의공학회지 : 제 15권, 제 3호, 1994
J. of KOSOMBE : Vol. 15, No. 3

연 구 논 문
94-15-3-14

Biodegradable Inorganic-Organic Composite Artiticial Bone Substitute

-Part 2. Collagen purification and its physical and biological propertjes-

Hwal Suh

=Abstract=

To develop an artificial bone substitute that is gradually degraded and replaced by the regenerated natural bone, the authors designed a composite that is consisted of calcium phosphate and collagen. To use as the structural matrix of the composite, collagen was purified from human umbilical cord. The obtained collagen was treated by pepsin to remove telopeptides, and finally, the immune-free atelocollagen was produced. The cross linked atelocollagen was highly resistant to the collagenase induced collagenolysis. The cross linked collagen demonstrated an improved tensile strength.

Key words : Bone substitute, Collagen, Collagenolysis, Tensile strength

INTRODUCTION

To restore bone defects oriented from either extrinsic or intrinsic factors, an artificial bone substitute was designed as an inorganic-organic composite that would be gradually degraded and replaced by natural bone after implantation. The composite consists of calcium phosphate, especially a carbonate apatite that comprises about 80% of the bone apatite, and type I collagen according to the main compositions of the natural bone. A carbonate apatite with similar crystallinity to the natural bone could be obtained and would be used as inorganic component of the composite as the authors' previous paper[1].

Type I collagen is the main organic component of bone that comprises about 23~25% in spongeous bone and 30 % in cortical bone in volume as protein[2].

Recently, demineralized bovine bone is widely used as bone substitute in practice with expecting that the regenerating osteoblasts adhere to the demineralized bone, which mainly consists of collagen structural matrix. It also contains bone growth factor known as the bone morphogenic protein[3]. This demineralized bone collagen demonstrates some advantages in clinics, for its spongeous matrix absorbs physiological stresses. Otherwise, the loss of inorganics in the collagen may decreases the compressive strength reciprocation. Its less stress resistant characteristics often produces postoperative deformities also[4].

Collagen is a natural protein, which acts as the interwining fibrous network in whole tissues of the vertebrates as the structural componenet of the body. Cells are living with being contact to collagen fibers, for it is an extracellular matrix and obtains cell adhesive property. Collagen demonstrates highly sensitive immunity by the telopeptides which exist at the both extremities of the molecule, while the immunity disappears by removing the telopeptides. The telopeptide-free collagen is called as an atelocollagen that has been known as immune-free.

To use a collagen as a bone substitute matrix, the aut-

Institute of Biomedical Engineering, College of Medicine, Seoul National University
통신저자: 서 활, (110-744) 서울특별시 종로구 연건동 28, Tel. (02) 740-8596, Fax. (02) 745-7870

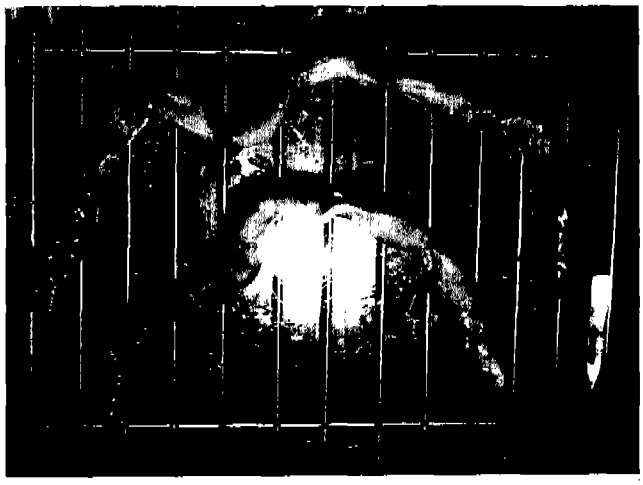

Fig. 1. Human umbilical cord fragments

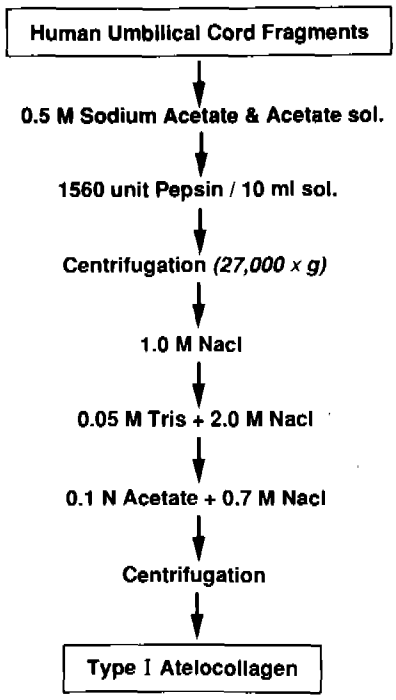

Fig. 2. Collagen purification process

hors extracted collagen from human umbilical cord, for the embryogenic collagen is less immunogenic[5]. In this study, qualification of the extracted collagen from human umbilical cord and its physical properties were investigated.

