Method to Extract Water-soluble Collagen from Induced Biphasic Commercial Gel

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Abstract

Water-soluble collagen extracted from commercial collagen gel could be a possible replacement for water-insoluble collagen fibrils, which are currently extracted from raw corium. If the water-soluble collagen proves to be a suitable replacement in the various water-insoluble collagen applications as mention in this paper, then the time-intensive milling process could be avoided. The first part of the process as described in this paper is a method to extract the water-soluble collagen. Water-soluble collagen was extracted from commercial collagen gel by mixing distilled water with the collagen gel, centrifugation, decanting, freezing and vacuum drying the supernate. Various ratios of commercial collagen gel to water ranging from 10% to 90% commercial collagen gel to water were examined based upon the extraction and performance of gravimetric analyses on the water-soluble collagen. The commercial collagen gel to water ratio had an effect on the mass fraction of extracted water-soluble collagen. Greater amounts of water-soluble collagen could be extracted by using lesser amounts of commercial collagen gel. The water-soluble collagen matrices had a mesh-like appearance similar to cotton, except that they could be flattened easily and could not retain their original shape. The water-soluble collagen matrices were formed into a chaotic, non-uniform manner caused by the re-constituting of fibers during the freezing and vacuum drying process.

Resumen

Colágeno soluble en agua extraído de gel de colágeno comercial podría ser un posible sustituto de las fibrillas de colágeno insolubles en agua, que actualmente se extraen del corium crudo. Si el colágeno soluble en agua prueba ser un sustituto adecuado en las varias aplicaciones para colágenos insolubles en agua como se menciona en este documento, entonces el largo proceso intensivo de molienda podría ser evitado. La primer parte del proceso como se describe en este documento es un método para extraer el colágeno soluble en agua. El colágeno soluble en agua se extrajo a partir de gel de colágeno comercial mediante la mezcla de agua destilada con el gel de colágeno, por centrifugación, decantación, congelación y secado al vacío del sobrenadante. Diversas proporciones de gel de colágeno comercial con el agua, que van desde 10% a 90% de gel de colágeno comercial con el agua se examinaron, basados en la extracción y el rendimiento de los análisis gravimétricos sobre el colágeno soluble en agua. La relación entre el gel de colágeno comercial y el agua tiene un efecto sobre la fracción de masa del colágeno soluble en agua extraído. Mayores cantidades de colágeno soluble en agua pueden ser extraídas mediante el uso de menores cantidades de gel de colágeno comercial. Las matrices del colágeno soluble en agua tenían un aspecto de una malla similar al algodón, excepto que podían ser fácilmente aplastadas, y no pudieron mantener su forma original. Las matrices de colágeno solubles en agua se formaron de una manera caótica, no uniforme, debido a la re-constitución de fibras durante el proceso de congelación y secado al vacío.

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Introduction

the current collagen milling processes in use, as described in various US patents, involves a time consuming process for unraveling the collagen fibers to obtain the collagen fibrils, which are then used for various applications, ranging from uses in the biomedical to environmental areas.[1] Some examples of the applications include water dispersing, flocculation and settling agents, cell immobilization for bioreactors and the controlled release of a drug. During the milling processes, water is used for washing the collagen, effectively removing the majority of water-soluble collagen. Therefore, to analyze water-soluble collagen, it must be separated directly from the raw corium. Commercial collagen gel is basically raw corium, which has been homogenized. Water-soluble collagen can be obtained by mixing the commercial collagen gel with water, providing agitation, and then centrifugation. The aqueous layer that forms above the collagen gel has water-soluble collagen dissolved in it. This water can be decanted, frozen and then vacuum-dried to obtain the water-soluble collagen.

Introduction to Collagen

Collagen is a classification that covers a wide range of protein molecules, which are helical in nature and form supramolecular matrix structures.[2] It is the most abundant protein in the animal kingdom, accounting for up 30% of all proteins in animals. The individual protein chains of collagen contain approximately twenty different amino acids and the exact composition of the chains vary between tissues.[3] This variation in the amino acid sequence accounts for the different types of collagen, i.e. Type I, Type II, up to Type XXIX. Type I and Type III collagen are the most common form characterized by fiber bundles in tissue.[3] Type I collagen is found mostly in bone, tendon and skin tissue. It can be readily extracted from the waste by-products found at slaughterhouses.

