed upon it by the

Table 1-1. The description of epidermal strata.

| Stratum | Description |
|---|---|
| Basale | Deepest layer, composed of single row of cuboidal or columnar keratinocytes that contain scatters tonofilaments (intermediate filaments). Stem cells undergo cell division to produce new keratinocytes, melanocytes, Langerhans cells, and Merkel cells. |
| Spinosum | Eight to ten rows of polyhedral keratinocytes with bundles of tonofilaments |
| Granulosum | Three to five rows of flattened keratinocytes in which organelles are beginning to degenerate. |
| Lucidum | Present only in skin of fingertips, palms, and soles, consists of three to five rows of clear, flat, dead keratinocytes with large amounts of keratin. |
| Corneum | Twenty-five to thirty rows of dead, flat keratinocytes that contain mostly keratin. |

Table 1-2. The description of dermal regions.

| Region | Description |
|---|---|
| Papillary | The superficial portion of the dermis (about one-fifth); consists of areolar connective tissue with elastic fibers; contains dermal papillae that house capillaries, corpuscles of touch, and free nerve endings. |
| Reticular | The deeper portion of dermis (about four-fifths); consists of dense irregular connective tissue with bundles of collagen and come coarse elastic fibers. Spaces between fibers contain some adipose cells, hair follicles, nerves, sebaceous glands, and sudoriferous glands. |

regular repeating units in the amino acid sequence, the highly specific alignment and packing of the molecules, and the axial and lateral cohesion afforded by the formation of intermolecular covalent crosslinking [11-15].

Among various types of collagen, type I collagen is the one most abundant, and has been frequently used as biomaterials because of its biological properties [16].

Type I collagen has been applied to soft tissue augmentation to overcome the limitations of synthetic biomaterials, such as injectable paraffin and silicone fluid, which have been associated with permanent undesirable textural changes of the skin and migration from the implantation site [17].

## 1.3. Collagenase resistant collagen for soft tissue augmentation

The anatomical structures of all soft tissues in the human body are maintained by an extracellular matrix containing protein such as collagen, elastin, and polysaccharides in glycosaminoglycan.

Conventionally, soft tissues anatomically damaged by inherited or acquired factors have been repaired by using a synthetic polymer as a bioprosthesis. A representative synthetic polymer is a silicone. A solid silicone with a molecular weight of 300,000 or more is mainly used. Occasionally, a methylmethacrylate polymer is also used. The bioprosthetic compound of silicone and methylmethacrylate polymer is highly biocompatible with the human body. However, they remain as permanent foreign substances in the human body and thus tend to undergo fibrosis

[18].

Meanwhile, there is a method for injecting only extracellular matrix components such as collagen and hyaluronic acid into the human body to encourage incorporation into auto-tissues. However, the injected collagen is degraded due to collagenase activity and the hyaluronic acid is also degraded with changes of the cell cycle of the surrounding tissues. As a result, the collagen and hyaluronic acid disappear within about six months after the injection. Therefore, re-injection becomes necessary.

Generally, collagen, as an extracellular matrix component, plays an important role in maintaining tension and flexibility of tissues due to its high water-binding property, as well as in serving as a structure which induces cellular infiltration. Because collagen is easily degraded by collagenase, its durability as a matrix structure to tissue cells is not long and thus it is difficult to expect an efficient tissue regeneration [18, 19].

In order to solve these problems, the crosslinking of collagen molecules was introduced [20]. However, in case of the of injection using a syringe, the crosslinked collagen molecules are injected in coagulated states, thereby causing pains.

For this reason, inhibitors for preventing the degradation of collagen by collagenase have been developed. For example, it was reported that ethylene diamine tetraacetate (EDTA) and dithiothreitol serve as inhibitors against the degradation of collagen by collagenase. In addition, it was reported that collagen or elastin crosslinked by flavonoid, a type of polyphenols, in the presence of a copper ion as a catalyst, is resistant to the activity of collagenase or elastinase [21, 22].

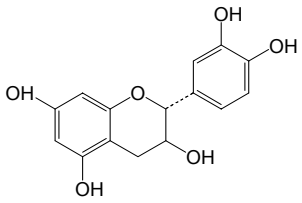
Recently, it was reported that some kinds of catechins such as (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epigallocatechin (EGC) have the activity of inhibiting collagen degradation and an antioxidative property

[23-26]. And it has been demonstrated that ECG and EGCG have the ability to inhibit the activities of some MMPs that are essential in the development and metastasis of cancer [27, 28]. The point that EGCG and ECG are different from other catechins is their having flexible galloyl rings on their backbone. The EGCG, a major component of green tea catechins, promotes stabilization of the collagen molecule when applied. However, the precise mechanisms for the inhibition of collagenase activity are not clear.

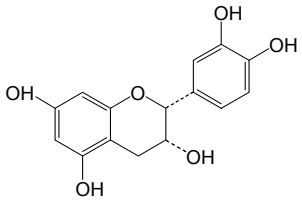
## 1.4. EGCG [(-)-Epigallocatechin-3-gallate]

Green tea is the unprocessed dried young leaves of *Camellia sinensis*, also known as *Thea sinensis L.* and is widely used as a beverage in oriental countries, including Korea, Japan, North America, and Europe [29, 30]. Polyphenols in green tea including catechins have strong biological and pharmacological activities, and also play important roles in metabolism and defense mechanism of plants [31-34].

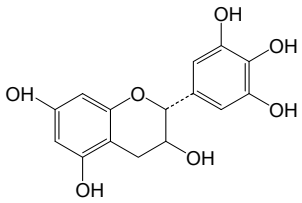
It was recently suggested that green tea has antimutagenic and anticarcinogenic properties which provide protection against various types of cancer. Yoshizawa et al. first reported that topical application of EGCG inhibited teleocidin-induced tumor promotion in the skin of mice previously initiated by 7, 12-dimethylbenz anthracene in 1987 [35]. And many researchers have studied biological and pharmaceutical activities of EGCG, such as the anticalcinogenic activity [36-39], inhibitory activity on the carcinogen-induced tumors of various organs in animals, including the duodenum [40], stomach [41-43], lung [44, 45], esophagus [46, 47], skin [35], and colon [48, 49], antioxidative activity [50-53], inhibitory activity of bacterial mutation [54], inhibition of HIV reverse transcriptase activity



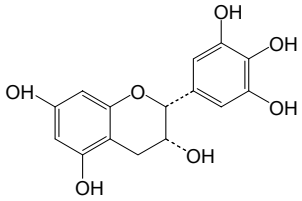

(+)-Catechin (C)



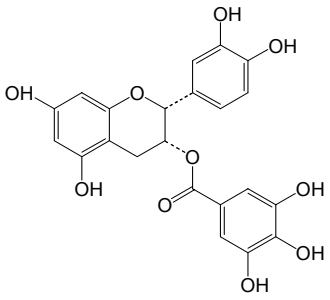

(-)-Epicatechin (EC)



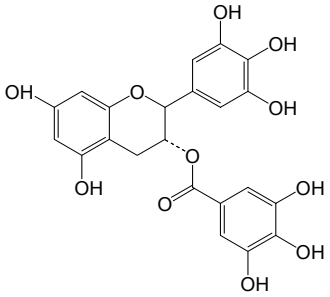

(+)-Gallocatechin (GC)



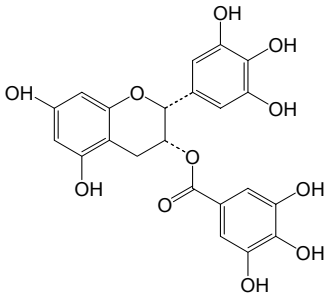

(-)-Epigallocatechin (EGC)




(-)-Epicatechin-3-gallate (ECG)




(-)-Epicatechin-3-gallate (GCG)




(-)-Epigallocatechin-3-gallate (EGCG)

Figure 1-2. The chemical structure of green tea polyphenols.

Table 1-3. The physicochemical properties of green tea polyphenols.

| Name | Molecular formula | Molecular weight | Melting point | Optical rotation |
|---|---|---|---|---|
| C | $C_{15}H_{14}O_6$ | 290 | 176 ℃ | $[a]_{D+}$18° |
| EC | $C_{15}H_{14}O_6$ | 290 | 242 ℃ | $[a]_{D-}$69° |
| GC | $C_{15}H_{14}O_7$ | 306 | 188 ℃ | $[a]_{D+}$15° |
| EGC | $C_{15}H_{14}O_7$ | 306 | 218 ℃ | $[a]_{D-}$50° |
| ECG | $C_{22}H_{18}O_{10}$ | 442 | 253 ℃ | $[a]_{D-}$177° |
| GCG | $C_{22}H_{18}O_{11}$ | 458 | 216 ℃ | $[a]_{D-}$179° |
| EGCG | $C_{22}H_{18}O_{11}$ | 458 | 254 ℃ | $[a]_{D-}$190° |

[55], anticaries activity [56], inhibitory activity of angiogenesis [57, 58], tyrosinase inhibition [59], antithrombotic activity [60], and deodoring activity [61-63].

(-)-Epigallocatechin-3-gallate (EGCG) is the one of the green tea polyphenols which are composed of seven kinds of catechin [56], (+)-Catechin (C), (-)-Epicatechin (EC), (+)-Gallocatechin (GC), (-)-Epigallocatechin (EGC), (-)-Epicatechin-3-gallate (ECG), (-)-Gallocatechin-3-gallate (GCG), and (-)-Epigallocatechin-3-gallate (EGCG). The chemical structure and some physicochemical properties such as molecular formula, molecular weight, melting point, and optical rotation of the green tea polyphenols are in figure 1-2 and table 1-3.

Because EGCG is a major bioactive constituent among green tea polyphenols, special interest has been directed to it. In 2000, Demeule M. et. al reported that green tea polyphenols and one of its constituents EGCG caused a strong inhibition of the gelatinolytic activities of MMP-2 and MMP-9 and of the elastinolytic activity of MMP-12 [64]. And R. Kuttan et. al reported that (+)-catechin treated collagen became resistant to the mammalian collagenase [65]. Other many researches have demonstrated that materials with high level of hydroxyl group like sugar could stabilize collagen molecule through hydrogen bonding with the collagen backbone [66, 67]. There are lots of hydroxyl groups in the backbone of green tea polyphenols, especially EGCG. And polyphenols have a significant affinity to extended proteins and peptides that contain a high proportion of proline residues in their sequences [68]. From these points, EGCG has been recognized as having a potential on the stabilization of collagen molecule.

The collagenase resistant collagen in this study is from structural stabilization by using EGCG that maintains its native biological functions

but provides enhanced anti-oxidation activity.

## 1.5. Purpose of This Study

Collagen, the most abundant protein in the human body, has been widely investigated as an attractive molecule for manufacturing biomaterials owing to its unique structural and functional characteristics. In addition, collagen was the one most frequently used out of the various components of extracellular matrix which maintained the anatomical structures of all soft tissues in the human body. Conventionally, bioprosthetic materials used to repair soft tissues damaged by inherited or acquired factors.

Because collagen is easily degraded by collagenase, its durability as an implantable bioprosthesis is not long. In order to solve these problems by giving collagenase resistance to collagen, EGCG was introduced to collagen, but the mechanism of collagenase resistance of EGCG treated collagen enhanced by EGCG treatment and chemical conformation between EGCG and collagen have not been clear and not the purpose of this study.

If collagen structure is changed by chemical modification, it is possible that the interaction with cells is disrupted due to loss of native conformational structure of collagen, suggesting that the conformational stability of collagen largely affect collagen on cellular activity. In this study, the thermal stability of EGCG treated collagen was evaluated, and the effects of EGCG treated collagen on cellular activity, such as cell adhesion and proliferation, was investigated. Also, toxicity test of EGCG treated collagen bioprosthesis using animal model, such as intradermal test, sensitization test, genetic toxicity test, and subacute toxicity test was investigated.

Briefly, purpose of this study is divided into four categories.

1. Preparation and characterization of collagen, EGCG treated collagen, and EGCG treated collagen bioprosthesis was investigated.

2. Maintenance of native conformational and thermal stability of EGCG treated collagen after modification at 37 ℃ of physiological temperature compared to collagen was investigated.

3. Collagenase resistance of EGCG treated collagen was investigated and the effects of EGCG treated collagen on cellular activity, such as cell adhesion and proliferation, was investigated.

4. Intradermal test, sensitization test, genetic toxicity test, and subacute toxicity test of EGCG treated collagen bioprosthesis was investigated using animal model.

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# 2. Preparation of EGCG-collagen

## 2.1. Introduction

The preparation of a collagen from calf skin generally involves several different steps [1, 2]. This process includes elimination of the noncollagenous components that are present in the extract, selective precipitation to remove alternative collagen types, ultra-filtration, and lyophilization.

In extraction of the collagen and its purification from calf skin, special precautions must be taken that tissues and derived extracts are maintained at temperatures between 0 ℃ and 10 ℃, because collagen readily becomes converted to gelatin or denatured. All of the process of extraction and purification requires treatment in cold room to keep temperature of 4 ℃. This minimizes bacterial growth and ensures the retention of native conformation of collagen in the solutions of preparative steps.

The removal of immunogenicity from the collagen is significant to use in biomaterials, especially implants. There are triple helical region and nonhelical telopeptide region that exists in both N- and C-terminal ends in a collagen molecule. The non-helical telopeptide region has been known as the host immune reaction providing site in collagen. To remove telopeptide and to prepare telopeptide free collagen, atelocollagen, without immunogenicity, pepsin digestion was processed [3, 4].

The obtained type I atelocollagen was used to prepare EGCG treated collagen. Maintenance of temperature between 0 ℃ and 10 ℃ must be taken on the way of process to prepare EGCG treated collagen, because

collagenase resistant EGCG treated collagen is prepared by solution reaction. The collagenase resistance is acquired through structural stabilization by using EGCG that provides its native biological functions and enhanced anti-oxidation activity.

## 2.2. Materials and method

### 2.2.1. Materials

Type I atelocollagen was prepared from calf skin using pepsin treatment with selective salt precipitation and stored at 4 ℃ before use. EGCG (molecular weight: 458.4) was supplied from Pharmafood Inc. (Kyoto, Japan). The other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2.2. Extraction and purification of collagen from calf skin

Calf skin from freshly killed cow was collected from the local slaughter house. The fur on the fresh calf skin was dissected away by shaving and the calf skin was washed with the distilled water for five times followed by ethanol washing. After washing, calf skin was cut into 3 cm × 10 cm followed by macerating and swelling in 5 % acetic acid solution at 4 ℃ for one night. Dermis tissue was obtained as a soft tissue collagenous specimen by separating epidermis and subcutaneous layer (hypodermis) from the organic acid solution swollen calf skin and weighed. After treatment in 2L of 70 % ethanol at 4 ℃ for overnight and washing in

distilled water (prechilled at 4 ℃) for 5 times. 800g of dermis tissue was minced with blender (Waring, 3,500 ~ 25,000 rpm) in 16L of distilled water (prechilled at 4 ℃), and homogenized in 50L of 0.5M acetic acid solution (pH 2.5). 109.86g of pepsin (903 unit/mg, Sigma Chemical Co.) was dissolved in the homogenized solution and digested for 16 hours at 4 ℃ to obtain atelocollagen. Pepsin treatment cleave the cross-linked bovine collagen at the telopeptide cross-links sites to provide monomeric collagen without damage to the rod-like structure (Figure 2-1).

After incubation, the solubilized component was collected by centrifugation (10,000 × g for 10 min. at 4 ℃) to remove insoluble residues. The supernatant solution including solubilized component was brought to 8.686L of 5M NaCl solution by slow addition to reach 0.7M final concentration of NaCl with stirring at 4 ℃ for 15 min. [5]. The precipitate was centrifuged (10,000 × g for 10 min. at 4 ℃) down, and was suspended in 8L of 0.02M urea solution and stirred overnight at 4 ℃. The solution was ultra-filtrated with 300KDa membrane filter to remove degraded and destroyed collagen chains. To remove impurities, the filtrate was transferred to several dialysis sacks (exclusion size 15KDa), and stirred at 4 ℃ against large volumes of prechilled distilled water. The prechilled distilled water, dialysis medium, was replaced every 3 hours by fresh one for 12 times. The dialyzed solution was adjusted pH 7.4 with adding 0.01M NaOH solution and frozen at -80 ℃. The frozen solution was lyophilized. During lyophilization, the sample panel and chamber was kept on 4 ℃. After 2~3 days of lyophilization, white fibrous atelocollagen was obtained. Figure 2-2 shows schematic diagram of the extraction of type I atelocollagen from dermis tissue of calf skin and purification.

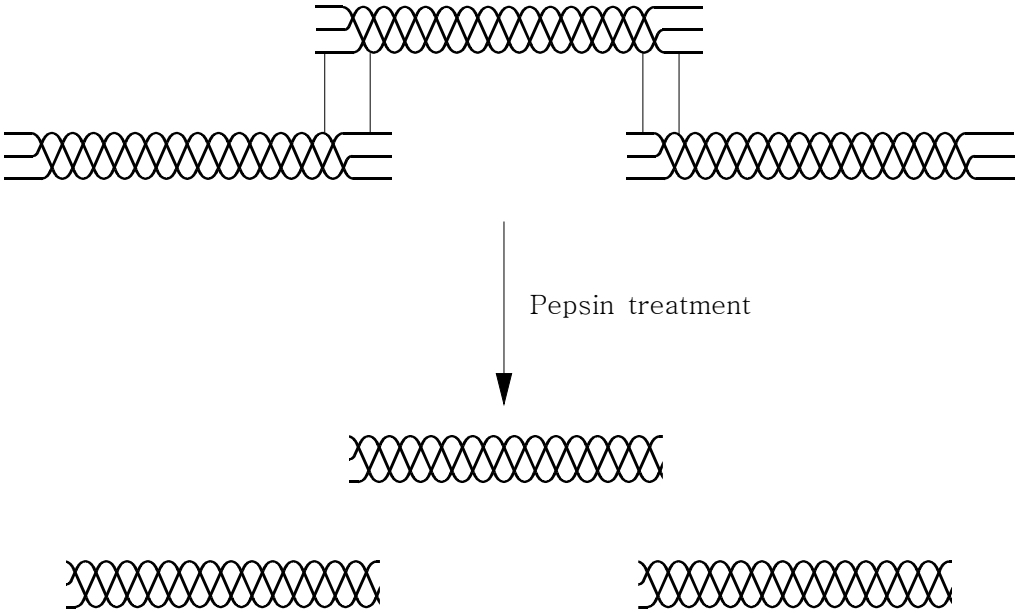


Figure 2-1. Cleavage of the crosslinked bovine collagen at the telopeptide crosslinks sites to provide monomeric collagen without damage to the rod-like structure.

