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Preparation of New Acellular Dermal Collagen Scaffolds *via* Combination of Phytoproteinases and Supercritical Carbon Dioxide Treatments

Wang-Yao Wu[1], Tse-wei Ciou[1], Kuan-Ta Chen[1], Tzu-hung Su[1], Meng-Jen Yang[2], Ing-Lin Chang[3] and Ching-Cheng Huang[1,3,4,*]

[1]Department of Biomedical Engineering, Ming Chuan University, Taoyuan, China
[2]PARSD Biomedical Material Research Center, Changzhou, China
[3]Department of Orthopaedics, Changhua Christian Hospital, Changhua, China
[4]Jiangsu University of Technology, Changzhou, China
[*]Corresponding author

***Abstract*—Tissue-engineering technology was important and popular which combine medical applications and engineering materials knowledge such as a cell extraction process, which was employed to remove the cellular components from porcine dermal, leaving a matrix of collagen, elastin, collagen, and tightly bound glycosaminoglycans(GAGs). Said cell extraction process was used to remove cellular components from porcine dermal materials. Different porosities and microstructure of the acellular tissues were could be created by using different concentrations of related enzymes and collagenase. In this study, the uniform porcine dermal would be obtained by using a designed machine. It get dermis exactly and quickly via a machine which was designed from our team(powerdriven dermatome, SIACIN international, SC-GST). The porcine dermal must remove fat and other impurities almost by supercritical fluid(SCF), otherwise adipose tissue will become rot immediately. For collagen scaffold samples, dermis were hydrolyzed by enzymes of different concentration, in this way it can gain stant completely and clearly. Electron microscopy revealed effect in extracellular matrix (ECM) of porcine dermal tissue via supercritical fluid and different concentrations enzymes. This suggests the enzymes concentration and when you put porcine dermal in SCF was mightily important for effect of the collagen.**

Keywords—ECM; enzymes; SCF

I. INTRODUCTION

Tissue and organ decellularization techniques are important to developed new biomedical materials for repair, replace and improve human's organ function[1]. The expected effects on the mechanical and biological properties of the extracellular matrix (ECM) are observed in orthopedics. Decellularized scaffolds had a lot of collagens, which constitute the main structural element of the extracellular matrix (ECM), provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development[2,3]. Dense connective tissue is an abundant source of materials with extracellular matrix (ECM), which could be obtained by using defatting and decellularizing procedures. Furthermore, the treatment of supercritical carbon dioxide fluids (SCF-CO_2) process could be employed as a defatting procedure and a decellularizing procedure. Also ethanol was used as a cosolvent in the supercritical carbon dioxide fluids (SCF-CO_2) process. The cosolvent would affect the solubility of extraction in supercritical carbon dioxide fluids (SCF-CO_2) system. The pressure and temperature of supercritical carbon dioxide fluids (SCF-CO_2) process could be controlled. The specific permeability and solubility of resulting material would be observed. In this study, the supercritical carbon dioxide fluids (SCF-CO_2) process was tried to be a suitable procedure to prepare new designed collagen materials having collagen molecules consist of a triple helix approximately 300 nm in length[4]. The acellular dermal collagen scaffolds would be obtained, analyzed and characterized by SEM, TGA, DSC, FTIR after freeze-dried procedure.

II. EXPERIMENTAL

A. Materials

The chemicals used in the enzymes of trypsin (Sigma-Aldrich Company), bromelain (Sigma-Aldrich Company), ficain (Sigma-Aldrich Company), 0.5M acetic acid and 20% alcohol and sodium dihydrogen phosphate and disodium hydrogen phosphate (First Chemical Works Company, Taiwan). Supercritical carbon dioxide fluid operating conditions: carbon dioxide's purity of 99.999%, pressure of 9 MPa, temperature of 50ºC, sample was washed time for 5 hours.

B. Preparation of the Porcine Dermal' Sample

Selection of ISO9001 quality certification, ISO14001 environmental certification porcine with the machine which self-made R & D, it can produce steadily the thickness of about 5mm of dermal tissue.