MATERIALS AND METHODS

1) Preparation of collagen

Human umbilical cord fragments were suspended in 75 % ethanol at 4 ℃, 24 hours, and the fragments were washed by distilled water for 24 hours at 4 ℃ (Fig. 1).

The fragments were ground in a homogenizer and placed in a 0.5M sodium acetate solution with or without EDTA at 4 ℃ for 16 hours. The wet precipitate was suspended in a 0.5M acetate solution. 3mg of Pepsin (1 : 10 000, 520unit/mg, P-7000, Sigma Co., St. Louis, MO, U.S. A.) was added to every 10 ml of the solution and stored at 4 ℃ for 16 hours and cetrifugation was performed at 27 000 × g.

The precipitate obtained from centrifugation was placed in 1.0M NaCl solution for one day. Centrifugation was followed, and the precipitate was dissolved in 2M NaCl containing 0.05M Tris buffer solution. The solution was centrifuged again, and the supernatant was mixed with 4.5M NaCl and controlled to pH 7.5 ± 0.1. After centrifugation, the precipitate was dissolved in 0.1N acetate contained 0. 7M NaCl solution at pH 7.5 ± 0.1 for one day. The solution was centrifuged, and the obtained precipitate was Type I atelocollagen (Fig. 2). All the procedures were performed at 4 ℃. After the extraction procedure, the collagen precipitates were lyophilized at -40 ℃ and -70 ℃, and reserved for the further use.

2) Strength enhancement by cross linking

1 %, 2 %, 3 %, 4 % and 5 % Type I atelocollagen solution was prepared by disolving in 0.001N HCl soltuion at 4 ℃ and buffered by 0.05M NaOH to pH 7.4. The atelocollagen solution was placed into an order made glass mold of 0.25 (height) × 10 (width) × 50 (length) mm. One experimental group was treated by 0.5 %, 1 %, 5 %, 10 %, 20 %, 30 % glutaraldehyde solution, while another group was irradiated by an ultraviolet ray with 254 nm wave length for 15, 30, 60 minutes, 2, 4 and 8 hours. Each group consisted of 4 specimens

After drying at 25 ℃ for 2 days, tensile strengths of the cross linked atelocollagen sheets were compared with a non-cross linked collagen sheet by a tensile strength measuring device (INSTRON Model 8511, Instron Corp., MA,

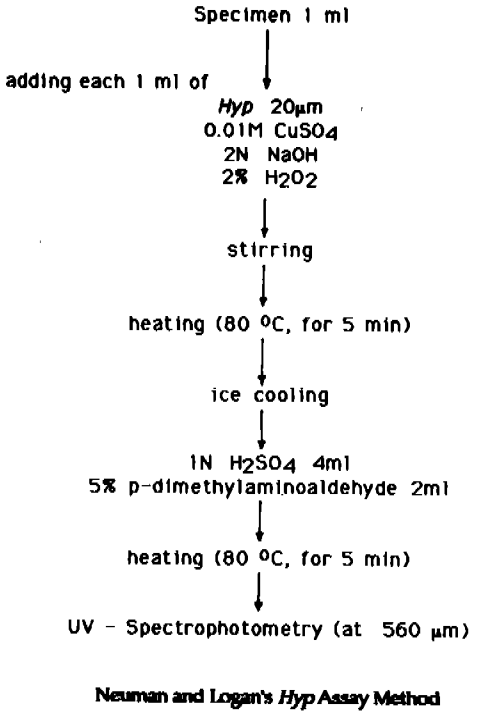

Fig. 3. Neuman and Logan's hydroxyproline assay process

U.S.A.). The sample was extended to break at a constant extension rate of 10 mm/min. The data were analyzed statistically using an analysis of variance (ANOVA) with multiple comparisons by a two-way analysis.