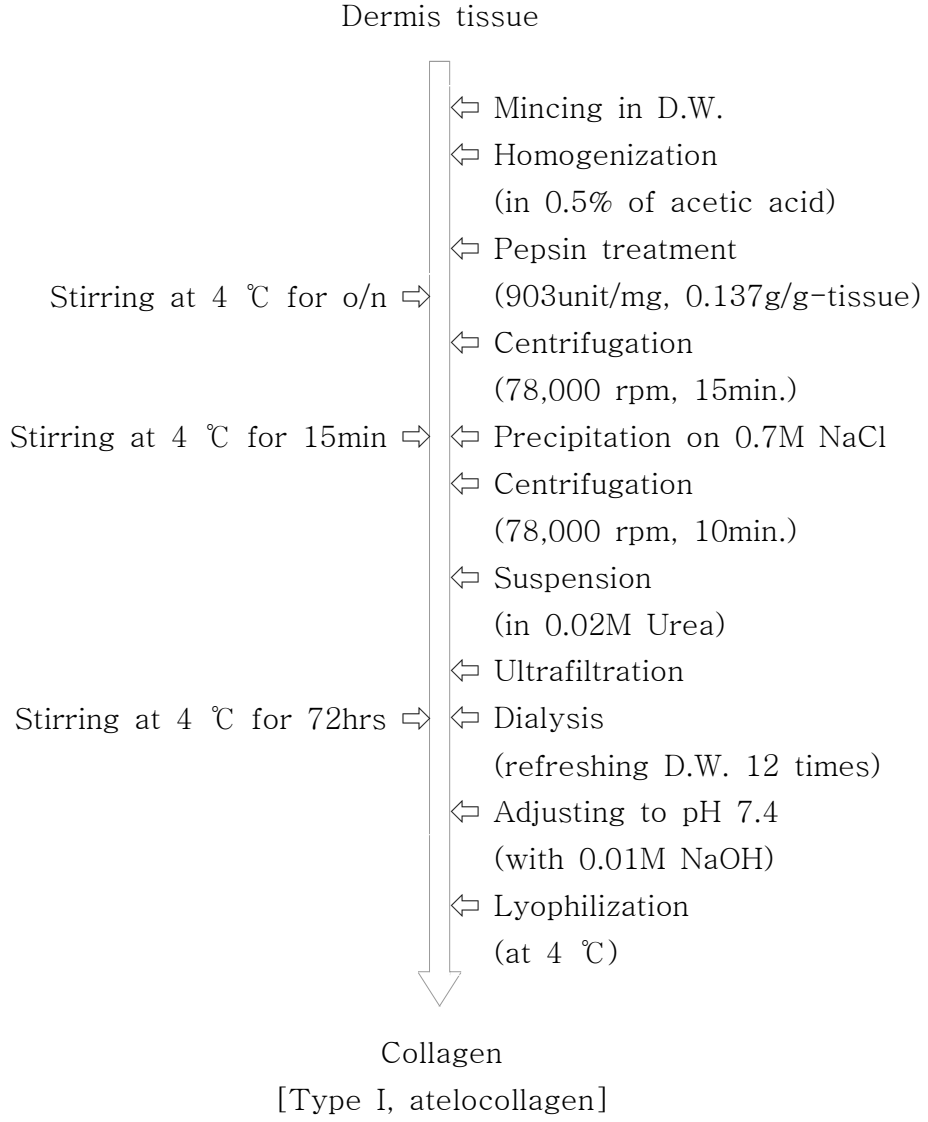


Figure 2-2. Schematic diagram of extraction of type I atelocollagen from dermis tissue of calf skin and purification.

### 2.2.3. Preparation of EGCG treated collagen

The lyophilized type I atelocollagen was dissolved in 0.5 mM Tris-HCl solution (pH 7.5) as 1 % (w/v) final concentration of collagen with stirring at 4 ℃ for 24 hours. After dissolving collagen, EGCG powder was added to 1 % atelocollagen solution as 0.1 mM final concentration of EGCG, and reacted with stirring at 4 ℃ for 24 hours. After reaction, EGCG treated collagen solution was diluted two times with distilled water, and then dialyzed to remove un-reacted EGCG at 4 ℃ for 72 hours with refreshing distilled water 12 times. Dialyzed EGCG treated collagen (E+Col) was frozen at -80 ℃ and freeze dried. Freeze-dried fibrous EGCG treated collagen was stored at 4 ℃ before use. Figure 2-3 shows schematic diagram of the preparation of EGCG treated collagen from type I atelocollagen extracted from calf skin.

### 2.2.4. Preparation of EGCG Treated Collagen Bioprosthesis

The lyophilized EGCG treated collagen was dissolved in a phosphate buffered saline solution as 3 % (w/v) final concentration of EGCG treated collagen with stirring at a rate of 150 rpm at 4 ℃ for one week. After dissolving EGCG treated collagen, hyaluronic acid was added to 3 % EGCG treated collagen solution as 0.75 % (w/v) final concentration of hyaluronic acid with stirring at a rate of 150 rpm at 4 ℃ for one week. The prepared bioprosthesis was stored at 4℃ before use. Figure 2-4 shows schematic diagram of the preparation of EGCG treated collagen bioprosthesis from EGCG treated collagen.

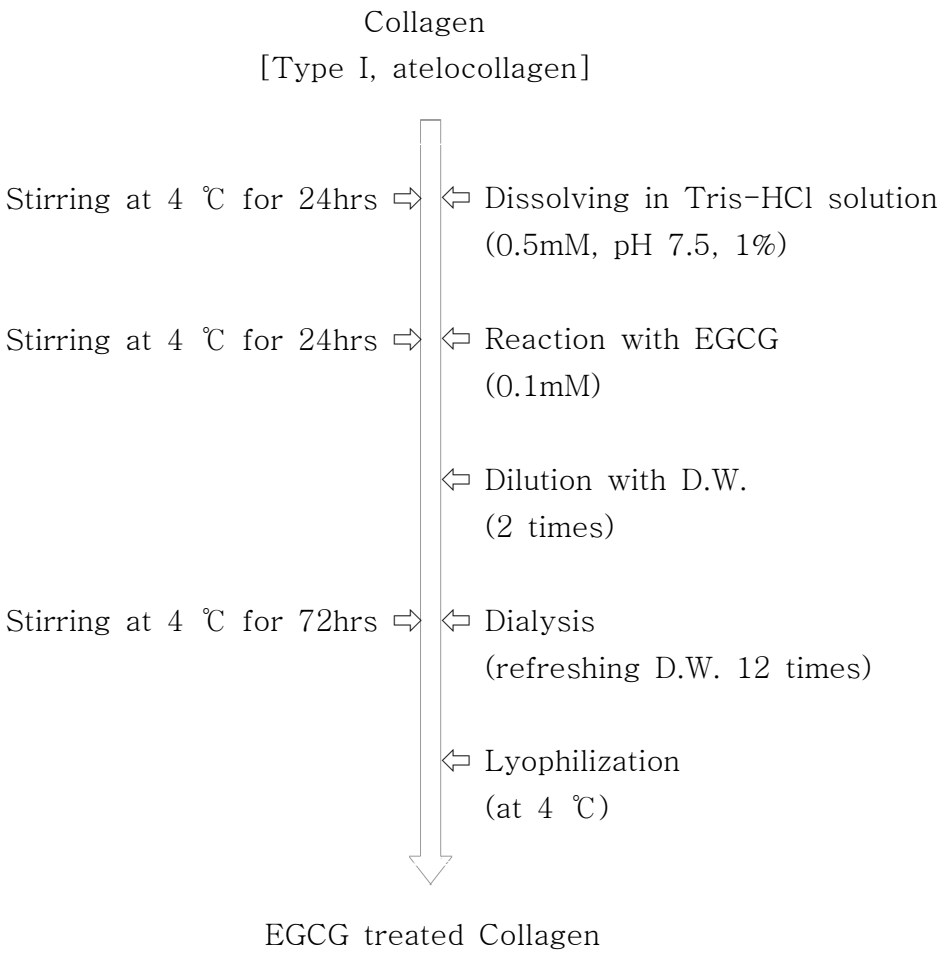


Figure 2-3. Schematic diagram of preparation of EGCG treated collagen from type I atelocollagen extracted from calf skin.

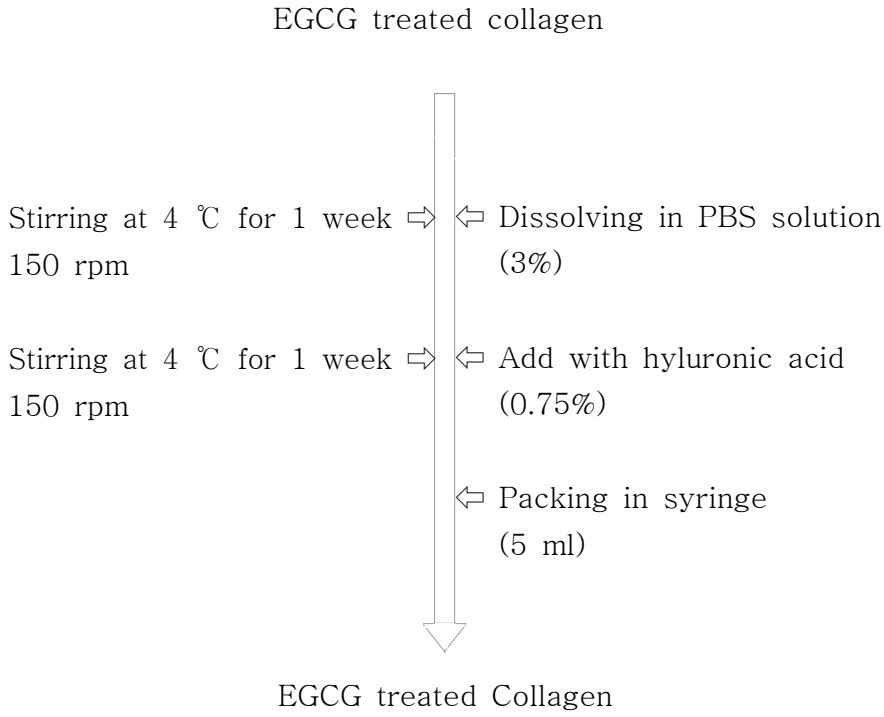


Figure 2-4. Schematic diagram of preparation of EGCG treated collagen bioprosthesis from EGCG treated collagen.

### 2.2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-page was used, according to the method of Laemmli [6]. Collagen samples were incubated for 5 min. at 100 ℃ in 62.5 mM Tris-HCl, pH 6.8, containing 2.0 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 10 % (v/v) glycerol and 0.02 % bromophenol blue. Samples were subjected to electrophoresis using a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA, USA) on a 0.1 % SDS-polyacrylamide gel (composed of 5 % stacking gel and a 6 % resolving gel) at 80V until the dye front ran off the gel at room temperature in a pH 8.3 running buffer containing 25 mM Tris, 192 mM glycine and 0.1 % SDS. When the electrophoresis is complete, the gels were then routinely stained with Coomassie Brilliant Blue R-250 (Sigma B-0149, St. Louis, U.S.A). The gel was taken photograph by CSC camera (Hama LP 5000K, Germany). The percent distribution of collagen among the different bands as a function of intensity was determined by scanning the gel and processed using the software IMAGE-PRO®.

### 2.2.6. Amino acid composition of collagen

Collagen samples were suspended in water (10 mg/ml) in hydrolysis tubes, and purged with nitrogen for 15 min. Nitrogen purged hydrochloric acid was added to give a final concentration of 6M HCl, sealed and incubated at 110 ℃ for 24 hours. The samples were then freeze-dried and dissolved in 0.2M citrate buffer pH 2.2, filtered through 0.2μm PVDF

filters and analysed using a Pharmacia AlphaPlus amino acid analyser®.

### 2.2.7. The conformational stability of collagen and EGCG treated collagen

To investigate the maintenance of the conformational triple helix structure on samples, circular dichroism (CD) analysis was used. Studies on the maintenance of triple helix structure were on collagen and EGCG treated collagen (E+Col). CD spectra of each sample were recorded on a Jasco J-715 dicrograph (Jasco Corp., Tokyo, Japan) using a 0.01 cm length thermostatized cuvette and using a Neslab RTE-111 thermostat®. Each sample was dissolved in 0.001N HCl with 3 mg/ml of concentration, and the samples were loaded at 25 ℃ into preheated CD cuvettes. CD spectra were recorded at 25 ℃ after 30 min. of incubation time for each sample. Protein concentration was determined by Bradford method [7] with triplicate samples taken out directly from the CD cuvette after the experiments.

## 2.3. Results and discussion

### 2.3.1 Sodium dodecyl sulphate(SDS)-polyacrylamide gel electrophoresis

Figure 2-5 shows the SDS-page electrophoresis patterns of the control (A: type I collagen from Sigma Co., B: type III collagen from Sigma Co.) and extracted type I collagen (C). Both displayed two α bands (α1[I] band

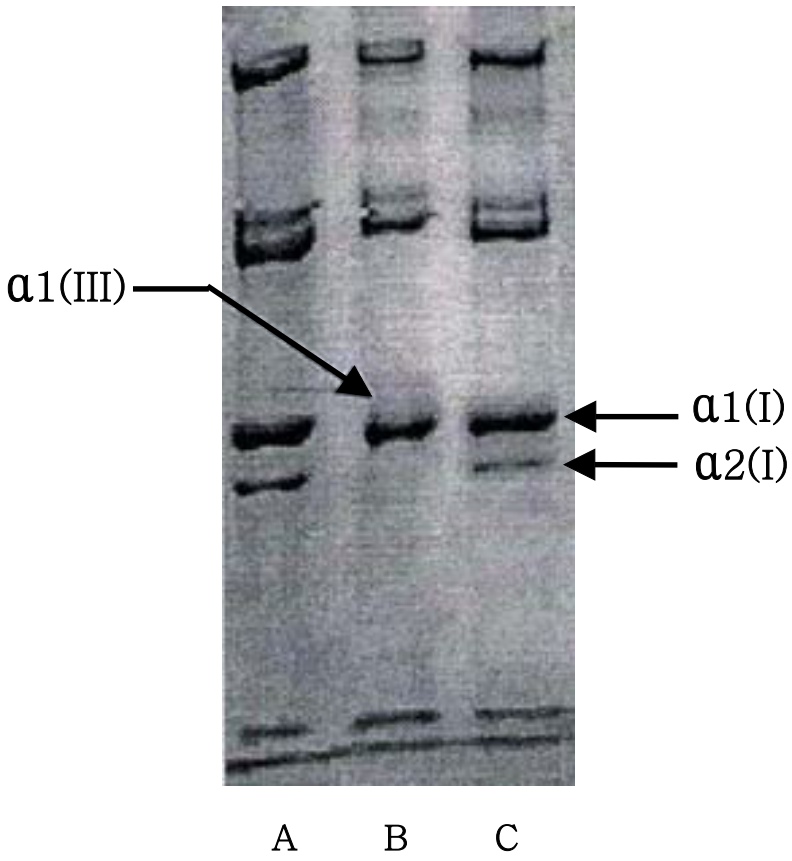


Figure 2-5. SDS-PAGE for characterization of extracted collagen.
(A) type I collagen (Sigma Co.), (B) type III collagen (Sigma Co.), (C) extracted type I collagen.

and α2[I] band), one β band, one γ band, and low molecular-weight bands. As shown in figure 2-5, α1[I] band of both type I collagen control and extracted type I collagen showed more dense in color density than α2[I] band from the percentage distribution among the bands of the collagen using one dimensional densitometer, because type I collagen consists of two α1[I] band and one α2[I] band. There are no differences between type I collagen control and extracted type I collagen. Type III collagen control showed the same patterns but α2 band.

### 2.3.2. Amino acid composition of collagen

Type I collagen consists of two identical α1 chains and one different α2 chain which is denoted as $[\alpha1(I)]_2\alpha2(I)$. And each chain consists of about 1,000 amino acid, the sequence of which is a typical feature of protein, determining its structure as a whole. Collagen has a distinctive amino acid composition and sequence Gly (glycine)-X-Y. Among the amino acids contained in collagen, Hyp (hydroxyproline) and Hyl (hydroxylysine) that do not occur in other proteins.

Amino acid composition of type I atelocollagen is shown in table 2-1. As shown in table 2-1, the amino acid composition of extracted collagen is characteristic of type I collagen. 327 residues among 1000 total residues are Gly. Hyp and Hyl residues are 92 and 9, respectively, and Pro (proline) residues, including Hyp residues, are over 20%.

Table 2-1. Amino acid compositions of extracted collagen.

| Amino acid | Residues |
|---|---|
| HYP (Hydroxy proline) | 92 |
| ASP (Aspartic acid) | 45 |
| GLU (Glutiamic a창) | 75 |
| SER (Serine) | 32 |
| GLY (Glycine) | 327 |
| HIS (Histidine) | 5 |
| ARG (Arginine) | 52 |
| THR (Threonine) | 16 |
| ALA (Alanine) | 110 |
| PRO (Proline) | 130 |
| TYR (Tyrosine) | 3 |
| VAL (Valine) | 23 |
| MET (Methionine) | 6 |
| ILE (Iso Leucine) | 11 |
| LEU (Leusine) | 25 |
| HYL (Hydroxy Lysine) | 9 |
| PHE (Phenylalanine) | 13 |
| LYS (Lysine) | 26 |

* Each value is expressed as residues/1000 total residues

### 2.3.3. The conformational stability of collagen and EGCG treated collagen

The circular dichroism (CD) spectra were applied to determine the maintenance of the conformational triple helix structure of collagen and EGCG treated collagen at temperature of 25 ℃. The CD spectra of the collagen and EGCG treated collagen at 25 ℃ are displayed in figure 2-6 and figure 2-7, respectively. The spectra were recorded in the absence of 2-mercaptoethanol.

As shown in figure 2-6, the CD spectrum of collagen shows a strong positive maximum peak at 225 nm with 5410000 deg×$cm^2$×$decimol^{-1}$ and a strong negative minimum peak at about 212 nm with -5960000 deg×$cm^2$×$decimol^{-1}$, which is characteristic of a triple helical conformation of the collagen.

And as shown in figure 2-7, the CD spectrum of EGCG treated collagen shows a strong positive maximum peak at 225 nm with 7680000 deg×$cm^2$×$decimol^{-1}$ and a strong negative minimum peak at about 212 nm with -9780000 deg×$cm^2$×$decimol^{-1}$, which is characteristic of a triple helical conformation of the protein.

From the CD analysis, the collagen shows the characteristic positive maximum peak at 225 nm and negative minimum peak at 212 nm, which means that collagen maintains the triple helix structure at 25 ℃ after extraction and purification. And the CD analysis of the EGCG treated collagen also shows the positive maximum peak at 225 nm and negative minimum peak at 212 nm same as collagen. It means that EGCG treated collagen maintains triple helix structure at 25 ℃.

Because the structural characteristic of collagen is importantly related

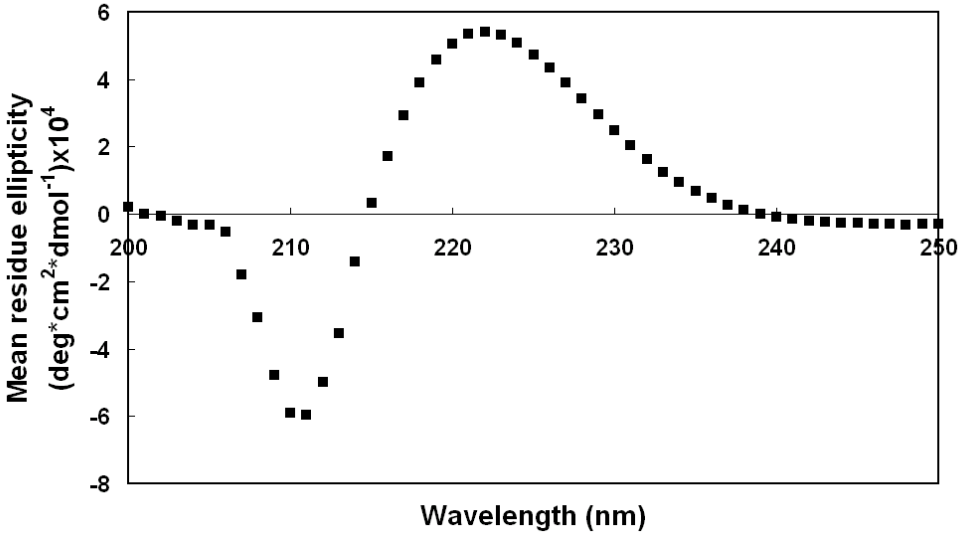


Figure 2-6. The conformational and thermal stability of collagen at 25 ℃ was analyzed using CD spectra. CD spectra of the dilute aqueous solutions of proteins were in the absence of 2-mercaptoethanol. The graph shows the ellipticity of each substrate after incubation at 25 ℃. The curves were recorded after the samples were kept at 25 ℃ for 15 min.