C. Decellularization by Using Trypsin and Supercritical carbon dioxide fluids (SCF-CO_2)

Sample XTp-S (X represented the concentration in reagent solution) were setting trypsin(0.1%,0.25%,0.5%,0.75%,1%, Sigma-Aldrich Company) aside at 27ºC for 12h. Then, decellularized sample S-XTp, XTp-S was washed with PBS(38 g sodium dihydrogen phosphate and 5.04 g disodium hydrogen phosphate in 1L dd water, both Sigma) for removal of residual fat, and organic substance. Finally, used supercritical carbon dioxide fluid(SCF), and analyzed by SEM, TGA, DSC, FTIR after freeze-dried.

D. Degreaser Treatments by Using Phytoproteinases and Supercritical Carbon Dioxide Fluid(SCF)

Samples XB-S, XF-S were setting bromelain and ficain (5, 10, 15, 30μg / ml, both Sigma) aside at 27ºC for 12h. Second, decellularized sample XB-S, XF-S was washed with PBS ,and DD water for 10 minutes. Third, used Acetic acid (0.5M) and Ethanol(20%) reagent solution concuss in Ultrasonic Cleaner for 2 and 1 hours, then washed with PBS for 10 minutes for removal of residual fat ,and organic substance. The resulting collagen scaffolds could be obtained, analyzed and characterized by SEM, TGA, DSC, FTIR after freeze-dried procedure.

E. The Treatments by Using Supercritical Carbon Dioxide Fluid(SCF)

Supercritical carbon dioxide fluid was used to pretreatment for degreaser treatments in the beginning. It also was used to be a Control Variables. Furthermore, cosolvent was considered in the treatments.

III. RESULTS AND DISCUSSION

In this study, a series of new collagen scaffolds were designed and prepared from porcine dermal sheets by using a combination of enzyme treatments and supercritical carbon dioxide fluid technology. The resulting scaffolds could be a potential application for minimally invasive surgeries in orthopedics. The new scaffolds could be prepared by using a series of combined procedures as listed in Table I and Table II. Furthermore, the Trypsin and a series of phytoproteinases such as bromelain and ficain were employed in this study.

TABLE I. PREPARATION OF COLLAGEN SCAFFOLD VIA TRYPSIN OF TREATMENTS

Sample Number	Reagent	Concentration, (μg / ml)	PreSCF[a)](hrs)	PostSCF[b)] (hrs)
0.1Tp-S	Trypsin	0.1%	X[c)]	5
0.25Tp-S		0.25%	X[c)]	5
0.5 Tp-S		0.5%	X[c)]	5
0.75Tp-S		0.75%	X[c)]	5
1Tp-S		1%	X[c)]	5
S-0.1Tp		0.1%	5	X[c)]
S-0.25Tp		0.25%	5	X[c)]
S-0.5Tp		5%	5	X[c)]
S-0.75Tp		0.75%	5	X[c)]
S-1Tp		1%	5	X[c)]

a) The SCF was employed before trypsin treatments
b) The SCF was employed after trypsin treatments
c) The SCF was not employed in the experiments

Bromelain is a crude extract from the pineapple that contains, among other components, various closely related proteinases, demonstrating, in vitro and in vivo, antiedematous, antiinflammatory, antithrombotic and fibrinolytic activities[10]. In addition, bromelain contains several proteinase inhibitors[10]. Stem-bromelain (EC. 3.4.22.32) is distinguished from fruit-bromelain (EC. 3.4.22.33), previously called bromelin [10]. Bromelain belongs to a group of proteolytic enzymes which are used as drugs for the oral systemic treatment of inflammatory and malignant diseases. For the applications of medical devices, bromelain could be an active molecule for preparing the biomaterialsand products. Apart from the plant cysteine-proteinases bromelain and papain, the group comprises proteinases from animal organs such as trypsin and chymotrypsin. These enzymes offer a wide spectrum of therapeutic efficacies such as in vitro and in vivo, antiedemateous, antiinflammatory, antithrombotic and fibrinolytic activities[10]. Bromelain was employed in this study for preparation of acellular dermal collagen matrix via a combinated procedure with supercritical carbon dioxide fluid(SCF-CO_2) as listed in Table II.