3) Collagenolysis

To investigate the collagenolysis rate of the cross linked collagen, Neuman and Logan's method of hydroxyproline assay was appplied. 1 wt% solution of human umbilical cord origin type I collagen was used. Cross linked collagens by 0.5%, 1, 5, 10, 20, 30% glutaraldehyde, and by UV ray irradiation for 15, 30, 60 minutes, 2, 4, 8 hours were testified. Each group consisted of 4 specimens. Bovine skin origin type I collagen (C 3511, Sigma Chemical Co., St. Louis, MO, U.S.A.) was the control specimen. 2 ml of the solution specimens were placed into a 10 ml of 0.05N $H_2NC(CH_2OH)_3$ [tris (hydroxymethyl) aminomethane] containing 2 ml of collagenase (C 0130, Sigma Chemical Co., St. Louis, MO, U.S.A.) controlled to 10, 20, 30, 40, 50 unit/ml in an experimental glass tube. After incubation at 37 ℃ for 5, 10, 15, 30, 60 min, 1 ml of ethanol was added

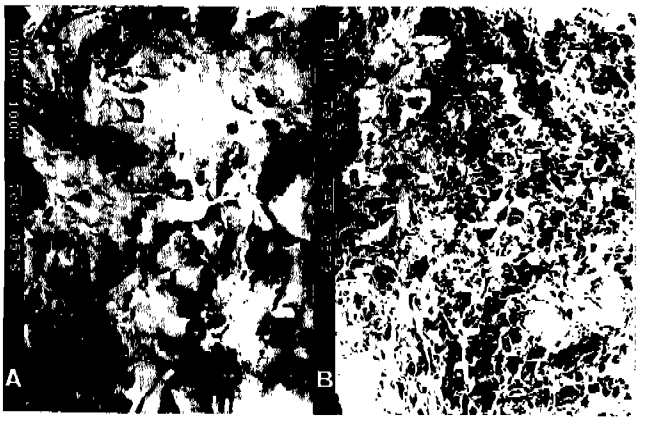

Fig. 4. Collagen matrix obtained from human umbilical cord by a SEM
A: lyophilized at -40 ℃ (× 100)
B: lyophilized at -70 ℃ (× 100)

to stop the collagenase digestion activity. 1 ml of the supernatant was obtained as a specimen, and mixed with 0.2 ml of 6N HCl and hydrolyzed in a dry constant temperature oven at 110 ℃ for 24 hours. The incubated liquid specimen containing derived hydroxyproline from the collagenase digested collagen solution was analyzed by an UV spectrophotometer (UV-150-02, Shimadzu Co., Tokyo, Japan) at 560 μm, and selected a 20 μg/ml hydroxyproline as a standard reagent (Fig. 3).

RESULTS AND DISCUSSION

The method used in this study to extract Type I collagen is a modification from the method applied by Niyibizi et al.,[6] and the purity is over than 99.8% as the author's previous report[7]. Lyophilization of the extracted collagen is an efficient storage method to keep the biochemical properties of the collagen. The extracted and lyophilized collagen at -40 ℃ was coarser than the -70 ℃ specimen without significant difference in the collagen property (Fig. 4).

Collagen is the most abundant protein in mammalians that consists about 30% of all body proteins. More than ten types have been isolated from type I to XI. Collagen consists of fibrillar proteins. Three polypeptide chains (α -chains) associate to form a collagen molecule that builds up a fibrillar unit. An α-chain contains about 1 000 amino acids with approximately 3 000 Å of length. Molecular