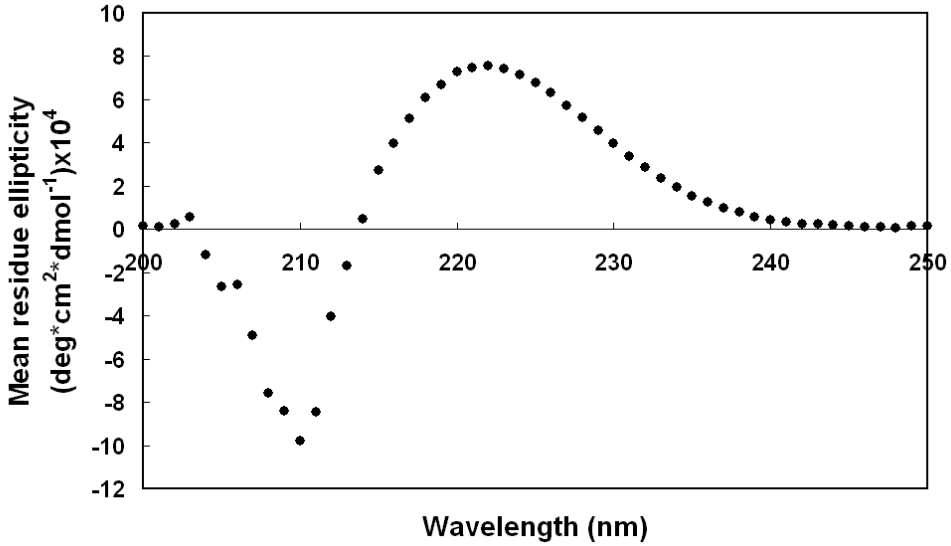


Figure 2-7. The conformational and thermal stability of EGCG treated collagen at 25 ℃ was analyzed using CD spectra. CD spectra of the dilute aqueous solutions of proteins were in the absence of 2-mercaptoethanol. The graph shows the ellipticity of each substrate after incubation at 25 ℃. The curves were recorded after the samples were kept at 25 ℃ for 15 min.

to it's biological function for the interaction with cell, maintenance of the conformational stability of EGCG treated collagen is important.

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# 3. Collagenase resistance of EGCG-collagen

## 3.1. Introduction

Collagen is the one most frequently used out of the various components of extracellular matrix, and is an attractive molecule for manufacturing biomaterials owing to its biological properties [1]. Collagen has its ability to support cell adhesion, and plays a crucial role in tissue remodeling [2, 3] In addition to its biological function, because collagen has the ability to persisting the body without developing a foreign body response that could lead to premature rejection, it has been extensively investigated as a biomaterial for artificial skin, tendons, blood vessels, cartilage, and bones [4, 5]. More than 20 types of collagen are already approved as an ingredient for hemostats, vascular sealants, tissue sealants, implant coatings, and artificial skin [6] However, biomaterials originated from collagen still have some limitations as to their use in human tissue due to inflammation through their biodegradation and relatively short durability. The extracellular degradation of collagens can occur both in nonhelical sites and through a triple helical cleavage [7]. Only the latter results in denaturation of the triple helix at physiological temperature. This is achieved by collagenases which belong to the family of endopeptidases called matrix metalloproteinases (MMPs). Collagenase-1 or MMP-1 (interstitial collagenase), collagenase-2 or MMP-8 (neutrophil collagenase), and collagenase-3 or MMP-13 are mammalian enzymes known to be able to initiate the intrahelical cleavage of triple helical collagen at neutral pH [8-10]. These collagenases cleave collagen at a single site

($Gly_{775}$-Leu/$Ile_{776}$) within each α chain of triple helical collagen molecule, approximately three quarters of the distance from the amino-terminal end of each chain, resulting in the generation of three quarter and one quarter length collagen fragments [11-13]. The cleaved collagen fragments spontaneously denature into nonhelical gelatin derivatives at physiological temperatures, thereby becoming susceptible to further degradation by other proteinases [14]. These proteolytic degradation of extracellular matrix components is involved in both physiological and pathological process, such as inflammation, aging, wound repair, angiogenesis, uterine involution, and tumor invasion. Although collagen is usually employed as the material for constructing artificial organs, collagen based biomaterials are usually stabilized either by physical or chemical crosslinking on a macroscopic level to control the rate of biodegradation of the material, to suppress its antigenicity and to improve the mechanical properties [15, 16]. For example, chemical treatment, such as succinylation, methylation and acetylation, has been applied to modification of collagen molecule in order to control the rate of proteolysis [17, 18]. Although the crosslinking of collagen results in enhancing mechanical property of collagen matrix through controlling the intermolecular interaction between collagen molecules, in a strict sense, collagen molecule may not be resistant to degradation by collagenase in the crosslinked collagen. Also, if collagen structure is changed by chemical modification, it is possible that the interaction with cells is disrupted. But, few researches have been done for the effect of chemical modification on the change of collagen structure, and for the interaction between chemically modified collagen and cells.

In other researches, many researches have demonstrated that collagen's thermal stability and resistance to enzymes could be enhanced through molecular stabilization using sugar [19]. Especially, it has been well known

that sugar could stabilize collagen molecule through hydrogen bonding with the backbone of collagen due to its high level of hydroxyl group [19, 20]. Like sugar, there are a lot of hydroxyl groups in the backbone of polyphenols, and it is predictable that polyphenols can play a role in stabilizing collagen molecule in a manner consistent with sugars. On the other hand, it has been well known that some polyphenols play a role of crosslinking in collagen molecules [21, 22], and many laboratory studies have demonstrated the inhibitory effects of tea polyphenols on tumor formation and growth [23]. Also, recently, it has been demonstrated that some kinds of catechins such as Epicatechin Gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG) have the ability to inhibit the activities of some MMPs that are essential in the development and metastasis of cancer [23, 24]. The point that EGCG and ECG are different from other catechins is their having flexible galloyl rings on their backbone. However, the precise mechanisms for the inhibition of collagenase activity are not clear.

This fact leaded us to investigate the role of EGCG, a major component of green tea catechins, on the stabilization of collagen molecule. In this study, type I atelocollagen was treated with EGCG, and the thermal stability of EGCG treated collagen was evaluated. Collagenase resistance of EGCG treated collagen was investigated, and the effects of EGCG treated collagen on cellular activity, such as cell adhesion and proliferation, was investigated. Also, free radical scavenging activity of EGCG treated collagen was investigated.

## 3.2. Materials and Method

### 3.2.1. Cells and Reagents

Normal adult human dermal fibroblasts from 50-year-old male donor were obtained by punch biopsy. Cells were maintained in Dubelcos' modified essential medium (DMEM) supplemented with 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in tissue culture flasks in a $CO_2$ incubator (5% $CO_2$, 95% humidity) at 37 ℃.

EGCG (molecular weight: 458.4) was obtained from Pharmafood (Kyoto, Japan), Hyaluronic acid (sodium salt, molecular weight : 1,000,000 ~ 3,000,000) was purchased from LGCI Co. (Icsan, Korea).

### 3.2.2. Collagenase resistance test (Collagen zymography)

#### 3.2.2.1. Collagenase resistance test of EGCG treated collagen

EGCG treated collagen were analyzed for collagenase resistance activity using collagen zymography. Zymogram gels and loading buffer [50 mM Tris HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue, 100 mM DTT] were prepared for the collagenase zymogram. 8% running gels containing 1.2 mg/ml of type I atelocollagen or EGCG treated atelocollagen as substrate gel were overlaid with a 5% stacking gel. The samples were prepared in a non-reducing buffer, and the gel was run at 150 V until the dye front ran off the gel with loading of 100 unit/ml bacterial collagenase (Sigma, St. Louis, Mo., USA). The gel

was removed from the glass plates, and washed with 2.5% hourson X-100 in a shaker for 30 min at room temperature. The gel was briefly washed with collagenase buffer and then incubated with collagenase buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.2% Brij-35, pH 7.6) for 24 hours at 37℃. Prior to staining, gel was briefly rinsed in distilled water. The gel was stained by Coomassie Brilliant Blue R250 (Sigma, St. Louis, USA) on a shaker at room temperature, and then de-stained. After completion of staining and de-staining, gel was briefly rinsed in distilled water and photographed by CSC camera. The intensities of the bands (clear bands) were quantified by densitometry.

#### 3.2.2.2. Matrix metalloproteinase resistance test of EGCG treated collagen

EGCG treated collagen were analyzed for MMP resistance activity using collagen zymography. In MMP resistance tests, MMP-1 (Sigma, St. Louis, Mo., USA), was used, and type I atelocollagen as control and EGCG treated collagen was used as substrate gel. Other process is same as above collagen zymography procedure.

#### 3.2.2.3. Study of the interaction between EGCG and collagenase or collagen using collagen zymography

To determine whether EGCG is a direct inhibitor for collagenase, collagenase was treated EGCG prior to gel loading into collagen gel as substrate, and, to investigate the brief mechanism of collagen stabilization by EGCG, succinylated collagen and EGCG treated succinylated collagen

were used collagen gel as substrate with loading of non-treated collagenase. Succinylated collagen was prepared by previously described method [25]. Other process is same as above collagen zymography procedure.

### 3.2.3. Circular dichroism analysis

Circular dichroism (CD) spectrum was used to determine the conformational stability of collagen. Studies of collagen stability were on 4 groups of collagen, EGCG treated collagen (E+Col), succinylated collagen (S-Col), and EGCG treated succinylated collagen (E+S-Col). CD spectra of each sample were recorded on a Jasco J-715 dicrograph (Jasco Corp, Tokyo, Japan) using a 0.01 cm length thermostatized cuvette and using a Neslab RTE-111 thermostat. Each sample was dissolved in 0.001N HCl with 3 mg/ml of concentration, and the samples were loaded at 25 ℃ into preheated CD cuvettes. Temperature was raised from 25 ℃ to 45 ℃ at the rate of 0.5 ℃/min, and CD spectra with 30 min. of incubation time at each temperature were recorded. Protein concentration was determined by Bradford method [26] with triplicate samples taken out directly from the CD cuvette after the experiments.

### 3.2.4. Macrophage adhesion assay

The study of macrophage adhesion was on 4 groups of collagen, EGCG treated collagen (E+Col), collagenase treated collagen, and collagenase treated E+Col. A gel block in 48-well tissue culture polystyrene plates was prepared by solidifying 1.5% agar and then coated with 2mg/ml each

substrate. Macrophage adhesion was assessed by measuring the level of the endogenous lysosomal enzyme, hexosaminidase [27]. $1.2 \times 10^6$ J774.1 murine macrophage cell line were seeded on each substrate, and cultured for 2 hours in RPMI 1640 without FBS at 37 ℃ and 5% $CO_2$. Unattached cells were removed by thoroughly washing 3 times with PBS prior to the addition of 200 μl/well of hexosaminidase substrate (3.75 mM ρ-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, St. Louis, Mo., USA), 0.25% Triton X-100, 0.05 M citrate buffer, pH 5.0). After 1 hour incubation, hexosaminidase activity was stopped and color developed by the addition of 150 μl/well of stop/development buffer (5 mM EDTA, 50 mM glycine, pH 10.4). Plates were read at 405 nm using ELISA reader. The number of adherent cells was determined from a standard curve of macrophages cultured on uncoated tissue culture plate.

### 3.2.5. Free radical scavenging activity test

Free radical scavenging activity of EGCG treated collagen was investigated using decolorization assay method of Roberta Re [28]. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, St. Louis, Mo., USA) was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to place in the dark at room temperature for 16 hours before use. The $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, to an absorbance of about 0.70 at 734 nm. Each samples were dissolved in PBS, and than 10 ml of each sample solution was added to 1.0 ml of diluted $ABTS^{\bullet+}$ solution. The mixed solutions were

reacted for 10 min at room temperature with shaking. After the reaction, mixed solutions were centrifuged, and then the supernatants were acquired. Free radical scavenging activity of the mixed solutions was determined by decolorization of $ABTS^{\bullet+}$, through measuring the reduction of the radical cation as percentage inhibition of absorbance at 734 nm by ELISA reader (Spectra Max 340, Molecular Device Inc., Ca., USA). The percentage inhibition of absorbance at 734 nm is calculated as a function of concentration of samples for the $ABTS^{\bullet+}$ solution.

### 3.2.6. Fibroblasts adhesion assay

Cell-substrate adhesion assays were on 4 groups of collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen. Briefly, 96-well tissue culture polystyrene plates were coated with 2 μg/ml of each substrate diluted in PBS, and non-coated area in plates was blocked with 10 mg/ml heat-denatured bovine serum albumin (BSA) (Sigma, St. Louis, Mo., USA) for 30 min. Adult human dermal fibroblasts adhesion was assessed by measuring the level of the endogenous lysosomal enzyme, hexosaminidase. Fibroblasts were treated with 100 μM RGDS peptides (Sigma, St. Louis, Mo., USA) in serum free DMEM, and RGDS treated fibroblasts or non-treated fibroblasts were seeded on each substrate at the concentration of about $1.0 \times 10^3$ cells, and cultured for 2 hours in DMEM without FBS at 37 ℃ and 5% $CO_2$. Unattached cells were removed by thoroughly washing 3 times with PBS prior to the addition of 100 μl/well of hexosaminidase substrate (3.75 mM ρ-nitrophenyl-N-acetyl-β-D-glucosaminide, 0.25% Triton X-100, 0.05 M citrate buffer, pH 5.0). After 1 hour incubation, hexosaminidase activity

was stopped and color developed by the addition of 75 μl/well of stop/development buffer (5 mM EDTA, 50 mM glycine, pH 10.4). Plates were read at 405 nm using ELISA reader. The number of adherent cells was determined from a standard curve of fibroblasts cultured on uncoated tissue culture plate.

### 3.2.7. Organization of the actin cytoskeleton

The study of F-actin filament characterization was on 4 groups of collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen. Each collagens were coated on chamber slides with the concentration of 2 μg/ml. Adult human dermal fibroblasts were plated on each substrate coated slide, and cultured for 2 hours in DMEM without FBS at 37 ℃ and 5% $CO_2$. Unattached cells were removed by thoroughly washing 3 times with PBS prior to fixation using 1% paraformaldehyde. The fixed cells were subjected to the indirect immunofluoresecence technique. After permeabilisation with 0.1% Triton X-100 in PBS, the fixed cells were washed in PBS and exposed in a moist chamber to 3% bovine serum albumin in PBS at 20 ℃. After washing with PBS, the fixed cells were stained 5 mg/ml fluorescent phalloidine conjugate (phalloidin-FITC (Sigma, St. Louis, Mo., USA)) solution in PBS with 1% DMSO for 1 hour at 20 ℃, and washed several times with PBS to remove unbound phalloidine conjugate. Cytoskeletal morphology was visualized with a laser scanning confocal microscopy system. Nuclei were stained with 4,6-diamine-2-phenylindole hydrochloride (DAPI: Sigma, St. Louis, Mo., USA) (100 ng/ml in PBS) for 5 min. and observed currently, and F-actin was visualized with cytochemical labeling with phalloidin-FITC.

### 3.2.8. Western blot analysis of β1 integrin subunit

The study of β1 integrin expression of fibroblasts was on 4 groups of collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen. Adult human dermal fibroblasts were plated at $5 \times 10^5$ cells on each substrate for 2 and 24 hours, trypsinized and the collected cell pellets were lysed in a 1% Triton X-100 solution in the presence of protease inhibitors (Sigma, St. Louis, Mo., USA) at 4 ℃ overnight. Total protein concentration was measured using Bradford method. Twenty mg of protein was fractionized by a SDS-page on a 6% gel and electrotransferred to nitcellulose paper. Non-specific protein-protein interactions were minimized by incubation of the blot in 3% bovine serum albumin in 0.5% Tween 20-Tris buffered saline solution for 2 hours. The blots were incubated with Integrin β1 (monoclonal, Chemicon Inc, USA), GAPDH (polyclonal, Trevigen, USA) for 2 hours at room temperature in blocking buffer. The blot was then washed and incubated with HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, United Kindom), was washed and then developed with ECL-Plus (Amersham Pharmacia Biotech, United Kindom). The intensities of the bands were quantified by densitometry.

### 3.2.9. Cell proliferation assay (DNA content assay)

Studies of cell proliferation were on two groups of collagen as the control and EGCG treated collagen. 48-well tissue culture polystyrene plates were coated with 2 μg/ml type I atelocollagen or EGCG treated type I atelocollagen in PBS. $2.0 \times 10^5$ adult human dermal fibroblasts were

plated on substrates coated plates supplemented with 5% FBS, and the medium was changed every 24 hours during incubation in a $CO_2$ incubator. Cell proliferation on each substrate was determined after 1, 4, 7, and 10 days. To remove unattached cells, substrates were washed by PBS solution. The DNA content, a measure of cell proliferation, was evaluated using the Hoechst 33258 fluorescent dye assay. Cells were digested in 250 μl 0.2 M NaCl, 0.1 M NaAc, 0.01 M L-cystein-HCl, 0.05 M EDTA-$Na_2$ (pH 6.0) containing 16 U papain for 16 hours at 65 ℃. 100 μl 0.02 M Tris-HCl (pH 8.0) containing 0.1% sodium dodecyl sulfate was added to 100 μl papain digest. The mixture was incubated for 30 min at 60 ℃. A 100 μl aliquot was analyzed for total DNA by the addition of 1ml Hoechst solution (0.2 μg Hoechst 33258/ml (Sigma, St. Louis, Mo., USA). The fluorescence of the samples was measured at an excitation of 365 nm and an emission of 458 nm. Calf thymusDNA (Sigma, St. Louis, Mo., USA) was used as standard.

### 3.2.10. Animal Test

#### 3.2.10.1. Intradermal test of EGCG treated collagen bioprosthesis

An intradermal test was performed according to the ISO 10993-10 (Biological Evaluation of Medical Devices, Part 10: Tests for irritation and sensitization). White rabbits (New Zealand) were used as an animal model and subcutaneous injection was generally used (sample solution: 0.2 ml). In addition to EGCG treated collagen bioprosthesis , a physiological saline solution was used as a negative control.

### 3.2.10.2. Sensitization test of EGCG treated collagen bioprosthesis

A sensitization test was performed according to the ISO 10993-10 (Biological Evaluation of Medical Devices, Part 10: Tests for irritation and sensitization). Guinea pigs were used as an animal model and the maximization test was generally used. In addition to the EGCG treated collagen bioprosthesis , a physiological saline solution was used as a negative control and 0.05% DNCB (2,4-dinitrochlorobenzene) was used as a positive control.

### 3.2.10.3. Genetic toxicity test of EGCG treated collagen bioprosthesis

A genetic toxicity test was performed according to the OECD Guideline for Testing of Chemicals 471 (Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay). Test strains were TA97, 98, 100, 102, and 1535. In addition to the EGCG treated collagen bioprosthesis , a physiological buffered saline solution was used as a negative control. As for a positive control, benzopyrene (BP) and 2-aminoanthracene (2-AA) were used in a S9 Mix. treated group and 4-nitroquinoline-N-oxide (NQNO), methylmethanesulfonate (MMS), and sodium azide (SA) were used in a S9 Mix. untreated group.

### 3.2.10.4. Subacute toxicity test of EGCG treated collagen bioprosthesis

A subacute toxicity test was performed according to the OECD Guideline for Testing of Chemicals 407 ("Repeated Dose Oral

Toxicity-Rodent: 28-day" ISO 10993-1 Biological Evaluation of Medical Devices, Part I: Guideline on selection of tests). Sprague-Dawley rats were used as an animal model. In addition to the EGCG treated collagen bioprosthesis , a USP polyethylene strip® (1 × 5 mm) was used as a negative control, a collagen solution (3%) was used as a comparative group I, and Restylane® (Q-Med. Inc., Uppsala, Sweden) was used as a comparative group II.