TABLE II. PREPARATION OF COLLAGEN SCAFFOLD VIA PLANT PROTEASE OF TREATMENTS

Sample Number	Reagent	Concentration (μg / ml)	PreSCF[a)](hrs)	PostSCF[b)](hrs)
5B-S	Bromelain	5	X[c)]	5
10B-S		10	X[c)]	5
15B-S		15	X[c)]	5
30B-S		30	X[c)]	5
5F-S	Ficain	5	X[c)]	5
10F-S		10	X[c)]	5
15F-S		15	X[c)]	5
30F-S		30	X[c)]	5
S-5B	Bromelain	5	5	X[c)]
S-10B		10	5	X[c)]
S-15B		15	5	X[c)]
S-30B		30	5	X[c)]
S-5F	Ficain	5	5	X[c)]
S-10F		10	5	X[c)]
S-15F		15	5	X[c)]
S-30F		30	5	X[c)]

a) The SCF was employed before plant protease treatments
b) The SCF was employed after plant protease treatments
c) The SCF was not employed in the experiments

A. The Micro-structure of Resulting Collagen Scaffolds

Phase behavior of solutes in supercritical fluids can be modified by the addition of small amounts of cosolvents such as methanol and ethanol. In this study, ethanol was employed for the treatment of supercritical carbon dioxide fluids (SCF-CO_2) process. The addition of ethanol would increase solubility selectively or non-selectively of supercritical carbon dioxide fluids(SCF-CO_2) processing and enhance solubility because of an increase in solvent density and/or intermolecular interactions. The ethanol could improve the feasibility of a process by improving solvent loading and/or selectivity of supercritical carbon dioxide fluids (SCF-CO_2) treatments[11]. The effect in ternary systems of lipid components such as fatty acids, ethanol and supercritical carbon dioxide fluids (SCF-CO_2) was quantified as the ratio of solubility obtained with ethanol addition to that without ethanol. The effects of operating conditions, ethanol concentration, and ethanol and

solute properties on the ethanol effect were identified. These behaviors could be attributed to "cosolvent effect in supercritical carbon dioxide fluids (SCF-CO_2) system" and ethanol could be considered as a kind of cosolvent. The cosolvent effect in supercritical carbon dioxide fluids (SCF-CO_2) system for fatty acids would be decreased with pressure. Specific intermolecular interactions (such as Hydrogen-bonding) between the fatty acids and ethanol contribute significantly to the cosolvent effect in supercritical carbon dioxide fluids (SCF-CO_2) system, which can be exploited to increase selectivity of a fractionation process. The treatment with supercritical carbon dioxide fluids(SCF-CO_2) before proteinases treatment would remove most lipids of porcine dermal' raw materials and provide friendly microstructure of materials for diffusion of proteolytic enzyme molecules. The collagen scaffolds would be easy to be obtained as shown in Figure I(B). The supercritical carbon dioxide fluids (SCF-CO_2) after proteinases treatment present lipid and tissue on the surface of materials, which would avoid the diffusion of proteolytic enzyme molecules in the proteolytic reaction . The collagen scaffolds would be relative difficult formed as shown in Figure I(A).