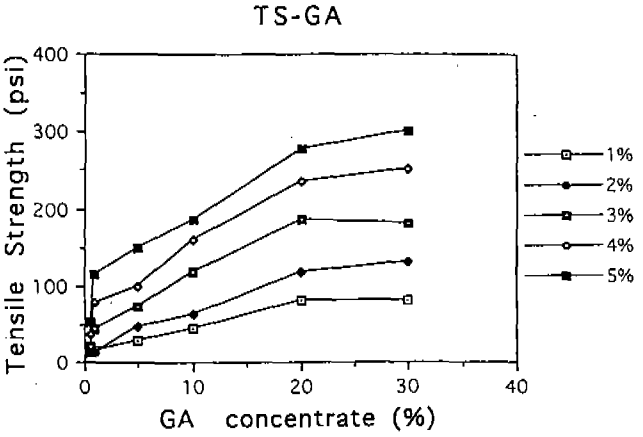

Fig. 5. Tensile strength of the glutaraldehyde treated collagens

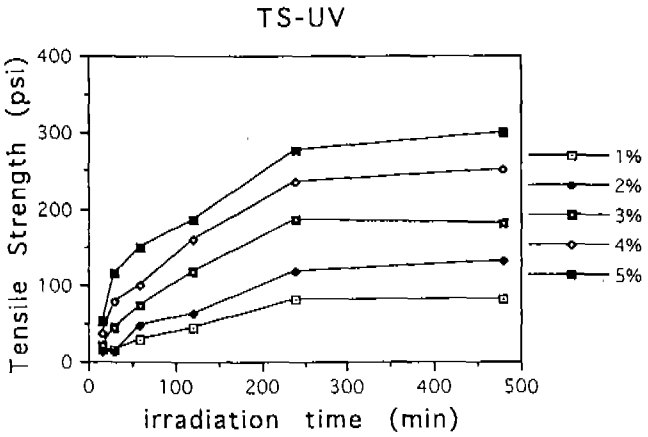

Fig. 6. Tensile strength of the UV ray irradiated collagens

weight of collagen is about 360 000, so the weight of an α -chain is about 120 000. At least 10 genetically distinct chains are introduced with differing in the composition and sequence of their amino acids. But their configurations are same, and glycine (Gly) occupies every third position in the amino acid sequence. An α-chain is a helical conformation of the amino acids. About 96% of the chain length is helical, and non-helical telopeptides are existing at both of the amino- and carboxyl- termini. These telopeptides are integrated each other to form a linear chain, and antigenic characteristics appear at these. The combination of 3 α-chains determines the type of the collagen molecule.

Collagen is degraded by proteolytic attack of collagenase, which breaks only the helical domains of the chains. Then smaller fragments become susceptible to proteolysis by other proteases, and the resulting fragments are resolved by phagocytosis. Collagenase is produced by fibroblast, polymorphonucleic lymphocyte (PMNL) and macrophage.

Collagen is an extracellular matrix, that is mainly produced and released by fibroblasts, and it provides favorable environment for cell adhesion and growth. When pepsin is applied to collagen, it breaks the telopeptide to dissociate the α-chain. The telopeptide -free collagen is called as an atelocollagen, which is known as antigenecity-free but keeps the other collagen characteristics.

These properties have leaded many challenges to use collagen as biomaterials. In cell culture, collagen is used as an additive medium to where cells adhere and grow in environment similar to nature by usually coating on the culture dish. Such a cell adhesive charactetistics is also applied to use collagen as hemostatic agent. Platelet adhesion to collagen accelerates blood clot formation, and the readily supplied fibrous network of the collagen reduces the natural clotting time. Some investigators suggested that a collagen coated artificial vessel graft is favorable to induce endothelial cell proliferation and adhesion, for which can enhance antithrombogenecity of an artificial vessel. Artificial skin is also a field where collagen matrix plays important roles. The epithelial cells cultured on collagen matrix or sheet is protecting the wound to the air, and the collagen is gradually degraded and replaced by natural skin[8,9].

In this study, type I atelocollagen was used as a biodegradable and natural tissue replacable matrix which would bind the inorganic substances and be periodically degraded during the regenerative natural bone replace the inorganic-organic composite bone substitute.

UV ray irradiated collagen matrix on the surface was more condensed than the non irradiated control specimen. It has been known that some chemicals, such as glutaraldehyde, or some light sources, such as γ-ray and ultraviolet ray enhance the cross-linkings of collagen without affecting physiological properties of the collagen[10]. The effect of glutaraldehyde and UV ray on enhancement of the collagen cross link was investigated in this study by measuring the tensile strength. The glutaraldehyde cross linked speci-

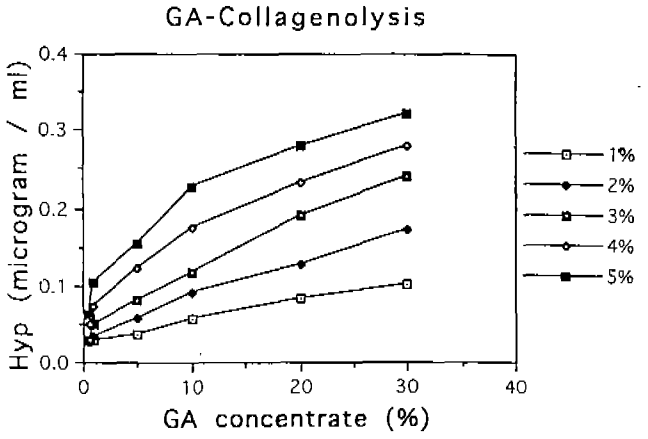

Fig. 7. Derived hydroxyproline amount after collagenolysis of the glutaraldehyde treated collagens