## 3.3. Results

### 3.3.1. Collagenase resistance of EGCG treated collagen

In the collagenase resistance test of EGCG treated collagen determined by collagen zymography, collagen degradation by collagenase was showed as clear area, and the intensities of the area were quantified by densitometric scanning and expressed in relative intensity to collagen as a substrate. The collagen zymography of collagen and EGCG treated collagen to analyse bacterial collagenase resistance of EGCG treated collagen is displayed in figure 3-1. Regarding clear band intensity of degraded collagen by collagenase as 100 after incubation for 24 hours, EGCG treated collagen was much less degraded by bacterial collagenase, which relative clear band intensity was 11.2 ± 2.7. From the collagenase resistance test, it was known that EGCG treated collagen had high resistance against degradation by collagenase up to about 90%. Also, in order to determine the effect of EGCG on collagenase activity, collagen zymography was assayed using non-treated collagen as a substrate and EGCG treated

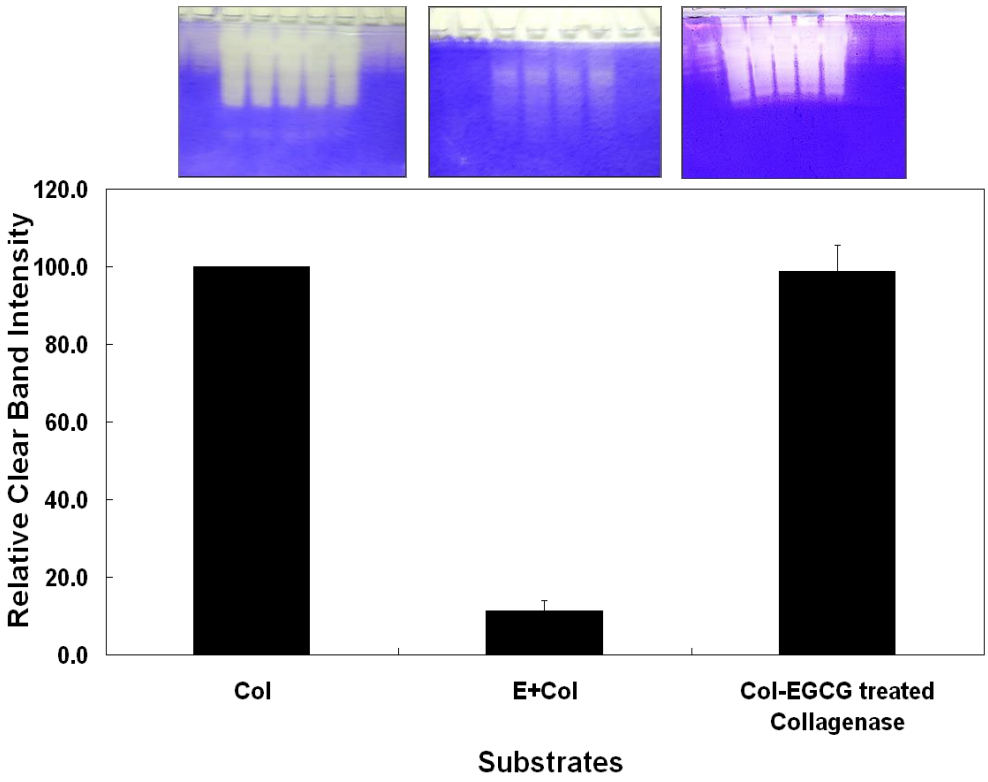



Figure 3-1. EGCG treated collagen were analyzed for collagenase resistance activity using collagen zymography. The intensity of clear bands degraded by bacterial collagenase was quantified by scanning densitometry, and collagenase resistance was expressed as relative band intensity considering the degraded clear band intensity in the use of collagen as substrate in zymography to be 100. Col: the use of collagen as substrate for bacterial collagenase, E+Col: the use of EGCG treated collagen as substrate for bacterial collagenase, Col+EGCG treated collagenase: the use of collagen as substrate for EGCG treated bacterial collagenase.

bacterial collagenase. Regarding clear band intensity of degraded collagen as 100 after incubation for 24 hours, collagen degradation with loading of EGCG treated collagenase showed no significant difference in degraded area from the collagen zymography with loading non-treated collagenase, which relative clear band intensity was 98.7 ± 6.9.

In order to determine the resistance of EGCG treated collagen to degradation by mammalian matrix metalloproteinase-1 (MMP-1: Collagenase-1) in comparison with resistance against bacterial collagenase, collagen zymography was used. The collagen zymography of collagen and EGCG treated collagen to analyse degradation by mammalian matrix metalloproteinase-1 of EGCG treated collagen is displayed in figure 3-2. As shown in figure 3-2, regarding clear band intensity of degraded collagen as 100 after incubation for 24 hours, EGCG treated collagen was not degraded by MMP-1, which relative clear band intensity was 0. The above results demonstrate that EGCG treated collagen show high resistance against both bacterial collagenase and MMP-1 in comparison with collagen.

In other experiments, to determine the effect of crosslinking though esteric bonding between hydroxyl groups of EGCG and carboxyl or hydroxyl groups of collagen on resistance to collagenase, collagen zymography was assayed using succinylated collagen and EGCG treated succinylated collagen as substrates. Regarding clear band intensity of degraded succinylated collagen as 100 after incubation for 24 hours, there was no significant difference in degraded area by bacterial collagenase between succinylated collagen and EGCG treated succinylated collagen, which relative clear band intensity was 100.1 ± 1.6 (Figure 3-3).

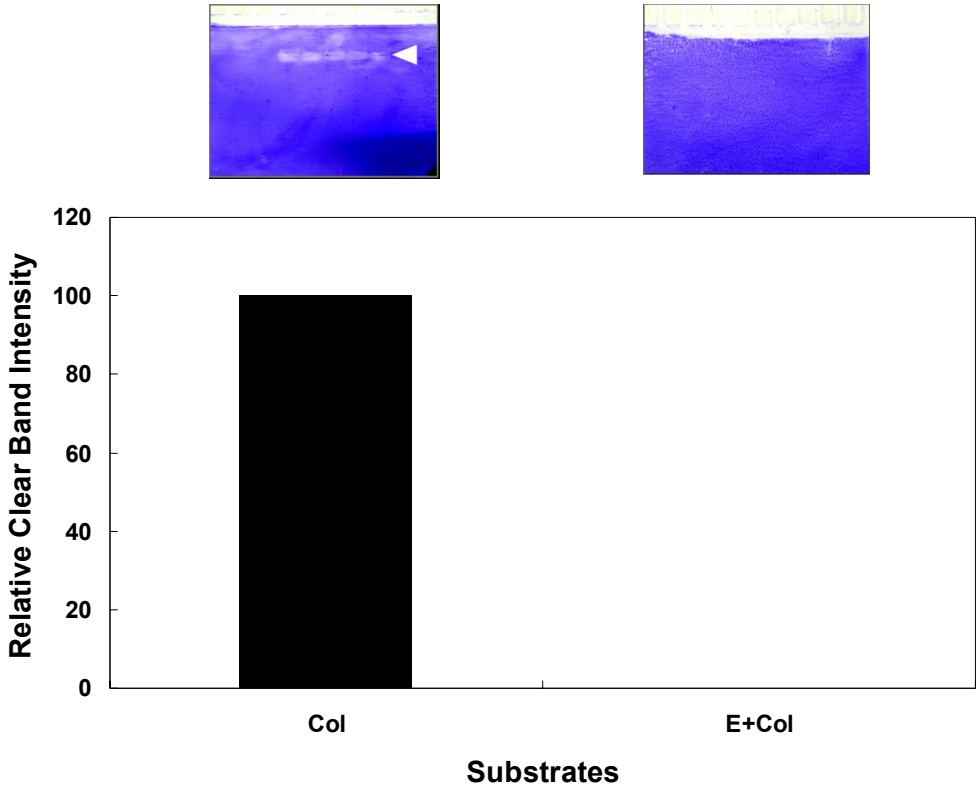



Figure 3-2. EGCG treated collagen were analyzed for MMP resistance activity using collagen zymography. The intensity of clear bands degraded by MMP-1 was quantified by scanning densitometry, and MMP-1 resistance was expressed as relative band intensity considering the degraded clear band intensity in the use of collagen as substrate in zymography to be 100%. Col: the use of collagen as substrate for MMP-1, E+Col: the use of EGCG treated collagen as substrate for MMP-1.

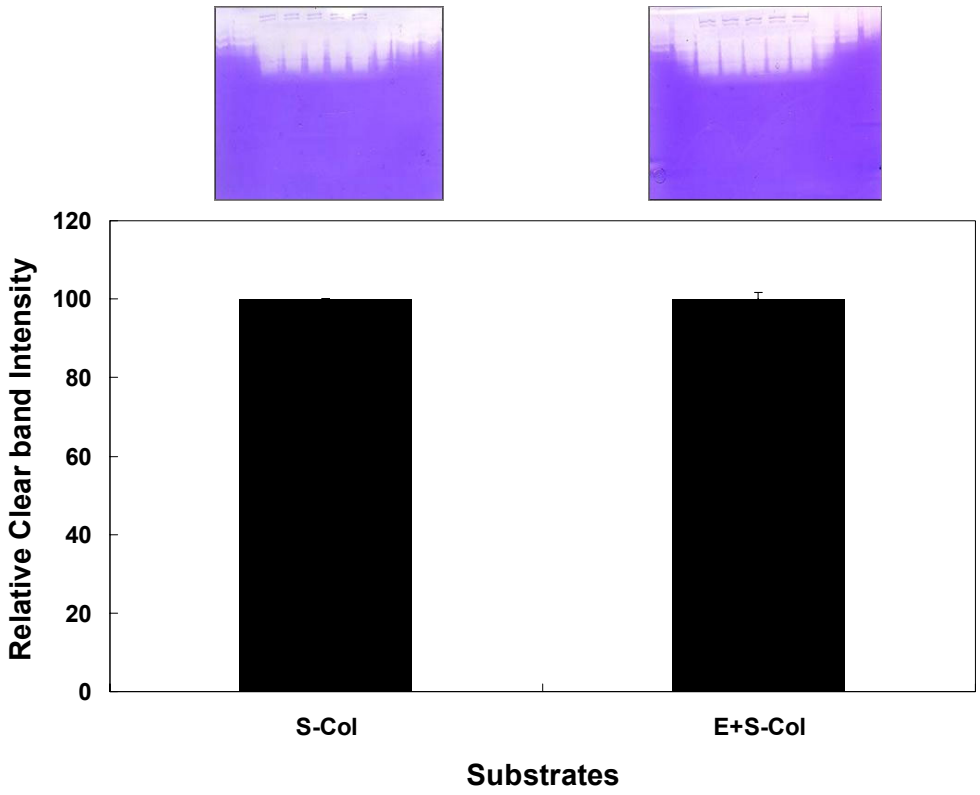



Figure 3-3. Succinylated collagen and EGCG pre-treated collagenase was used to determine the role of EGCG as direct inhibitor of collagenase and crosslinker of collagen in collagen zymography. The intensity of clear bands degraded by bacterial collagenase was quantified by scanning densitometry, and collagenase resistance was expressed as relative band intensity considering the degraded clear band intensity in the use of collagen as substrate in zymography to be 100 %. S-Col: the use of succinylated collagen as a substrate for bacterial collagenase, E+S-Col: the use of EGCG treated succinylated collagen as a substrate for bacterialcollagenase.

### 3.3.2. The conformational stability of EGCG treated collagen at physiological temperature

The CD spectra were applied to investigate the structural or conformational stability of collagen, succinylated collagen and EGCG treated collagen at different temperatures. The CD spectra of the collagen, EGCG treated collagen, and succinylated collagen at 25 ℃ and 37 ℃ are displayed in figure 3-4 and figure 3-5, respectively. The spectra were recorded in the absence of 2-mercaptoethanol. The collagen and EGCG treated collagen showed a strong positive maximum peak at 225 nm with 5410000 and 7680000 deg×$cm^2$×$decimol^{-1}$ and a strong negative minimum peak at about 212 with -5960000and -9780000 deg×$cm^2$×$decimol^{-1}$ at 25 ℃, which was characteristic of a triple helical conformation of the protein. But, succinylated collagen showed highly decrease of negative peak at 212 nm in spite of strong positive peak at 225 nm at 25 ℃. At 37 ℃ of physiological temperature, EGCG treated collagen showed retaining of strong positive and negative peak at 225 and 212 nm, but both collagen and succinylated collagen showed relatively decreased negative peak at 212 nm.

### 3.3.3. Macrophage adhesion to EGCG treated collagen

To investigate the effect of collagen resistance to degradation on macrophage adhesion, macrophage adhesion was assayed on 4 different groups of collagen, EGCG treated collagen, collagen degraded by collagenase, and EGCG treated collagen degraded by collagenase.

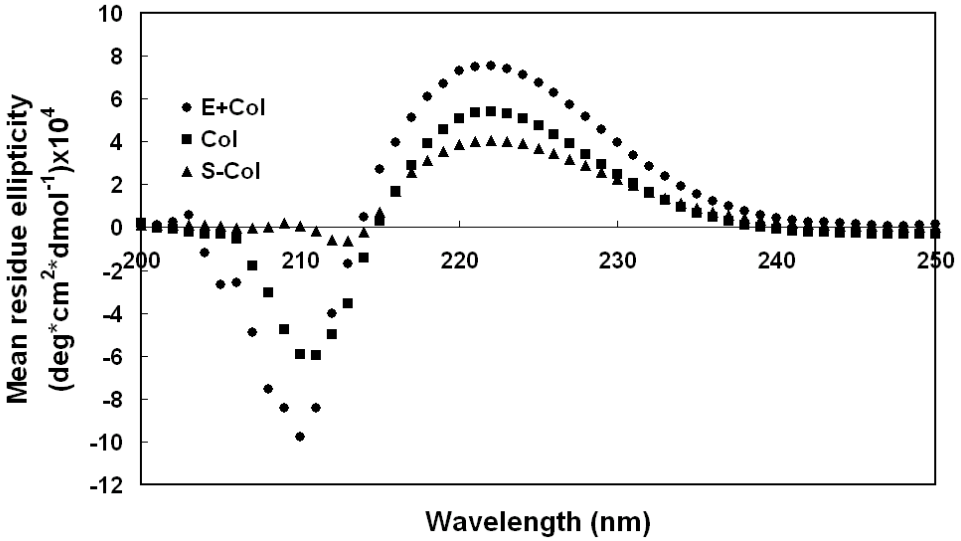



Figure 3-4. The conformational and thermal stability of EGCG treated collagen at 25 ℃ was analyzed using CD spectra. CD spectra of the dilute aqueous solutions of proteins were in the absence of 2-mercaptoethanol. The graph shows the ellipticity of each substrate after incubation at 25 ℃. The curves were recorded after the samples were kept at 25 ℃ for 15 min. Col: collagen, E+Col: EGCG treated collagen, S-Col: succinylated collagen.

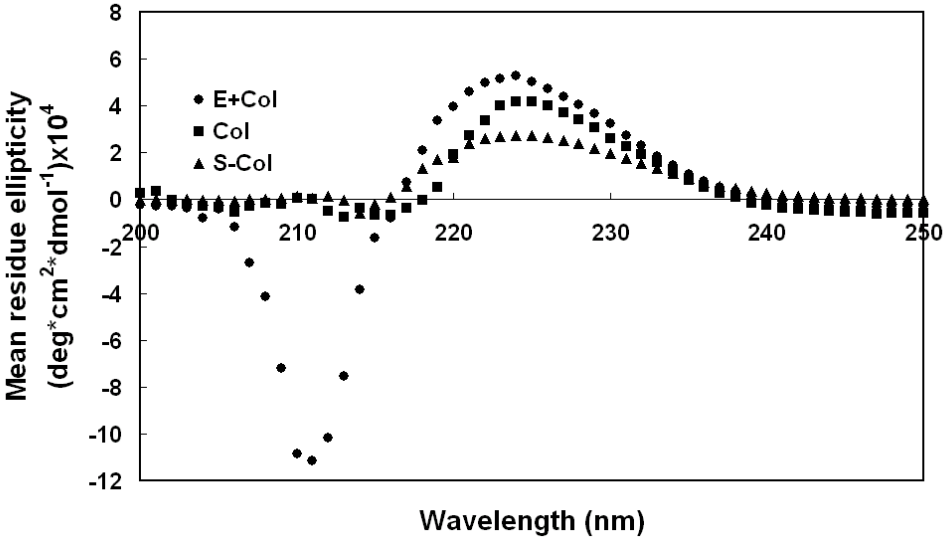



Figure 3-5. The conformational and thermal stability of EGCG treated collagen at 37 ℃ was analyzed using CD spectra. CD spectra of the dilute aqueous solutions of proteins were in the absence of 2-mercaptoethanol. The graph shows the ellipticity of each substrate after incubation at 37 ℃. The curves were recorded after the samples were kept at 37 ℃ for 15 min. Col: collagen, E+Col: EGCG treated collagen, S-Col: succinylated collagen.

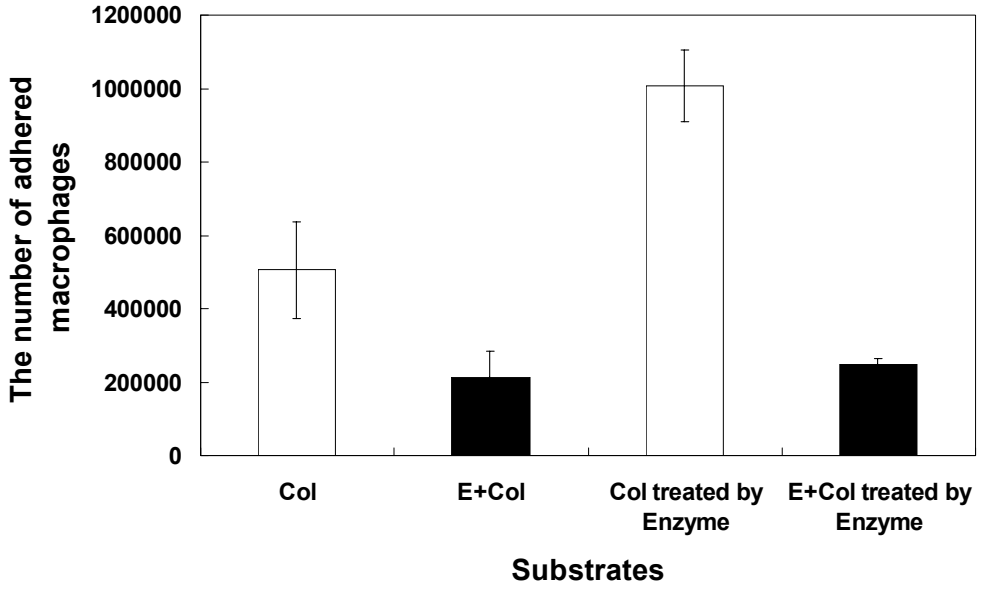



Figure 3-6. Adhesion of macrophage (J774.1 murine macrophage cell line) on 4 different substrates. Col: collagen, E+Col: EGCG treated collagen, Col treated by Enzyme: Collagen degraded by bacterial collagense, E+Col treated Enzyme; EGCG treated collagen degraded by bacterial collagenase. Each point represents the mean SEM cells in 6 samples. (*: significant difference in macrophage adhesion between Col and Col treated by Enzyme, *: $P<0.05$)

The density of adhered cells on each substrate was determined by measuring the level of the endogenous lysosomal enzyme, hexosaminidase after culture for 2 hours. Adhesion of macrophage (J774.1 murine macrophage cell line) on 4 different substrates such collagen, EGCG treated collagen, Collagen degraded by bacterial collagense, and EGCG treated collagen degraded by bacterial collagenase are displayed in figure 3-6. The number of macrophage adhere on collagen degraded by collagenase was significantly higher than that on collagen, which the number of macrophage was 1006600 ± 97880 cells and 505300 ± 132445 cells ($P<0.05$). In comparison with collagen, there was no significant difference in the number of adhered macrophage between EGCG treated collagen and EGCG treated collagen degraded by collagenase, which were 213000 ± 70188 cells and 250100 ± 12334 cells.