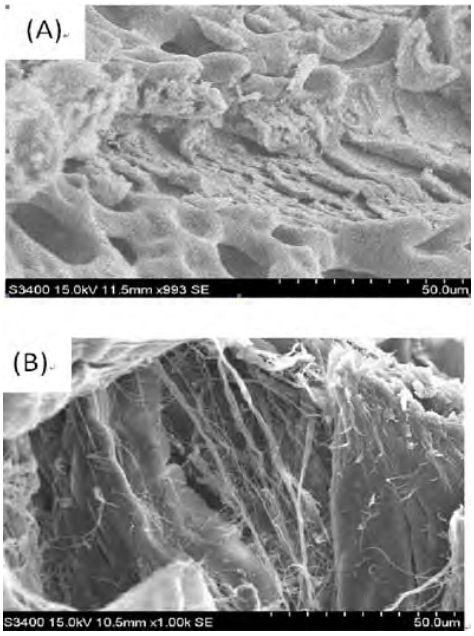

FIGURE I. SCANNING ELECTORN MICROGRAPHS OF DECELLULARIZATION SAMPLE (A) 0.5TP-S (B) S-0.5TP

B. Thermal Stability of Resulting Collagen Scaffolds

The decomposition behaviors of resulting collagen scaffolds as well as their composite membranes were analyzed by the TG technique. Thermal stability of resulting collagen scaffolds could be characterized by TGA and DSC. In usual, the collagen peptide decomposed and showed main peak mass loss rates at about 305ºC. In this study, the resulting collagen scaffolds showed high T_{dMax} than 350 ºC, which are a kind of biomaterials with the relative high thermal stability than collagen peptide. The results of the porcine dermal samples were shown in Table III. The glass transition temperature (Tg) of each sample after removal of fat by the supercritical carbon dioxide fluid would result in a slight decrease in the glass transition temperature (Tg) as listed in Table 4. Most of the Tg values could be determined between 72 and 95 ºC. The resulting collagen scaffolds would be soft when the temperature higher than 70ºC.

TABLE III. THE TGA RESULTS OF RESULTING COLLAGEN SCAFFOLDS

Sample Number	Tdi,ºC	Tdmax, ºC
5B-S	221.5	354.6
10B-S	222.3	353.9
15B-S	221.9	352.7
30B-S	222.5	352.1
5F-S	225.6	380.1
10F-S	226.3	375.4
15F-S	226.1	373.2
30F-S	225.8	371.6
S-5B	232.6	362.1
S-10B	230.5	361.2
S-15B	228.9	360.9
S-30B	230.1	359.5
S-5F	228.2	358.3
S-10F	227.9	356.8
S-15F	227.5	355.2
S-30F	228.4	354.7
S-0.1Tp	230.2	359.6
S-0.25Tp	228.1	358.2
S-0.5Tp	229.4	357.8
S-0.75Tp	227.6	356.4
S-1Tp	228.7	355.8

TABLE IV. THE DSC RESULTS OF RESULTING COLLAGEN SCAFFOLDS

Sample Number	Tg(ºC)	Sample Number	Tg(ºC)
0.1Tp-S	79	S-0.1Tp	90
0.25 Tp-S	76	S-0.25Tp	92
0.5 Tp-S	77	S-0.5Tp	93
0.75 Tp-S	80	S-0.7Tp	89
1 Try-S	82	S-1Tp	91
5B-S	72	S-5B	85
10B-S	75	S-10B	88
15B-S	72	S-15B	89
30B-S	74	S-30B	86
5F-S	79	S-5F	95
10F-S	76	S-10F	93
15F-S	82	S-15F	91
30F-S	78	S-30F	94

C. *Fourier Transform Infrared Spectroscopy Analysis of Resulting Collagen Scaffolds*

From the FTIR analysis(Figure II), absorptions bands at 1,454, 1,403, 1,340, 1,282, 1,240, and 1,205 cm^{-1} were observed. These bands would be attributed to the groups of CH_2, CH_3, C–N, and N–H absorptions of resulting acellular dermal collagen scaffolds. Amides I and Amides II absorptions were observed at 1,650 and 1,550 cm^{-1} respectively[9]. The absorption band at 3100 cm^{-1} for C-H of the fatty acid tend to slow down, demonstrating the effectiveness of the supercritical carbon dioxide fluid.

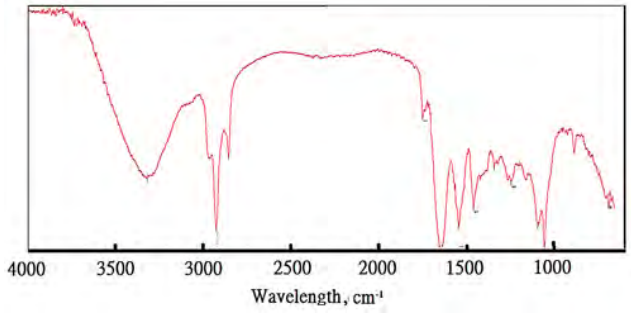

FIGURE II. THE FOURIER TRANSFORM INFRARED SPECTROSCOPY ANALYSIS OF 10B-S

IV. CONCLUSIONS

In this study, a series of new collagen scaffolds were obtained from porcine dermal matrix by using enzymes and supercritical carbon dioxide fluid technology. This experiment provides a simple and time-saving method process for decellularized tissue and form collagen scaffold structurally intact. The Tdmax of the resulting collagen scaffolds were observed over 350ºC. The collagen scaffolds with high thermal stability were obtained. The resulting scaffolds could be a potential application for minimally invasive surgeries in orthopedics.

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