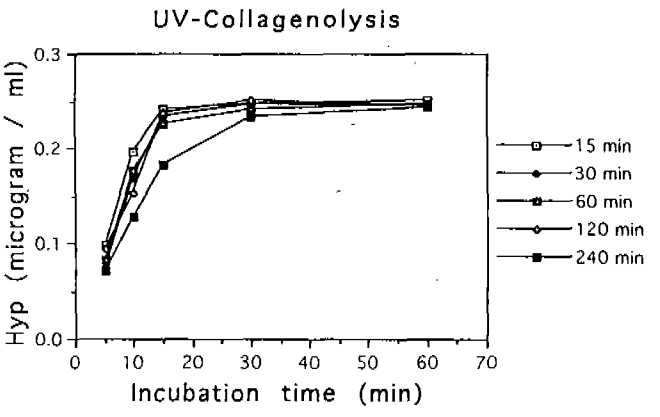

Fig. 8. Derived hydroxyproline amount after collagenolysys of the UV ray irradiated collagens

men demonstrated the higher resistance than the UV ray cross linked one to the strength (Figs. 5 and 6). But concerning to the reaction of the residual aldehyde to tissues, UV ray irradiation would be better to use, for the specimen of 4 hours UV irradiation revealed sufficient tensile strength as much as the 20% glutaraldehyde treated one.

Hydroxyproline is a typical amino acid that exists in the collagen α chain, and Neuman and Logan's assay has been an well known efficient tool to measure the released hydroxyproline's amount during collagenolysis by collagenase. In this study, the collagenolysis rate decreased as the cross link rate increased. The less cross linked collagens demonstrated the earlier rapid degradation, though the early period within 10 minutes demonstrated similar amount of the released hydroxyproline (Figs. 7 and 8). UV irradiation for over than 60 minutes was an efficient method to prevent the collagenolysis. There was no difference in the collagenolysis rate between the control bovine skin origin type I collagen and the specimen extracted from human umbilical cord.

CONCLUSION

To obtain a collagen that would be used as a structural matrix in an organic - inorganic composite artificial bone substitute, the authors extracted type I collagen from human umbilical cord. To enhance cross link of the collagen to increase the resistance against the physical tensile strength and biological collagenolysis, UV irradiation was conferred. The followings are conclusions.

1. UV irradiation is an efficient tool to increase collagen cross link as much as a glutaraldehyde treatment.
2. Collagen's resistance to the tensile strength and collagenolysis increases as the cross link rate increases
3. Cross link by an UV ray irradiation for over than 4 hours provides reliable physical and biological stability to collagen.

ACKNOWLEDGMENT

Dr. Choon ki Lee, Department of Orthopaedics, Seoul National University, is a coworker of this study.

The authors appreciate Miss Sue-Jin Ahn for her outstanding assistance on experiments in this study.

This research has been supported by the Korea Science and Engineering Foundation (Grant No. 93-13-00-01-3).

REFERENCES

1. Lee, C. K., Suh, H.: Biodegradable Inorganic-Organic Composite Artificial Bone Substitute. Part 1. Synthesis and Crystallinities of Inorganic Substances. J KOSOMBE, 15:195-199, 1994.
2. Fox, S. I.: Human Physiology, 4th ed., p 35-39, Wm. C. Brown Publishers, Iowa, 1993.

3. Guglielmotti, M. B., Alonso, C., Itoiz, M. E., Cabrini, R. L.: Increased osteogenesis in alveolar wound healing elicted by demineralized bone powder. J Oral Maxillofac Surg, 48: 487-490, 1990.
4. Amonti, U., Petit, J. C., Moehl, T., van den Heever, B., van Wyk, J.: Immediate reconstruction of massive cranio-orbito-facial defects with allogenetic and alloplastic matrices in baboons. J Craniomaxillofac Surg, 21: 302-308, 1993.
5. Ohno, M.: Ideal biomaterial-Embryonic collagen. Proceedings, The 25th International Biomaterials Symposium, p 48, Birmingham, AL, April, 1993.
6. Niyibizi, C., Fietzek, P. P., van der Rest, M.: Human placenta type V collagens. Evidence for the existence of an α1(V) α2 (V) α3(V) collagen molecule. J Biol Chem, 259: 14170-14174, 1984.
7. Suh, H.: Method of type I collagen purification from human umbilical cord. Pat Pend 94-9735, Korea Patent Bureau, 1994.
8. Quteish, D., Singh, G., Dolby, A. E.: Development and testing of a human collagen graft material. J Biomed Mater Res, 24: 749-760, 1990.
9. Nimni, M. E.: Collagen. CRC Press, Florida, 1988.
10. Nimni, M. E., Cheung, D., Strates, B., Kodama, M., Sheikh, K.: Chemically modified collagen: biomaterial for tissue replacement. J Biomed Mater Res, 21: 741-771, 1987.