### 3.3.4. Free radical scavenging activity of EGCG treated Collagen

To determine free radical scavenging activity of EGCG treated collagen, antioxidant activity of EGCG treated collagen was investigated in accordance with various concentrations of EGCG treated collagen in comparison with collagen using decolorization assay method. Figure 3-7 shows that EGCG treated collagen has much higher free radical scavenging activity that collagen at each concentration. The percentage of inhibition for oxidation from free radical in EGCG treated collagen was 14.21 ± 1.67, 31.90 ± 1.29, 65.00 ± 4.14, 91.34 ± 1.11, 94.12 ± 0.79, and 94.58 ± 0.87% at the concentrations of 0.125, 0.25, 0.50, 0.75, 1, and 3%, while in collagen was 5.93 ± 2.01, 15.56 ± 2.17, 30.37 ± 1.81, 55.56 ± 1.23,

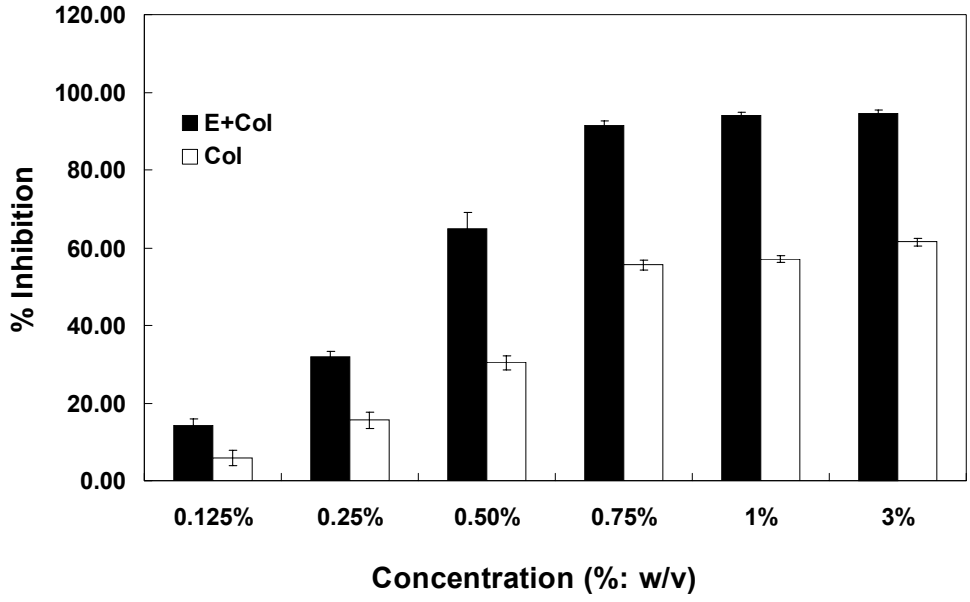



Figure 3-7. Free radical scavenging activity of EGCG treated collagen was investigated using decolorization assay. The percentage inhibition of absorbance at 734 nm is calculated as a function of concentration of samples for the $ABTS^{\bullet+}$ solution. Col: Collagen, E+Col: EGCG treated collagen. Each point represents the mean  SEM cells in 5 samples.

56.94 ± 0.84, and 61.44 ± 1.05% at the concentrations of 0.125, 0.25, 0.50, 0.75, 1, and 3%.

### 3.3.5. Human dermal fibroblasts adhesion to EGCG treated collagen

To determine the effect of enhanced structural stability of EGCG treated collagen on human dermal fibroblasts adhesion, fibroblasts adhesion was assayed using on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagenas substrates for cell adhesion with or without RGDS treatment of cells. The density of adhered cells on each substrate was determined by measuring the level of the endogenous lysosomal enzyme, hexosaminidase, after culture for 2 hours in DMEM without FBS. In the adhesion assay, non-specific cell adhesion except though substrates was blocked with albumin coating on substrate coated culture plate. From the result of fibroblasts adhesion assay (Figure 3-8), there is no significant difference in the number of adhered cells without RGDS treatment except 37 ℃ incubated collagen among collagen, EGCG treated collagen, and 37 ℃ incubated EGCG collagen, which were 961 ± 73, 1111 ± 136, and 939 ± 92 cells. However, the number of adhered fibroblasts on 37 ℃ incubated collagen was significantly lower than other groups, which was 373 ± 144 cells. The figure 3-8 also shows that there was no significant difference in the number of adhered fibroblasts in each group except collagen between cells without RGDS treatment and cells with RGDS treatment. In the fibroblasts adhesion on collagen, the number of RGDS pre-treated fibroblasts was relatively lower than that of non-treated fibroblasts, which were 961 ± 73 and 779 ± 103

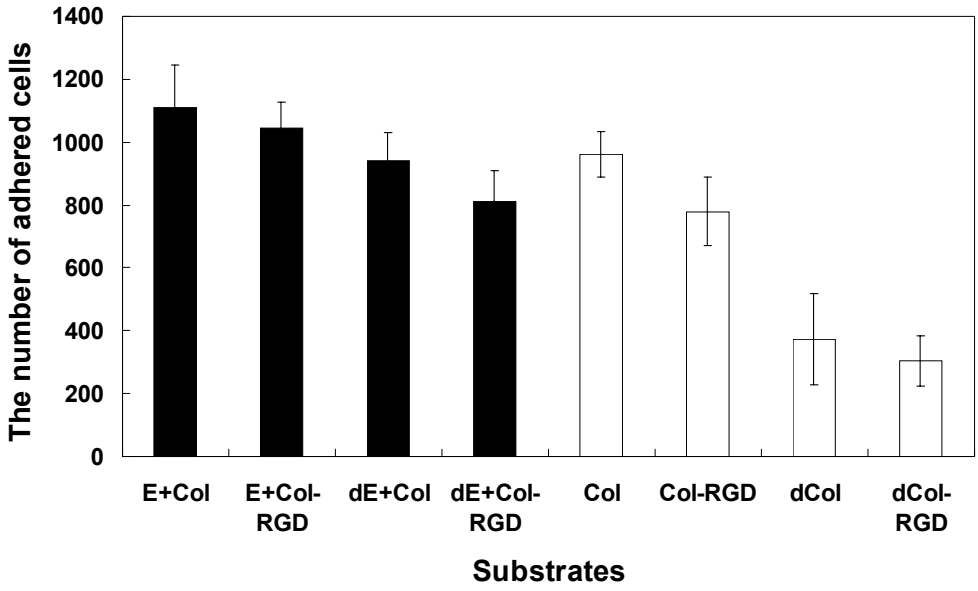



Figure 3-8. Adult human dermal fibroblasts adhesion was assayed using on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen as substrates for cell adhesion with or without RGDS treatment of cells. E+ Col: EGCG treated collagen, E+ Col-RGD: RGDS treated fibroblasts culture on EGCG treated collagen, dE+ Col: 37 ℃ incubated EGCG treated collagen, dE+ Col-RGD: RGDS treated fibroblasts culture on 37 ℃ incubated EGCG treated collagen, Col: Collagen, Col+ RGD: RGDS treated fibroblasts culture on collagen, dCol: 37 ℃ incubated collagen, dCol-RGD: RGDS treated fibroblasts culture on 37 ℃ incubated collagen. Each point represents the mean SEM cells in 6 samples. (*: significant difference in cell adhesion between without RGDS treatment and with RGDS treatment, *: $P<0.05$)

cells ($P<0.05$).

### 3.3.6. β1 integrin expression of adhered fibroblasts on EGCG treated collagen

To investigate the effect of EGCG treated collagen on fibroblasts integrin expression related with cell adhesion through collagen receptor, β1 integrin expression was assayed after 2 and 24 hours of culture on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen in DMEM without FBS using western blot assay, and the intensities of the bands for β1 integrin were quantified by densitometric scanning and expressed as normalized intensity by band intensity of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) of each sample.

Figure 3-9 displayed β1 integrin expression after 2 and 24 hours cultured on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen in DMEM without FBS using western blot assay.

There was no significant difference in β1 integrin expression of cells cultured on each substrate as shown in figure 3-9. In the β1 integrin expression, collagen, 37 ℃ incubated collagen, EGCG treated collagen, and 37 ℃ incubated EGCG treated collagen showed the relative band intensity of 1.00, 0.79, 1.10, and 0.93 after 2 hours of culture. And collagen, 37 ℃ incubated collagen, EGCG treated collagen, and 37 ℃ incubated EGCG treated collagen showed the relative band intensity of 1.24, 1.24, 1.19, and 0.92 after 24 hours of culture.

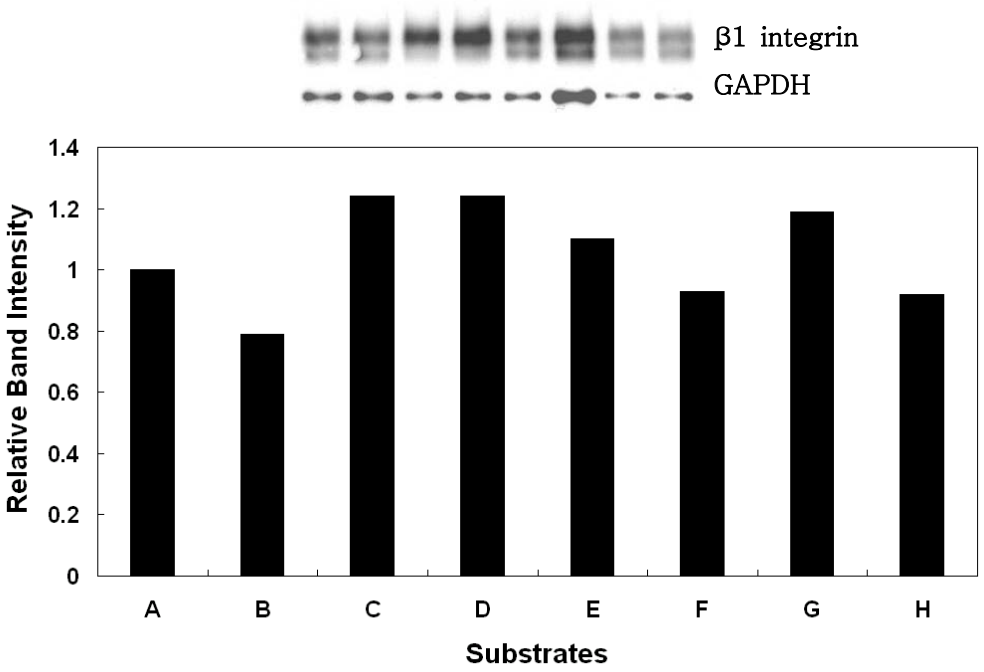



Figure 3-9. β1 integrin expression was assayed after 2 and 24 hrs of culture on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen in DMEM without FBS using western blot assay. (A) after 2 hrs of culture on collagen, (B) after 2 hrs of culture on 37 ℃ incubated collagen, (C) after 24 hrs of culture on collagen, (D) after 24 hrs of culture on 37 ℃ incubated collagen, (E) after 2 hrs of culture on EGCG treated collagen, (F) after 2 hrs of culture on 37 ℃ incubated EGCG treated collagen, (G) after 24 hrs of culture on EGCG treated collagen, (H) after 24 hrs of culture on 37 ℃ incubated EGCG treated collagen.

### 3.3.7. Actin cytoskeleton organization of fibroblasts

To investigate cell morphological change of fibroblasts related to structural stability of EGCG treated collagen, actin filament expression was observed using immunofluorescence assay. We used FITC labeled phalloidin to visualize potential difference in the organization of cytoplasmic filamentous actin in fibroblasts on collagen, EGCG treated collagen, 37 ℃ incubated collagen, and 37 ℃ incubated EGCG treated collagen. As shown in figure 3-10, the appearance of cytoplasmic actin filament correlated with the capacity of the fibroblasts on substrates: fibroblasts on collagen (Figure 3-10, Col), EGCG treated collagen (Figure 3-10, E+Col), and 37 ℃ incubated EGCG treated collagen (Figure 3-10, dE+Col) generally exhibited long actin filaments that were oriented in parallel with the long axis of the cells after culture of 2 hours. In contrast, the cytoskeletal actin of fibroblasts on 37 ℃ incubated collagen (Figure 3-10, dCol) that exhibited a poor capacity to spread formed shorter filaments with a variety of orientations.

### 3.3.8. Fibroblasts proliferation on EGCG treated collagen

Human dermal fibroblasts proliferation on collagen and EGCG treated collagen was observed for 1, 4, 7, and 10 days (Figure 3-11). The DNA content, a measure of cell proliferation, was evaluated using the Hoechst 33258 fluorescent dye assay. There was no significant difference in cell growth between collagen and EGCG treated collagen until 7days of culture ($P > 0.05$). The total DNA content in the fibroblasts culture on collagen for 1, 4, and 7days was 92.0 ± 10.7, 140.4 ± 8.9, and 194.6 ± 8.6 ng,

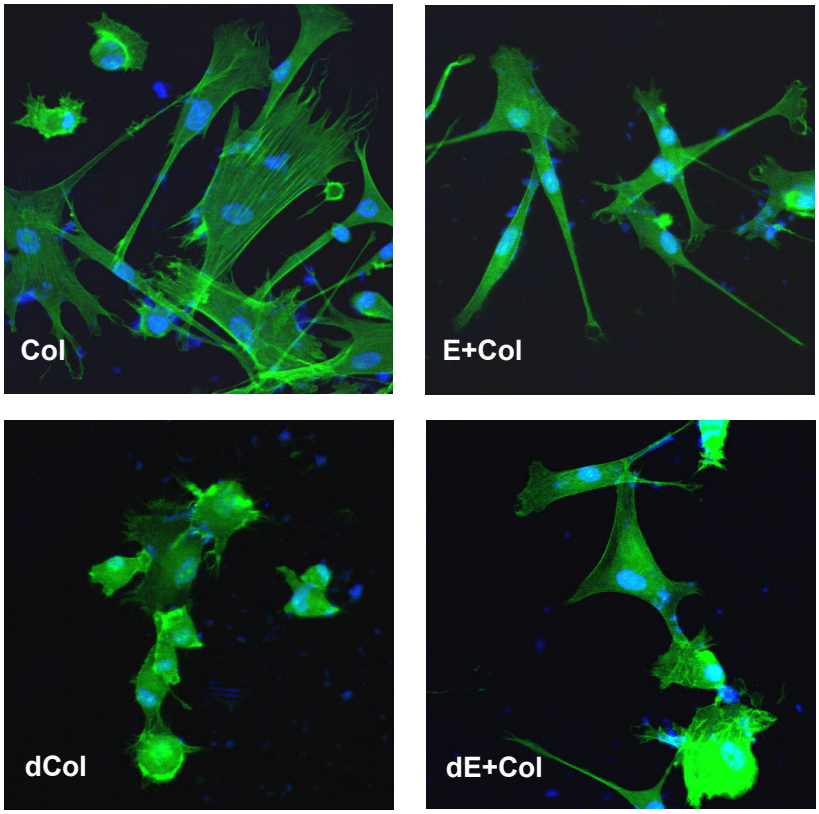



Figure 3-10. Morphological characterization of adult human dermal fibroblasts on different substrates in 2 hours of adhesion was observed by immunofluoresence staining. Actin filament was visualized as green fluorescence through FITC-phalloidin conjugation, and cell nucleus was stained by DAPI. Col: culture on collagen, E+Col: culture on EGCG treated collagen, dCol: culture on 37 ℃ incubated collagen, dE+Col: culture on 37 ℃ incubated EGCG treated collagen.

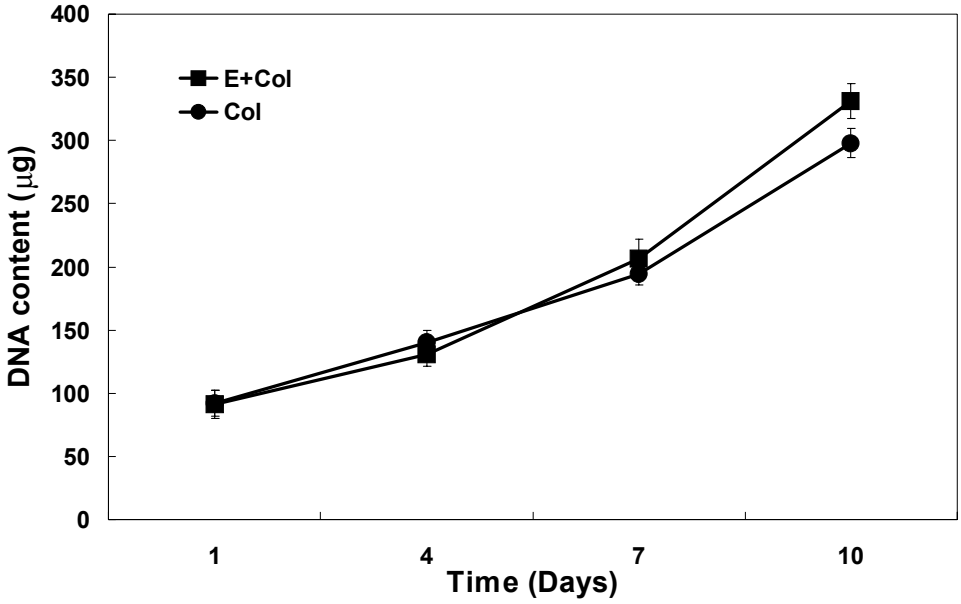
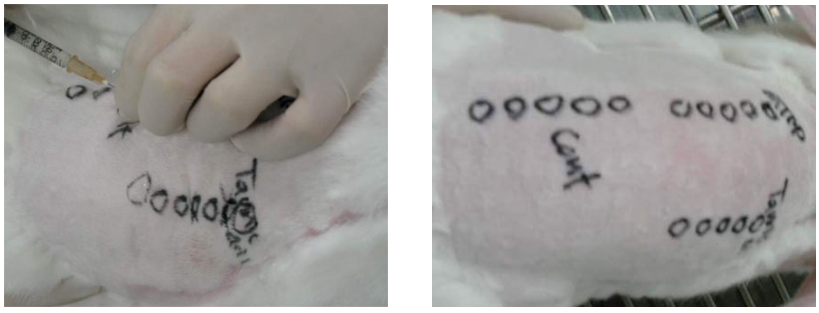



Figure 3-11. Human dermal fibroblasts proliferation on collagen and EGCG treated collagen was observed for 1, 4, 7, and 10 days. The DNA content, a measure of cell proliferation, was evaluated using the Hoechst 33258 fluorescent dye assay. Col: culture on collagen, E+Col: culture on EGCG treated collagen. Each point represents the mean SEM in 6 samples. (*: significant difference in cell proliferation between Col and E+Col, *: $P<0.05$)

and on EGCG treated collagen was 91.0 ± 11.4, 130.6 ± 9.6, and 206.8 ± 15.3 ng. In contrast, the absolute number of cells proliferated on EGCG treated collagen in 10 days of culture was slightly higher than on collagen (P0.05). The total DNA content in the fibroblasts culture on collagen in 10 days was 297.8 ± 11.5 and 331.0 ± 13.7 ng on collagen and EGCG treated collagen.