Collagen has unique properties, which can allow it to be used for various applications. Collagen has the ability to retain many times its mass in water, as well as the ability to disperse water. Collagen dispersions can be used for coagulation of particles suspended in water, allowing them to be filtered out easily.[1] The collagen fibers in collagen dispersions have great surface areas, which allow for the collagen to interact with foreign objects in water, causing separation from water to take place. This can be illustrated by using collagen dispersion to decrease the turbidity of water, removing particles that are too small to settle on their own. Collagen has also been used in many biomedical applications, such as a hemostatic agent, wound-dressing, intradermal augmentation, adhesives, absorbent pads for surgical use, sponges, tubes, sutures and drug delivery systems.[4] It is also used to make implantable prostheses such as heart valves, vascular grafts, tendons, ligaments and pericardium.[5] Collagen is also purified for use in the manufacturing of cosmetics, using acid-soluble collagen. It can also be formed into matrices, which can be used as a structure for cells in bioreactors to latch onto and grow.

Water-Soluble Collagen

In nature, triple-helical protein structures, such as collagen, are generally not soluble in water. Collagen is mostly insoluble and water absorbent, although, there is a small percentage of collagen that does dissolve in water. One common type of water-soluble collagen is gelatin. It occurs when Type I collagen is added to boiling water for a duration long enough for the collagen to denature. The gel that is formed is water-soluble, but makes the water very viscous.[5] Heating collagen in water causes competition between the water molecules within the heated water and the water molecules in the helical structure. This competition results in hydrogen bonds forming between the alpha-chains, distorting the helical structure.

Experimental

Experimental Overview

Before extraction of water-soluble collagen could begin, centrifugation studies were conducted in order to determine the necessary speed and duration to successfully separate the commercial collagen gel from the water-soluble collagen/water mix after the two were shaken together. A series of commercial collagen gel to water ratios ranging from 10% to 90% commercial collagen gel based on weight were tested. The centrifuge used was a Beckman model TJ-6, which required the use of 250mL centrifuge bottles to hold the Fisher brand polypropylene 50mL graduated vials.

Since the 10% commercial collagen gel to water ratio mixture proved to lead to a bi-phasic separation, more tests centered on the 12% to 2% commercial collagen gel to water ratios. The tests were designed to examine if any differences in these commercial collagen gel to water ratios would affect the amount of water soluble collagen that could be extracted, while holding the agitation and centrifuging settings constant. In order to perform the gravimetric analysis on the extracted water-soluble collagen, a few preliminary measurements on the commercial collagen gel were performed. The total mass fraction of collagen in the commercial collagen gel was determined, in order to calculate the mass fraction of collagen extracted from the samples.

Materials

The commercial collagen gel was received from a local company to be used to extract water-soluble collagen. This material is sold to various companies for making sausage casings and other edible food additives. The mass composition of the material requested was 6% collagen in the gel. In order to get meaningful results, the mass composition of collagen had to be pre-determined. A weighted sample of the collagen was heated to 37 °C at full vacuum in a Fisher Scientific Isotemp vacuum oven model 282A for approximately 24 hours. The change in weight of the commercial collagen gel after vacuum heating allowed the mass composition to be determined.

Centrifugation Study

Commercial collagen gel and distilled water were mixed together in vials based on a set of ratios ranging from 10% to 90% commercial collagen gel to water based on weight. Vials of these mixtures were first shaken for 10 minutes at 10 shakes/second using the Burrell wrist action shaker model 75, which was sufficient to give a good mixture, ensuring that the majority of the water-soluble collagen within the commercial collagen gel sample had a chance to become dissolved in the water. The mixture was determined to be acceptable for this study when there no longer was a distinction between the collagen gel sample and the water. Then, these mixtures were centrifuged based on the maximum allowable speed determined by the plastic vials used with the setup inside the centrifuge. Capped vials were inserted into 250mL Nalgene centrifuge bottles to hold them in place within the centrifuge. This setup proved to be effective at holding the vials in place, but certain higher centrifugal speeds would distort and/or break the vials. The vials were able to withstand a maximum speed of 2,000 rpm before the plastic vials and centrifuge bottles would begin to distort and break. Vials that would distort and break would leave shards of plastic that would fall into the collagen mixtures. A duration of 20 minutes provided a sufficient bi-phasic separation between the solid collagen gel and the soluble collagen/water mixture. All subsequent mixtures were centrifuged at 2,000 rpm for 20 minutes.