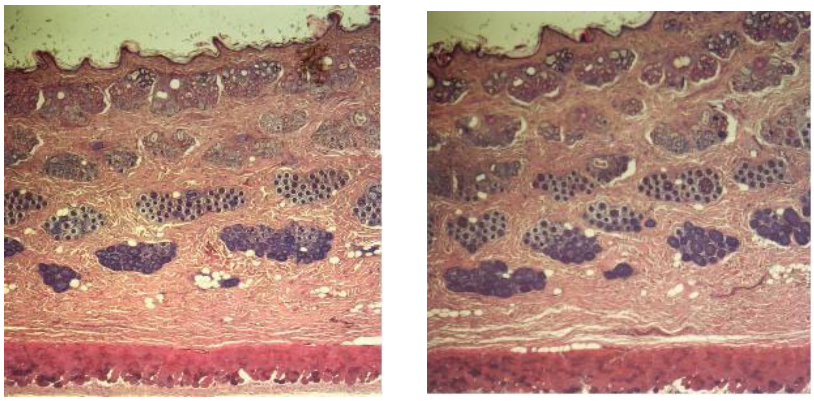
### 3.3.9. Intradermal test of EGCG treated collagen bioprosthesis

Intradermal test of EGCG treated collagen bioprosthesis was observed after subcutaneous injection on white rabbits (New Zealand) used as an animal model. Subcutaneous injection procedure of EGCG treated collagen bioprosthesis and skin response after the subcutaneous injection are displayed in figure 3-12, respectively (A: subcutaneous injection procedure of EGCG treated collagen bioprosthesis, B: skin response after the subcutaneous injection). As shown in figure 3-12, skin response such as erythema and edema was not visualized after the subcutaneous injection of EGCG treated collagen bioprosthesis from the naked eye observation. Figure 3-13 shows skin biopsy results after the intradermal test of negative control (A) and EGCG treated collagen bioprosthesis (B), respectively. According to the skin biopsy results, as shown in figure 3-13, EGCG treated collagen bioprosthesis did not exhibit specific histological indications such as inflammatory cell infiltration and fibrosis, comparison with the negative control.



(A) (B)

Figure 3-12. Subcutaneous injection procedure of EGCG treated collagen bioprosthesis on white rabbits (New Zealand) and skin response after the subcutaneous injection, respectively. (A) subcutaneous injection procedure of EGCG treated collagen bioprosthesis, (B) skin response after the subcutaneous injection.



(A) (B)

Figure 3-13. Skin biopsy after the intradermal test on white rabbits (New Zealand). (A) negative control (physiological saline solution), (B) EGCG treated collagen bioprosthesis.

### 3.3.10. Sensitization test of EGCG treated collagen bioprosthesis

To test for irritation and sensitization of skin, sensitization test of EGCG treated collagen bioprosthesis was observed. Guinea pigs were used as an animal model and the maximization test was generally used.

Figure 3-14 shows the skin response observed after the sensitization test of physiological saline solution as a negative control, EGCG treated collagen bioprosthesis, and 0.05% DNCB (2,4-dinitrochlorobenzene) as a positive control, respectively (A: physiological saline solution as negative control, B: EGCG treated collagen bioprosthesis, and C: 0.05% DNCB as a positive control). As shown in figure 3-14, in case of the EGCG treated collagen bioprosthesis and negative control except for the positive control, both the fraction response (FR) (positive number of animals/total number of animals) and the mean response (MR) (summation of score/total number of animals) after the challenge phase was zero, which indicates no sensitization.

### 3.3.11. Genetic toxicity test of EGCG treated collagen bioprosthesis

To test for the genetic toxicity of EGCG treated collagen bioprosthesis, Ames test was performed. Results of the Ames test in the S9 untreated group and the S9 treated group are shown in Table 3-1. And figure 3-15 shows the photographs of the results of the Ames test in S9 untreated strains of TA98, TA100, TA102, and TA1535, respectively, figure 3-16

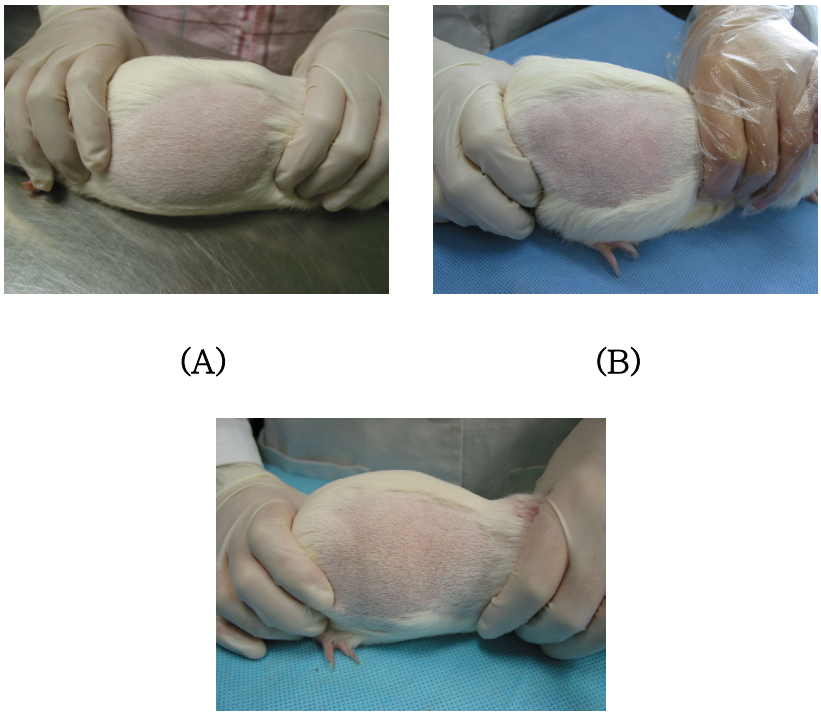
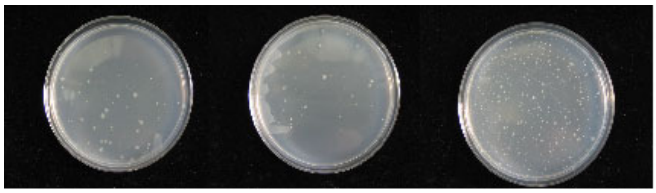



Figure 3-14 Skin response after the sensitization test on Guinea pigs. (A) negative control (physiological saline solution), (B) EGCG treated collagen bioprosthesis, (C) positive control (0.05% DNCB (2,4-dinitrochlorobenzene)).

Table 3-1. Ames test results in the S9 treated group and the untreated group.

| Strain/presence(+) or absence(-) of S9 treatment | Revertants/Plate | | | |
|---|---|---|---|---|
| | Negative control | EGCG treated collagen bioprosthesis | Positive control | Collagen |
| TA98/S9(-) | 40 | 37 | 240 | N/A |
| TA100/S9(-) | 59 | 55 | 276 | N/A |
| TA102/S9(-) | 42 | 36 | 184 | N/A |
| TA1535/S9(-) | 47 | 32 | 108 | N/A |
| TA97/S9(+) | 30 | 560 | 270 | ≥1,000 |
| TA98/S9(+) | 56 | ≥1,000 | 195 | ≥1,000 |
| TA100/S9(+) | 30 | ≥1,000 | 120 | ≥1,000 |
| TA1535/S9(+) | 36 | 520 | 162 | ≥1,000 |



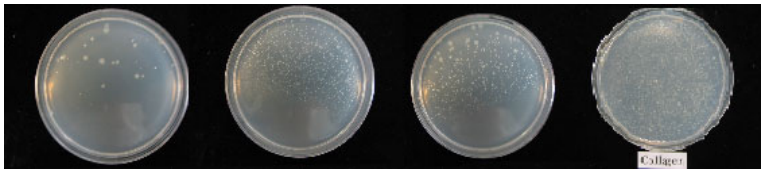
TA98 (-)S9 Negative control EGCG treated collagen Positive control(NQNO)

TA100 (-)S9 Negative control EGCG treated collagen Positive control(MMS)

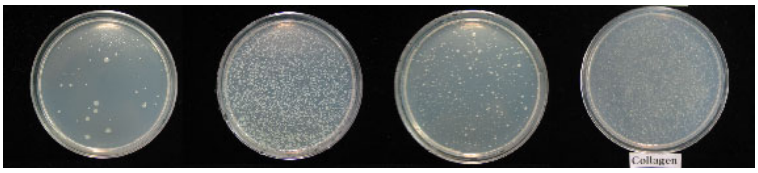
TA102 (-)S9 Negative control EGCG treated collagen Positive control(MMS)

TA1535 (-)S9 Negative control EGCG treated collagen Positive control(SA)

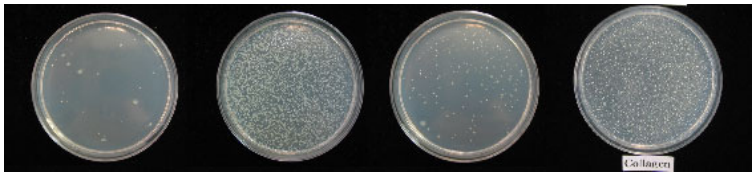
Figure 3-15. Photographs of the Ames test in S9 untreated strains of TA98, TA100, TA102, and TA1535, respectively. Physiological buffered saline solution was used as a negative control, and 4-nitroquinoline -N-oxide (NQNO), methylmethanesulfonate (MMS), and sodium azide (SA) were used as positive control.



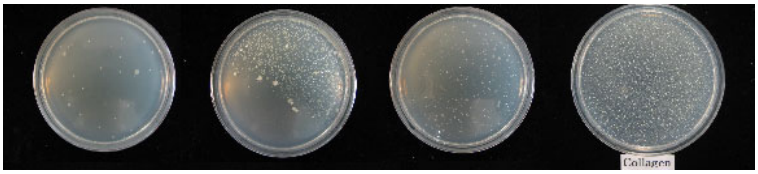

TA97 (+)S9 Negative control EGCG treated collagen Positive control(BP) Collagen




TA98 (+)S9 Negative control EGCG treated collagen Positive control(2-AA) Collagen




TA100 (+)S9 Negative control EGCG treated collagen Positive control(2-AA) Collagen




TA1535 (+)S9 Negative control EGCG treated collagen Positive control(2-AA) Collagen

Figure 3-16. Photographs of the results of the Ames test in S9 treated strains of TA97, TA98, TA100, and TA1535, respectively. Physiological buffered saline solution was used as a negative control, and benzopyrene (BP) and 2-aminoanthracene (2-AA) were used as positive control.

shows the photographs of the results of the Ames test in S9 treated strains of TA97, TA98, TA100, and TA1535, respectively.

As shown in table 3-1 and figure 3-15, in the S9 untreated *Salmonella typhimuriumstrains* of TA98, TA100, TA102, and TA1535, the EGCG treated collagen bioprosthesis did not induce mutagenicity, like the negative control as shown in table 3-1 and figure 3-15. In case of the S9 treated *Salmonella typhimuriumstrains* of TA97, TA98, TA100, and TA1532, significant growth of colonies was observed in all test strains of negative control, EGCG treated collagen bioprosthesis, and positive control as shown in table 3-1 and figure 3-16. This result is likely to be due to non-specific degradation of collagen by the S9 enzyme system, since a main component of the EGCG treated collagen bioprosthesis is collagen. Due to such a false positive reaction, the result from the Ames test is not to be trusted. Meanwhile, in order to confirm the false positive reaction, Ames test was performed using collagen in the S9 treated strains of TA97, TA98, TA100, and TA1535. As a result, growth of colonies was observed in all the test strains, suggesting that the false positive reaction of the EGCG treated collagen bioprosthesis was demonstrated.

### 3.3.12. Subacute toxicity test of EGCG treated collagen bioprosthesis

To test for the subacute toxicity of EGCG treated collagen bioprosthesis, the changes in the body weight, food consumption, relative weights of the internal organs, hematological values, biochemical values, and pathological values of Sprague-Dawley rats which were used as an animal model were performed.

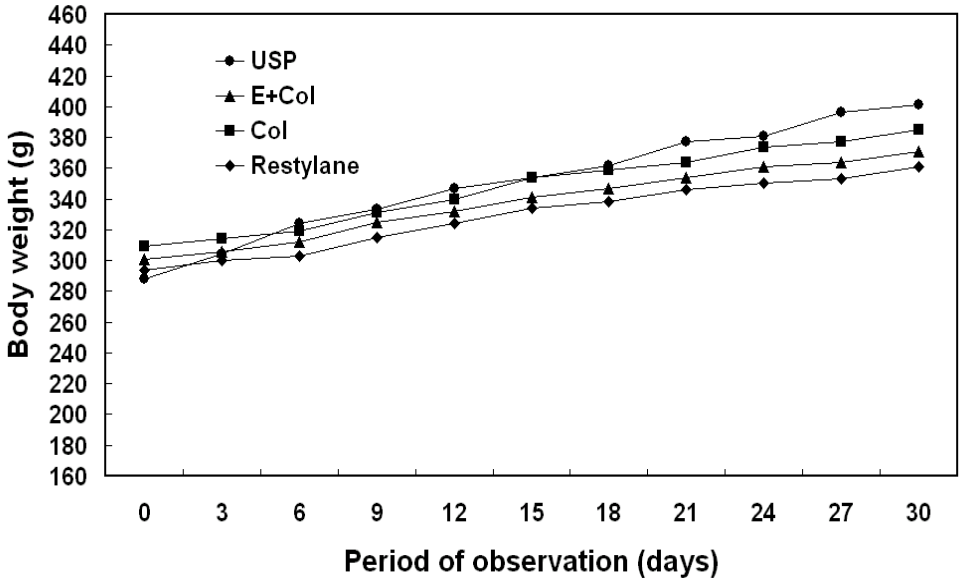



Figure 3-17. The changes in the body weight of rats during the subacute toxicity test on Sprague-Dawley rats. USP: USP polyethylene strip® as a negative control, E+Col: EGCG treated collagen bioprosthesis, Col: collagen solution (3%) as a comparative group I, Restylane: Restylane® as a comparative group II.

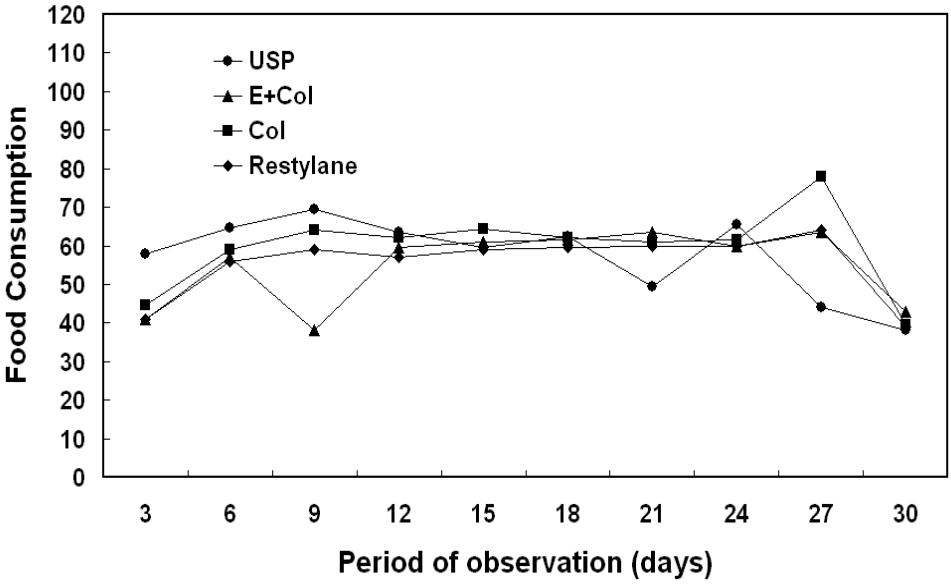



Figure 3-18. The changes in the food consumption of rats during the subacute toxicity test. USP: USP polyethylene strip® as a negative control, E+Col: EGCG treated collagen bioprosthesis, Col: collagen solution (3%) as a comparative group I, Restylane: Restylane® as a comparative group II.

Table 3-2. Relative weights of the internal organs of rats as a result of the subacute test.

Unit : g

| Internal organ | collagen | | USP polyethylene strip® | | Restylane® | | EGCG treated collagen bioprosthesis | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Adrenals | 0.02 | 0.005 | 0.02 | 0.005 | 0.02 | 0.005 | 0.02 | 0.005 |
| Brain | 1.27 | 0.068 | 1.29 | 0.100 | 1.30 | 0.155 | 1.19 | 0.247 |
| Kidneys | 2.71 | 0.301 | 2.43 | 0.170 | 2.48 | 0.194 | 2.59 | 0.075 |
| Liver | 14.0 | 2.240 | 13.4 | 0.850 | 12.63 | 1.432 | 13.2 | 0.055 |
| Lungs | 2.78 | 0.808 | 1.57 | 0.066 | 2.28 | 0.242 | 2.13 | 0.105 |
| Spleen | 0.97 | 0.064 | 0.84 | 0.060 | 0.92 | 0.115 | 0.82 | 0.094 |
| Testes | 4.66 | 1.847 | 5.18 | 0.215 | 5.54 | 0.365 | 5.32 | 0.566 |
| Thymus | 2.25 | 0.155 | 2.03 | 0.045 | 2.06 | 0.145 | 2.13 | 0.306 |
| Thyroid | 0.77 | 0.190 | 0.54 | 0.192 | 0.68 | 0.061 | 0.68 | 0.028 |

The changes in the body weight and food consumption of rats during the subacute toxicity test are displayed in figure 3-17 and figure 3-18, respectively. Relative weights of the internal organs of rats as a result of the subacute test are shown in table 3-2. In comparison with USP polyethylene strip® used as a negative control, EGCG treated collagen bioprosthesis almost never influenced on the body weight and food consumption, and on the relative weight of each of the internal organs of rats. Also, in case of the EGCG treated collagen bioprosthesis , macroscopic changes in the liver, kidneys, lungs, spleen, adrenals, and testes of rats were not observed. And collagen solution (3%) used as a comparative group I and Restylane® used as a comparative group II also almost never in influenced on the body weight and food consumption, and on the relative weight of each of the internal organs of rats.

Hematological values of rats measured after 4 weeks from the beginning of subacute toxicity test are shown in table 3-3, and biochemical values of rats measured after 4 weeks are shown in table 3-4. There was almost little difference between the EGCG treated collagen bioprosthesis and the negative control in terms of the hematological values, and clinical biochemical values and serum electrolyte values. And both comparative group, collagen solution (3%) and Restylane® also show almost little difference with the negative control and the EGCG treated collagen bioprosthesis in the hematological values, and clinical biochemical values and serum electrolyte values.

Pathological values of rats measured during 3 months of the subacute toxicity test are shown in table 3-5, and results of the Mann-Whitney test of statistical significance according to changes of the pathological values as a result of the subacute toxicity test are shown in table 3-6. Figure 3-19 shows pathological results, after 3 months after injection, during the

Table 3-3. Hematological values of rats measured after 4 weeks from the beginning of the subacute toxicity test.

| Hematological values | Collagen | | USP polyethylene strip® | | Restylane® | | EGCG treated collagen bioprosthesis | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Hb | 14.5 | 0.1 | 14.4 | 0.2 | 15.3 | 0.6 | 14.25 | 1.2 |
| HCT(%) | 50.4 | 0.3 | 52.6 | 1.2 | 55.2 | 1.7 | 51.2 | 3.7 |
| WBC ($10^3/mm^3$) | 7.375 | 0.7 | 7.17 | 0.7 | 7.61 | 1.8 | 6.7 | 1.6 |
| RBC ($10^6/mm^3$) | 5.19 | 0.2 | 4.84 | 0.3 | 4.40 | 0.1 | 6.075 | 0.4 |
| Platelet count ($10^3/mm^3$) | 672.5 | 103.5 | 473.66 | 130.4 | 582.0 | 157.2 | 710 | 131.0 |
| MCV ($\mu m^3$) | 68.3 | 1.1 | 73.5 | 4.9 | 72.4 | 1.8 | 76.3 | 0.7 |
| MCH (pg) | 19.65 | 0.4 | 20.2 | 0.9 | 20.1 | 0.7 | 21.25 | 0.1 |
| MCHC (g/dl) | 28.8 | 0 | 27.5 | 0.9 | 27.8 | 0.2 | 27.9 | 0.3 |

Table 3-4. Biochemical values of rats measured after 4 weeks from the beginning of the subacute toxicity test.

| Biological values | collagen | | USP polyethylene strip® | | Restylane® | | EGCG treated collagen bioprosthesis | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Albumin | 2.75 | 0.1 | 3.0 | 0.5 | 2.7 | 0.1 | 2.7 | 0.0 |
| Globulin | 3.1 | 0.1 | 3.0 | 0.1 | 2.9 | 0.0 | 3.05 | 0.1 |
| Total bilirubin | 0.45 | 0.1 | 0.9 | 0.7 | 0.36 | 0.1 | 0.35 | 0.1 |
| Cholesterol | 94 | 17.0 | 94.6 | 4.9 | 89.3 | 17.0 | 94 | 0.0 |
| SGOT (U/L) | 173 | 9.9 | 193 | 61.5 | 163.3 | 16.0 | 155 | 14.1 |
| SGPT (U/L) | 42 | 2.8 | 50 | 16.8 | 39.6 | 4.0 | 41.5 | 4.9 |
| LDH | 2653 | 164.0 | 2231.3 | 873.9 | 2194.3 | 235.3 | 2178 | 72.1 |
| ALP | 313.5 | 24.7 | 353.3 | 20.7 | 363.3 | 22.2 | 412.5 | 119.5 |
| CPK | 1336.5 | 75.7 | 1671 | 1210.3 | 1328.6 | 80.7 | 1128 | 79.2 |
| BUN | 26.15 | 3.5 | 20.9 | 3.2 | 21.6 | 1.4 | 21.75 | 3.2 |
| Alkaline phosphatase | 313.5 | 24.7 | 353.3 | 20.7 | 363.3 | 22.2 | 412.5 | 119.5 |
| r-GTP | 1.5 | 0.7 | 1.7 | 1.2 | 1 | 0.0 | 2 | 0.0 |
| Creatinine | 0.75 | 0.1 | 0.6 | 0.2 | 0.7 | 0.0 | 0.65 | 0.1 |
| Na | 139.5 | 0.7 | 138.6 | 2.1 | 139.3 | 0.6 | 138 | 1.4 |
| K | 10.35 | 0.1 | 11 | 1.9 | 11.63 | 0.5 | 11.25 | 0.6 |
| Cl | 94.5 | 0.7 | 94.6 | 1.5 | 95.3 | 0.6 | 94.5 | 2.1 |
| Ca | 11.25 | 0.1 | 11.8 | 0.6 | 10.9 | 0.7 | 11.45 | 0.5 |
| Triglyceride | 47 | 15.6 | 62.3 | 22.4 | 34.3 | 17.2 | 72 | 4.2 |

Table 3-5. Pathological values of rats measured during 3 months of the subacute toxicity test.

| Samples | Test rigion | Duration of Subacute toxicity test | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Days | | | Weeks | | | Months | |
| | | 0 | 1 | 3 | 1 | 2 | 4 | 2 | 3 |
| USP polyethylene strip® | Skin and Subcutaneous tissue | + | ++ | ++ | + | + | + | 0 | 0 |
| | | + | ++ | ++ | + | + | + | 0 | + |
| | | + | ++ | ++ | + | + | + | 0 | 0 |
| | | + | + | + | + | + | + | + | 0 |
| | | | ++ | + | + | ++ | + | 0 | 0 |
| Collagen | Skin and Subcutaneous tissue | + | + | +++ | + | + | + | 0 | 0 |
| | | + | + | + | + | + | + | 0 | 0 |
| | | | | | + | + | + | | 0 |
| | | ++ | + | +++ | ++ | + | + | 0 | + |
| | | ++ | + | +++ | ++ | ++ | + | 0 | + |
| | | ++ | | ++ | ++ | + | + | 0 | 0 |
| Restylane® | Skin and Subcutaneous tissue | + | + | + | + | + | + | 0 | + |
| | | + | + | 0 | + | + | + | 0 | + |
| | | | | | + | + | | | 0 |
| | | + | + | ++ | + | + | | + | 0 |
| | | + | + | + | + | + | + | + | 0 |
| | | + | + | + | ++ | + | + | 0 | 0 |
| | | | | | + | | | 0 | 0 |
| EGCG treated collagen bioprosthesis | Skin and Subcutaneous tissue | + | ++ | + | + | + | + | 0 | 0 |
| | | + | + | ++ | + | + | + | 0 | 0 |
| | | | | | ++ | | | | 0 |
| | | + | ++ | ++ | + | + | 0 | + | 0 |
| | | + | + | + | + | + | + | 0 | 0 |
| | | + | ++ | + | | ++ | 0 | 0 | 0 |

0 : Very rare infiltration of inflammatory cells

0~1+ : rare infiltration of inflammatory cells

1+ : Focal infiltration of inflammatory cells

2+ : Diffuse infiltration of inflammatory cells

3+ : Abscess formation

Table 3-6. Results of the Mann-Whitney test of statistical significance according to changes of the pathological values as a result of the subacute toxicity test.

| | Duration of Subacute toxicity test | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Days | | | Weeks | | | Months | |
| | 0 | 1 | 3 | 1 | 2 | 4 | 2 | 3 |
| USP®-Collagen | · | P≤0.05 | · | · | · | · | · | · |
| USP®-Restylane® | · | P≤0.05 | · | · | · | · | · | · |
| USP®-EGCG treated collagen bioprosthesis | · | · | · | · | · | · | · | · |
| Collagen-Restylane® | · | · | P≤0.05 | · | · | · | · | · |
| Collagen-EGCG treated collagen bioprosthesis | · | · | · | · | · | · | · | · |
| Restylane®-EGCG treated collagen bioprosthesis | · | · | · | · | · | · | · | · |

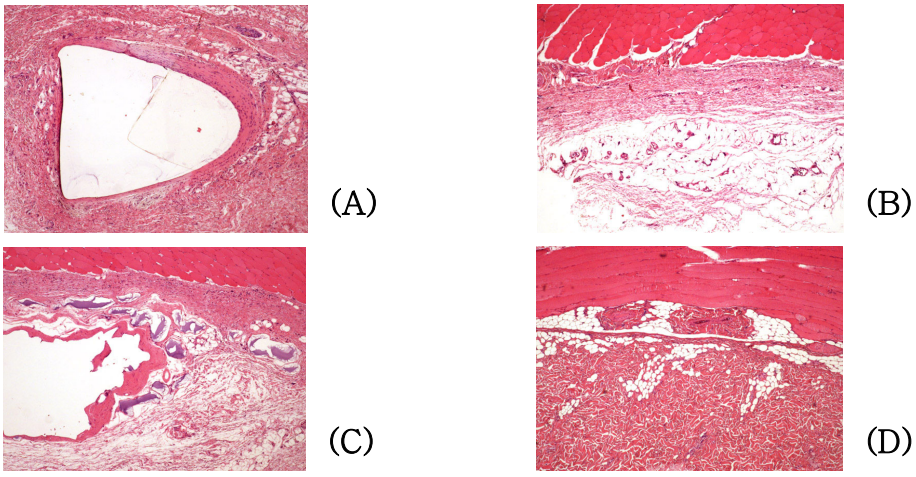



Figure 3-19. Pathological results, after 3 months later from injection. (A) USP polyethylene strip® as a negative control, (B) collagen solution (3%) as a comparative group I, (C) Restylane® as a comparative group II, (D) EGCG treated collagen bioprosthesis.

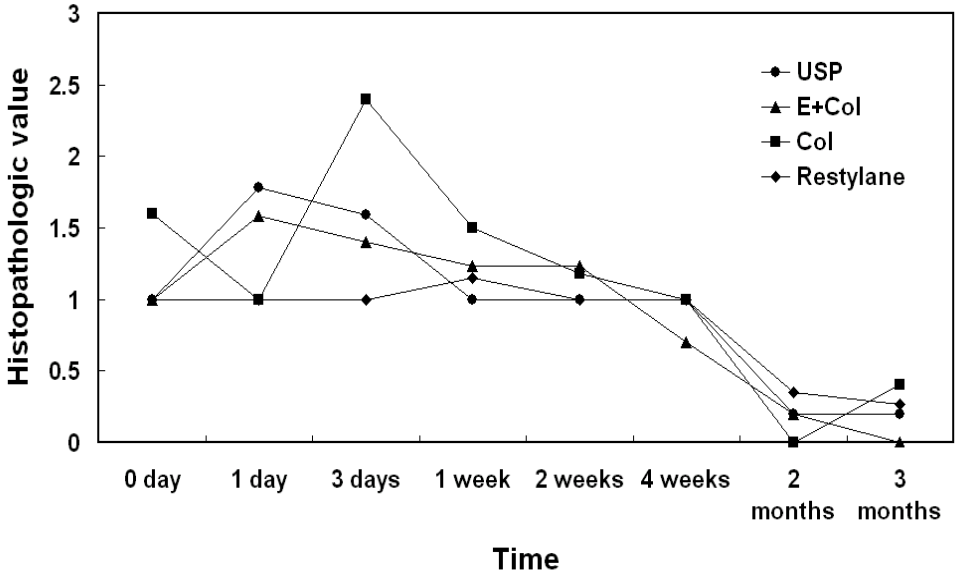



Figure 3-20. The changes in pathological values of rats during the subacute toxicity test. USP: USP polyethylene strip® as a negative control, E+Col: EGCG treated collagen bioprosthesis, Col: collagen solution (3%) as a comparative group I, Restylane: Restylane® as a comparative group II.

subacute toxicity test and figure 3-20 shows the change in pathological values of rats, during the subacute toxicity test. In addition, the EGCG treated collagen bioprosthesis did not exhibit a distinct difference to the negative control, as shown in pathological results. Therefore, it was confirmed that EGCG treated collagen bioprosthesis induces no systemic toxicity even when administered over an extended period of time. From the results of the Mann-Whitney test of statistical significance according to changes of the pathological values in the subacute toxicity test, the USP-collagen® and the USP-Restylane® exhibited significant difference at 1 day and the collagen-Restylane® exhibited significant difference at 3 days on a condition that of $P \leq 0.05$. On the other hand, in case of the comparison with other groups, the differences were not statistically significant.

## 3.4. Discussion

The connective tissue made principally of a network of tough protein fibers embedded in a polysaccharide gel, and this extracellular matrix is secreted mainly by fibroblast. As two main types of extracellular protein fiber are collagen and elastin, collagen is an extracellular matrix (ECM) that has cell adhesive property. When cells are supplied to a matrix consisting mainly of type I collagen, the cells adhere to the collagen fibers and contract the initially loose network to a dense tissue-like structure. This process is accompanied by a fundamental reprogramming of cell morphology and metabolism [29-32]. In spite of various biological roles of collagen, collagenous tissues from allografts is generally degraded by host collagen degrading enzymes after implantation due to their poor durability,

and, to increase their durability, collagen based biomaterials for implantation have been extensively stabilized in macroscopic level by crosslinking using chemicals or irradiations. Although it is known that inter-molecular crosslinking by chemical treatment enhances mechanical properties of collagen-based scaffold, the effect of crosslinking by chemical treatment on structural integrity and biological function of collagen is not been well known. In other researches, many researches demonstrated that collagen's thermal stability and resistance to enzymes could be enhanced through various techniques such as site-directed mutagenesis or molecular stabilization using sugar [7, 19]. Especially, it has been well known that sugar could stabilize collagen molecule through hydrogen bonding with the backbone of collagen due to its high level of hydroxyl group [19, 20]. Like sugar, there are a lot of hydroxyl groups in the backbone of polyphenols, especially in EGCG. This fact leaded us to investigate the role of EGCG on the stabilization of collagen molecule. In this study, we developed collagenase resistant collagen through structural stabilization using EGCG with maintaining native biological functions and enhanced anti-oxidation activity.

There might be several possible mechanisms, which may account for the stabilization of collagen by EGCG. The possible mechanisms might be mainly divided into macroscopic stabilization and microscopic stabilization. One possible mechanism in macroscopic level is the inter-molecular crosslinking of collagens. It has been reported that some polyphenols induce crosslinking through the formation of aldehyde groups on substrate only in the presence of copper ion [21], and it is also suggested that polyphenols are capable of the inter-molecular crosslinking between collagen molecules through the formation of multiple hydrogen or esteric bonds [22]. However, these mechanisms were not well elucidated yet.

Other possible mechanism in microscopic level is that the addition of EGCG may restrict the conformational space available to the collagen backbone like sugar in a way similar to that of proline, in which the steric restrictions imposed by pyrrolidine ring stabilizes the conformation of the helix [19, 33]. It is also suggested that glycosylation may influence the stability of the native state, through hydrogen bonds from sugar to the polypetide backbone [19], and hydroxyl groups of sugars is found to be decisive factors for their stabilizing effect on collagen structure [20]. It has been reported that EGCG and Epicatechin Gallate (ECG) are most effective for the inhibition of collagenase activity among green tea catechins [34]. The point that EGCG and ECG are different from other catechins is due to their having flexible galloyl rings on their backbone. The study for interaction of tannin with proline rich proteins (PRPs) demonstrates that an important aspect of the interaction is the stacking the gallic acid moieties of tannins against pyrrolidine rings of proline residues [35, 36]. In this study, collagen zymography and CD spectra were used to investigate the interaction of EGCG with collagen. EGCG has seven hydroxyl groups on the ring, and its hydroxyl groups might easily react with carboxyl group in the collagen molecule, resulting in the formation of the esteric bond. It was hypothesized that collagen molecules might be connected or crosslinked through such esteric bonds. In order to investigate precisely the interaction of EGCG with carboxyl groups of collagen, collagen was treated by succinyl anhydride to form succinylated collagen, resulting in the replacement of almost all amine groups of collagen molecule by carboxyl groups, which is previously described [25]. From the study, esteric bond formation between hydroxyl groups of EGCG and carboxyl groups of collagen molecules was not a major factor for resistance to degradation by collagenase. However, it might be

questionable that succinylated collagen should have also stabilized by EGCG in microscopic level, resulting from the formation of EGCG-succinylated collagen complex in the same manner with collagen. CD analysis reveals that succinylated collagen did not have identical triple helix structure of collagen molecule in comparison with non-treated collagen and EGCG treated collagen, resulting in the loss of collagen stability in succinylation. Such loss of collagen stability in succinylated collagen might prevent EGCG from enhancing the stability through the formation of succinylated collagen-EGCG complex. In comparison with succinylated collagen, collagen and EGCG treated collagen have a CD spectrum with a positive peak at 225 nm and a stronger negative peak at about 212 nm at 25 ℃, which are defining characteristics of a collagen triple helix. The ellipticity of collagen decreases with increasing temperature, which is characteristic of denaturation of the triple helix [37], but the ellipticity of EGCG treated collagen remained constantly at high temperature. From these results, it might be suggested that EGCG enhance the molecular stability of collagen resulting in increasing thermal stability. Additionally, EGCG treated collagen showed respectively high resistance to the degradation by collagenase and MMP-1 in comparison with collagen. The fibrillar triple helical collagens are cleaved by vertebrate collagenases at an appreciable rate under physiological conditions. The collagenases cleave their substrates in a highly specific manner, which cleave collagen at a single site (Gly775-Leu/Ile776) within each α chain of triple helical collagen molecule, approximately three quarters of the distance from the amino-terminal end of each chain, resulting in the generation of three quarter and one quarter length collagen fragments. Although there are several sites with same sequence (Gly-Leu or Gly-Ile) within collagen molecule, only 775-776 site can be

cleaved by collagenase. It is well known that the 775-776 site resides in the region with relatively loose triple helical structure, and that this flexibility of the site plays a role in collagenase recognition [38]. In other words, the structural stability of triple helix might be related to the resistance for collagenase. This can be supported by our results that EGCG treated collagen showed higher stability of triple helical structure of collagen molecule, resulting in relatively high resistance for collagenase. From the study of structural stability and collagenase resistance of EGCG treated collagen, it might be suggested that EGCG may restrict the conformational space available to the collagen backbone through stacking against collagen molecule, and several hydroxyl groups of EGCG might provide hydrogen bonding to collagen backbone, resulting in increasing the structural stability of collagen triple helix, and makes collagen be highly resistant to the degradation by collagenase. In other hands, it has been suggested that EGCG is a portent inhibitor for collagenase itself. From this study, EGCG alone could not inhibit the collagen degradation by bacterial collagenase itself. The role of EGCG as an inhibitor for collagenase needs to be investigated more.

Collagen degradation might be related to the immune response and durability of implanted collagenous matrix. Once collagen is cleaved by collagenase, resulting in two large fragment, the cleaved collagen fragments spontaneously denature into non-helical gelatin derivatives at physiological temperatures, thereby becoming susceptible to further degradation by other proteinases and altering their native conformation [14]. These proteolytic degradation of collagen is involved in both physiological and pathological process, such as inflammation. Macrophages are multifunctional immune cells that are involved in the regulation of immune and inflammatory responses. It has been reported that

marcrophage can recognize and adhere remarkably to denatured collagens that result from increased proteolytic activity at sites of inflammation [39]. From our study of macrophage adhesion assay, EGCG treated collagen was less susceptible to the degradation by collagenase than non-treated collagen, and there was no significant difference in macrophage adhesion between EGCG treated collagen (E+Col) and collagenase treated E+Col. Although collagen has been widely used in biomedical application, collagen still have some limitations as to their use in human tissue due to inflammation through their biodegradation and relatively short durability. It is reported that the rate of collagen degradation is related to humoral immune response and cell-mediated reaction [40]. For the EGCG treated collagen is far from degradation by collagenase, it might be predictable that the implanted EGCG treated collagen produces less inflammatory response due to the less degraded collagen substances.

Oxidative damage of proteins by free radicals involves increased susceptibility to proteolysis and fragmentation. It is implicated in various physiological and pathological processes [41, 42]. Roles of for the oxidative destruction of collagens in inflammatory as well as in age-related processes have also been proposed [43, 44]. Collagen fragmentation may occur either as direct result of an oxidation attack, or as a consequence of the enhanced susceptibility of oxidized collagen to enzymatic hydrolysis [45, 46]. It is also reported that thermal stability of type I collagen significantly reduced by oxidation [47]. It is well known that flavonoids such as catechins can be protective against cancer and inflammatory diseases, and these protective effects are largely due to their inhibition of some enzymes and antioxidative activities by scavenging free radicals [48]. In this study, free radical scavenging activity of EGCG treated collagen was investigated in comparison with collagen. Although

non-treated collagen also had a high free radical scavenging activity, free radical scavenging activity increased significantly in EGCG treated collagen. From the result of free radical scavenging activity assay, it might be known that EGCG plays a role of scavenging free radical even in the complex of EGCG-collagen in addition to enhancing collagen stability.

Collagen has been modified chemically to improve its biological and physical properties [49]. For example, collagen based biomaterials are usually stabilized either by physical or chemical crosslinking to control the rate of biodegradation of the material [50, 51], and succinylated collagen has been applied in cardiovascular implant coating in order to let the implants resistant to thrombogenesis [52]. However, the effect of these chemical treatments on molecular structural characteristics of collagen related to induce cell function has not been well investigated. The interactions of collagens with cells are important in a variety of biological events such as adhesion, migration, growth and differentiation [53, 54]. Cellular interactions with collagens have been shown to be mediated by cell surface receptors that belong to the integrin family. The two conformation-dependant integrins α1β1 and α2β1 represent to date the major receptors for collagen. The two integrins mediate the transmembrane signal transduction that ultimately leads to changes in gene expression patterns of fibroblasts, affecting cell functions such as cell proliferation, collagen synthesis, and collagenase expression. The mechanism of cell adhesion in this case may involve the formation of focal contacts through a clustering of cell integrin receptor on the cell surface that interact with collagen [29, 55]. It has been reported that cell adhesion to collagens via α1β1 and α2β1 integrins is strongly conformation dependant and that native and denatured collagens were not equivalent in their ability to support cell adhesion. A variety of approaches has shown

that disruption of the triple helical structure of collagen greatly affects cell adhesion level and disrupts the binding sites recognized by α1β1 and α2β1 integrins [46, 57]. The RGD (Arginine-Glycine-Aspartic acid) sequence, which commonly occurs in collagens, is considered to be a classical integrin-recognition motif. However, when included in the rigid collagenous triple helical conformation, this adhesive motif appears to be either not accessible to cells or exhibits a specific conformation which cannot be recognized by a RGD-dependant integrins. Therefore, cell interaction with denatured type I collagen has been postulated to be mediated by αvβ3 integrin rather than α1β1 and α2β1 integrins, which binds to proteins via RGD motifs [58]. It is reported that fibroblasts from aged donor exhibited fewer RGD-dependant integrins and decreased function of the integrins on substrates, but surface expression of α1β1 and α2β1 integrin was not affected by age [59]. Also, the quantity and quality of type I collagen in connective tissues is known to decrease with age [60], and it is probable that aged tissues have a reduced availability of specific matrix ligands that support cellular adhesion. These observations could emphasize the importance of structural aspect of collagen in the use of biomaterials. Our fibroblasts adhesion data suggest that the structural stability of collagen might be an important factor for cell adhesion. As known from adhesion assay, distinctively, 37 ℃ incubated collagen showed a very poor capacity for cell adhesion, and EGCG treated collagen might retain its structural integrity even after denaturation process at 37 ℃, resulting in favorable cell adhesion. Also, there was no significant difference in cell adhesion between RGDS treat fibroblasts and non-treated fibroblasts in each substrate except collagen, but the reduction in adhesion on collagen of RGDS treated fibroblasts might be related to partial denaturation of collagen at culture condition such as 37 ℃ incubation, which could be

supported by CD data. However, there was no significant difference in β1 integrin expression among substrates. This result might be explained that β1 integrin expression is generally balanced in a proper level in cells without specific signaling such as the induction by TGF β [61]. The acquisition of adhesive function by integrins may be a consequence of conformational changes in these receptors that result in an increased ligand binding affinity. The conformational change of their external domain, mediated by the integrin cytoplasmic domain, allows the integrins to bind to their ligand with high affinity without modifying the overall cell surface expression. In addition, cells can control integrin-mediated adhesion through other mechanisms, including receptor clustering and association to cytoskeleton, phenomena that regulate the avidity of integrins for ligand molecules without altering their monovalent affinity [62]. Regulation of cell adhesion can occur at several levels, including affinity modulation, clustering, and coordinated interactions with the actin cytoskeleton [63]. From our immunofluorescence staining assay, fibroblasts on collagen and EGCG treated collagen, retaining triple helical structure, could show well developed actin filament with spindle shape, but fibroblasts on 37 ℃ incubated collagen, losing triple helical structure, showed poor capacity to express their fibroblastic phenotype, which it could be known that structural integrity of collagen play important roles for cell morphology related to actin filament expression in spite of no significant difference in β1 integrin expression. In addition to cell adhesion on collagen, recent researches demonstrate that cell proliferation can be affected by the conformational stability of collagen. It is reported that cell proliferate poorly on denatured or fragmented collagen in comparison with collagen having natural structure [64]. In our cell proliferation assay on collagen and EGCG treated collagen, although it was expected that collagen might

be more easily denatured at culture condition of 37 ℃ incubation than EGCG treated collagen resulting in poor fibroblasts proliferation, no significant difference in cell proliferation was found. This might be due to the effect of serum, which is necessary for in vitro long-term cell culture, added in culture medium. FBS containing various proteins such as fibronectin and vitronectin might be absorbed on substrates, and absorbed various proteins might affect the adhesion and proliferation of fibroblasts [65]. These observations underscore the need for native, triple helical collagen conformation as a prerequisite for integrin mediated binding and functions of cells. Consequently, enhancing the stability of collagen molecule for biomedical application using collagen may be an important aspect in guiding the formation of tissue with certain structural and cellular functions.

EGCG treated collagen bioprosthesis did not exhibit distinct negative results in the safety tests such intradermal test, sensitization test, genetic toxicity test, and subacute toxicity test. From the observation of the Intradermal test of EGCG treated collagen bioprosthesis, according to the skin biopsy results, EGCG treated collagen bioprosthesis did not exhibit specific histological indications such as inflammatory cell infiltration and fibrosis. In the sensitization test of EGCG treated collagen bioprosthesis, the fraction response (FR) (positive number of animals/total number of animals) and the mean response (MR) (summation of score/total number of animals) after the challenge phase was zero, which indicates no sensitization. And in the genetic toxicity of EGCG treated collagen bioprosthesis, he EGCG treated collagen bioprosthesis did not induce mutagenicity. In addition, the EGCG treated collagen bioprosthesis did not exhibit any subacute toxicity in the test. These observations show the safety of the EGCG treated collagen bioprosthesis.

## 3.5. Conclusion

Collagenase resistant collagen has been developed through EGCG treatment of type I atelocollagen molecules. Collagen and EGCG treated collagen was prepared and characterized. The native conformational structure of collagen was maintained after preparation and purification of it, and so do EGCG treated collagen after treated EGCG.

Collagen zymography showed high collagenase resistance of EGCG treated collagen against degradation by bacterial collagenase and mammalian collagenase MMP-1. And, collagenase zymography of EGCG treated bacterial collagenase degradation on collagen showed that the effect of collagenase resistance was not the effect of EGCG by itself but that of EGCG treating on collagen though the mechanism of collagenase resistance of EGCG treated collagen and chemical conformation between EGCG and collagen was not elucidated. In addition, EGCG treated collagen molecule may not be resistant to degradation by collagenase due to the crosslinking of collagen by EGCG from collagen zymography of succinylated collagen and EGCG treated succinylated collagen. However, it might be questionable that corsslinking by succinylation of collagen could be the direct evidence of the collagenase resistance effect of crosslinking of EGCG on collagen, because succinylated collagen already lost identical triple helix structure of collagen molecule in comparison with non-treated collagen and EGCG treated collagen, resulting in the loss of native conformational structure of collagen. EGCG might be restrict the conformational space available to the collagen backbone through stacking against collagen molecule, and several hydroxyl groups of EGCG might provide hydrogen bonding to collagen backbone, resulting in increasing

structural stability of collagen triple helix, and make collagen be highly resistance to the degradation by collagenase. The role of EGCG as an inhibitor for collagenase needs to be investigated more.

CD spectra showed that EGCG treated collagen retained it's identical triple helical structure in comparison with collagen after denaturation process of incubation at 37 ℃ of physiological temperature due to its conformational stability enhance by EGCG. Free radical scavenging activity significantly in EGCG treated collagen, although collagen also had a free radical scavenging activity, suggesting that EGCG plays a role of scavenging free radical even in the complex of EGCG-collagen.

Macrophage adhesion assay showed that EGCG treated collagen was less susceptible to the degradation by collagenase than collagen. There was no significant difference in fibroblast adhesion between EGCG treated collagen and collagen, but for 37 ℃ incubated condition, the number of fibroblast adhered to EGCG treated collagen was significantly high than that of collagen. In addition, fibroblasts on collagen, EGCG treated collagen, and 37 ℃ incubated EGCG treated collagen, retaining triple helical structure, showed well developed actin filament with spindle shape, but fibroblasts on 37 ℃ incubated collagen, losing triple helical structure, showed poor capacity to express their fibroblastic phenotype. This results suggested that the structural stability of collagen play an important roles for cell adhesion and cell morphology related to actin filament expression. There was no significant difference in β1 integrin expression among collagen, 37 ℃ incubated collagen, EGCG treated collagen, and 37 ℃ incubated EGCG treated collagen substrates, suggesting that β1 integrin expression is generally balanced in a proper level in cells. In cell proliferation assay on collagen and EGCG treated collagen, significant difference in cell proliferation was not found.

From the toxicity test of EGCG treated collagen bioprosthesis using animal model, the results did not show toxicity. From the intradermal test, skin response such as erythema and edema was not visualized after the subcutaneous injection of EGCG treated collagen bioprosthesis from the naked eye observation, and EGCG treated collagen bioprosthesis did not exhibit specific histological indications such as inflammatory cell infiltration and fibrosis, comparison with the negative control. In case of the sensitization test, EGCG treated collagen bioprosthesis and negative control, both the fraction response (FR) (positive number of animals/total number of animals) and the mean response (MR) (summation of score/total number of animals) after the challenge phase was zero, which indicates no sensitization. The Ames test of genetic toxicity test showed that the EGCG treated collagen bioprosthesis did not induce mutagenicity in spite of false positive reaction owing to native character of collagen. And, there was no subacute toxicity on EGCG treated collagen bioprosthesis.

In view of the results so far achieved, these studies suggest that EGCG treated collagen assume to be a suitable biomaterial for biomedical application, especially for soft tissue augmentation.

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## 국문초록

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콜라겐은 가장 유용한 생체재료로 인식되어 왔다. 콜라겐이 지니고 있는 생분해성과 비교적 낮은 항원성과 같은 생물학적 특성에 기인한 생체적합성과 안전성으로 인하여 콜라겐은 생체의학분야에 응용되고 있는 기본적인 재료가 되었다. 이러한 생물학적 특성과 더불어, 콜라겐은 생체에서 거부반응을 일으키는 이물반응이 적어 인공피부, 인공근육, 인공혈관, 인공연골 및 인공뼈 등의 생체재료로서 많은 연구가 진행되어 왔다.

콜라겐은 체내삽입형 보형재료로서 사용하기 위한 목적으로, 생체 내에서의 콜라겐의 지속성을 증가시키기 위하여 가교된 형태의 콜라겐으로 많이 이용되어 왔다. 콜라겐을 화학적으로 개질하는 것은 콜라겐이 지니고 있는 삼중 나선 구조를 파괴하여 콜라겐 분자의 구조적 본질에 영향을 준다. 이러한 콜라겐의 구조적 특성은 세포와의 상호작용을 위한 생물학적 기능과 중요한 연관성을 지니고 있다.

본 연구에서는, 녹차 추출 성분의 하나인 EGCG 처리에 의하여 콜라겐의 구조적 안정성을 증가시켜 박테리아 콜라겐 분해효소와 생체의 콜라겐 분해효소에 의한 콜라겐 분해에 저항성을 지니도록 하였으며, 이는 자이모그라피 실험방법에 의해 확인하였다. 원편광 이색성 분광분석법 (Circular dichroism) 으로 분석한 결과에 의하면, EGCG가 처리된 콜라겐의 삼중 나선 구조는 생체온도인 37 ℃에서도 유지하는 것을 확인하였다. 그리고 콜라겐에 EGCG를 처리함으로써 높은 자유

라디칼 제거능을 확인할 수 있었다. 또한, EGCG가 처리된 콜라겐을 이용한 섬유아세포 배양실험에서, EGCG가 처리된 콜라겐의 구조적인 안정성은 세포 점착능, 액틴 필라멘트 발현, 세포 증식 등의 세포의 기능을 좋은 상태로 유지할 수 있도록 하고 있다. EGCG가 처리된 콜라겐은 콜라겐보다 대식세포에 대한 점착능이 낮아 콜라겐 분해효소에 의한 분해에 대한 저항성을 보이고 있으며, EGCG가 처리된 콜라겐과 EGCG가 처리된 콜라겐에 콜라겐 분해효소가 처리된 것에 대한 대식세포 점착능은 큰 차이가 없다.

게다가, EGCG가 처리된 콜라겐 보형재료의 동물모델을 이용한 피내반응 검사, 감작성 검사, 유전독성 검사 및 아급성 독성 검사와 같은 독성 검사에서 독성이 나타나지 않았다. 피내반응 검사에서, EGCG가 처리된 콜라겐 보형재료의 피하삽입 후 육안적 평가시 홍반이나 부종과 같은 피부반응을 확인할 수 없었고, 조직사진에서도 음성대조군과 비교하여 염증세포 침윤이나 섬유화 현상과 같은 특이한 조직학적 징후를 나타내지 않았다. 감작성 검사결과, EGCG가 처리된 콜라겐 보형재료와 음성대조군은 모두 첼린지 페이스 후의 FR (fraction response, positive number of animals/total number of animals) 값과 MR (mean response, summation of score/total number of animals) 값이 모두 0으로서 감작성을 확인할 수 없었다. 유전독성 검사에서는 EGCG가 처리된 콜라겐 보형재료는 콜라겐 자체의 특성으로 인한 거짓 양성반응이 나타남에도 불구하고, 변이원성을 일으키지 않는 것으로 확인되었다. 또한 EGCG가 처리된 콜라겐 보형재료의 아급성 독성이 없음을 확인하였다.

이러한 연구 결과들은 콜라겐이 지니고 있는 고유의 삼중 나선 구조가 인테그린이 매개한 세포 점착능과 세포의 기능을 위한 필요조건으로서 필요하다는 것을 시사하고 있다. 본 연구에 따르면, EGCG가 처리된 콜라겐은 그 구조적인 특성을 유지함으로써 콜라겐 분해효소에 의한 콜라겐의 분해 억제능을 지니고 있으며, 생체재료로서, 특히 보형재료로서 유용한 재료가 될 것이다.

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핵심되는 말: 콜라겐; EGCG가 처리된 콜라겐; 보형재료; 콜라겐 분해효소 억제능; 자유라디칼 제거능; 삼중 나선 구조

# 감사의 글

세월이 유수와 같이 흐름을 아는 늦은 나이가 되서야 박사학위를 받는 감회가 새롭습니다. 하나님께서 많은 돕는 자들을 예비하여 주시고, 박사학위논문을 할 수 있도록 인도하여 주셨습니다. 제가 잘 한 일이라곤 고통과 고난 속에서도 예수님의 옷자락은 놓지 않겠다고 선포하였던 것 이외에는 없습니다. 지난 세월 속의 어려웠던 일들과 힘들었던 상황들이 오늘 주마등처럼 뇌리를 스치지만, 그 속에서도 많은 분들께서 제게 힘을 주시고, 사랑을 베풀어 주셨습니다. 지면으로나마 그 분들께 감사함을 전하고자 이 글을 작성합니다.

가장 먼저, 능력 없고, 지식 없음에도 불구하고 학위를 예비하여 주신 하나님 아버지께 감사와 영광을 드립니다. 동경 유학시절부터 지금까지 지켜주신 하나님 아버지께서 계시지 않았다면 불가능할 일을 위로와 채찍으로 함께 하신 하나님은 언제나 저의 가장 큰 지원자이셨습니다. 오랜 세월 자식을 위하여 헌신하시며, 사랑으로 이끌어 주신 어머님께 불초한 자식이 머리를 조아려 진정한 감사의 마음을 전합니다. 언제나 한결같이 옆에서 지켜보며 자신을 낮추고 내조를 아끼지 않으며 마음고생을 많이 한 사랑스러운 아내에게 큰 고마움을 전합니다. 동경 유학시절에 하늘로 간 큰 딸 예인이, 언니를 대신하여 우리 가정의 큰 기쁨이 되고 있는 태정이, 그리고 앞으로 태어날 아들에게도 고마움을 전합니다. 여식을 훌륭하게 키워 배필로 주신 장인어른과 장모님께 감사함을 전합니다. 늘 힘이 되어 준 처제 하 옥선님과 동서인 김 재석님, 그리고 하 동찬님, 하 동훈님에게도 감사를 전합니다.

때로는 채찍질로 때로는 격려로, 그리고 때로는 사랑으로 이끌어 주신 서 활선생님, 지난 몇 년간 실력 없는 제자를 지도하시느라 마음고생이 심하셨습니다. 항상 온화한 모습으로 부족한 후배를 진정한 사랑으로 이끌어 주신 한국과학기술연구원의 조 현남 박사님의 말씀은 언제나 가슴에 새기고 살겠습니다. 두 분께 진정한 감사함을 전합니다. 아주대학교의 박 기동 박사님과 동경유학시절 후원자이신 동경여자의과대학의 Teruo Okano 교수님, 지도교수이셨던 동경대학교의

Kazunori Kataoka 교수님께도 감사함을 전합니다.

논문이 완성되기까지 많은 도움을 주었던 같은 연구실 후배 황 유식님과 마음으로 응원하여 준 후배 윤 주영님, 그리고 도움을 아끼지 않은 차 태호님께 특별한 고마움을 전합니다.

후배 사랑을 몸소 실천하시며 격려를 아끼지 않으셨던, 한국과학기술연구원의 홍 순만 박사님, 한 동근 박사님 박 기홍 박사님, 한양대학교의 김 종만 교수님, 중앙대학교의 손 태일 교수님, 태준제약 중앙연구소의 나 성범 소장님께 감사를 드립니다. 어려웠던 유학시절 정을 베풀어 주신 김 필동 목사님과 사모님, 그리고 오 충식 목사님과 사모님께 감사함을 전합니다.

많은 도움을 주신 연세대학교의 박 종철 박사님, 동거동락을 해 오면서, 도움을 아끼지 않은 연구실의 박 시내 박사, 송 민정님, 김 형범님, 설 아람님, 최 유석님, 차 재민님과 이 동휘 박사, 한 동욱님, 박 봉주님께 감사를 드립니다.

Kyoto대학교의 정 주은 박사와 그 남편인 Motoichi Kurisa 박사, 동생 같은 아주대학교의 김 제범님, 지 경수님, 김 용수님, 배 진우님, 안 상수님, 정 명철님, 정 윤기님, 대학시절부터 지금까지 우정을 나누어 온 생산기술연구원의 허 훈 박사, 삼성에 있는 김 형준 박사, 한국타이어의 김 종수님, 후배 한국과학기술정보연구원의 이 준우 박사, 금호공대의 권 오형 박사, 늘 따뜻한 말씀을 해 주신 한국과학기술연구원의 김 정봉 선생님과 이 명남 선생님께 감사를 드립니다.

기도로써 힘이 되어 준 김 희정님, 김 안나님, 이 지현님, 에벤에셀 식구들께 감사를 드립니다. 주의 종 손 석일 전도사님은 많은 위로로 참 마음의 평화를 찾아 주었습니다. 하나님의 예비하심에 용기 있는 결정을 내렸기에 그의 앞길에 주님이 함께 하실 것을 믿습니다.

부족한 오빠를 위해서 성심성의껏 도와 준 사촌동생 김 경희님께도 감사를 드립니다. 또한 물심양면으로 응원을 아끼지 않으신 작은 아버님들과 고모님들께도 감사를 드립니다.

끝으로, 심사위원을 맡아 주시며 논문을 많은 조언을 아끼시지 않으신 서 활 선생님과 조 현남 선생님, 연세대학교 의과대학의 양 승철 선생님, 연세대학교의 김 중현 교수님, 김 현민 교수님께 다시 한번 감사를 드립니다.

하나님께서 저와 함께 하심이 정녕 제게 큰 힘이 되었으며, 제 앞길이 그 분께서 예비하신 대로 가기를 원하며, 제 영혼이 항상 순종함과 기도함으로 아버지 하나님께 영광을 돌리기를 원합니다.

“네가 물 가운데로 지날 때에 내가 함께할 것이라 강을 건널 때에 물이 너를 침몰치 못할 것이며 네가 불 가운데로 행할 때에 타지도 아니할 것이요 불꽃이 너를 사르지도 못하리니” 「이사야 43장 2절」

“눈물을 흘리며 씨를 뿌리는 자는 기쁨으로 거두리로다. 울며 씨를 뿌리러 나가는 자는 정녕 기쁨으로 그 단을 가지고 돌아 오리로다” 「시편 126장 5-6절」