Extraction Process

The vials and caps used were initially weighted, to be able to determine the weight of the material within the vials. Commercial collagen gel and distilled de-ionized water were weighed individually and then mixed together based on the indicated water-to-commercial collagen gel ratio. It was then shaken using the Burrell Wrist Action Shaker Model 75 for about 10 minutes at 10 shakes/second. Following the shaking step, the vials were centrifuged, based on the pre-determined settings, to create two distinct layers: insoluble collagen gel and soluble collagen/water mixture. The water mixture layer was then decanted and transferred to another vial using a transfer pipette. At every stage of the experiment, the collagen gel, water, and variations of each were weighed. The extracted water mixture layer was placed in the freezer for about 2-3 days at roughly –80 °C to ensure that the water was completely frozen, while the insoluble collagen layer was discarded. The frozen water mixture sample was then placed in a Labconco Stoppering Tray Dryer for 1-3 days, based on the number of samples placed in the machine, in order to remove the frozen water via vacuum drying. For one experiment, consisting of 10 samples, roughly 1 day was required to remove all traces of water, leaving behind a water-soluble collagen matrix in each of the vials. The water-soluble collagen matrix was weighed and microscopy was performed utilizing a Scanning Electron Micrograph to analyze the structure.

RESULTS AND DISCUSSION

Collagen Composition of Commercial Collagen Gel

The commercial collagen gel was a flexible, fibrous gel containing water and collagen. A few samples were dried-out in the vacuum oven to remove all traces of water. Upon completion of vacuum drying, the collagen samples became much smaller in physical volume and were rigid and brittle. The average mass composition of the collagen in the commercial collagen gel was calculated to be 7.01% with a standard deviation of 0.0676. The commercial collagen gel was listed as containing 6% collagen, although, testing revealed that the average composition was around 7%.

Mass Fraction of Water-soluble Collagen Matrices

The mass fraction of water-soluble collagen matrices extracted was analyzed at various commercial collagen gel to water ratios. The average mass fraction, as well as the standard deviation, of water soluble collagen to collagen in the commercial collagen gel is shown in Table 1 & 2. Four vials at each commercial Collagen Gel to water ratio were tested.

TABLE I
Mass Fractions for Water Soluble Collagen Matrices

Commerical Collagen Gel to Water Ratio	2/98	4/96	6/94	8/92	10/90
Average	16.782	14.722	8.438	6.032	4.896
Standard Deviation	3.537	2.577	6.587	2.427	2.083

TABLE II
Mass Fractions for Water Soluble Collagen Matrices

Commerical Collagen Gel to Water Ratio	12/88	13/87	15/85	17/83
Average	4.002	1.274	0.373	0.571
Standard Deviation	0.601	0.468	0.318	0.076

A trend of the average mass fractions at each commercial collagen gel to water ratio used is shown in Figure 1. It indicates that the amount of water-soluble collagen extracted depended on the amount of commercial collagen gel used. As the percentage of commercial collagen gel increased, the percentage of water used decreased, which allowed less water-soluble collagen to be extracted. At a ratio of 2% commercial collagen to water, the mass fraction was much greater than that of the ratio of 10% commercial collagen to water.

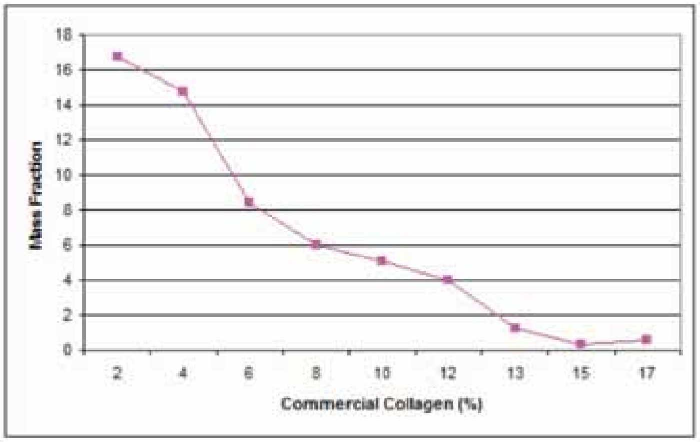

Figure 1. Average Mass Fractions at Various Commerical Collagen Ratios.

Microscopy of Commercial Collagen Gel

The commercial collagen gel was a white, flexible, fibrous material that had a texture similar to that of strands of hair within a gel. It could be formed into different shapes, such as spheres and cylinders, and could be pulled apart very easily. As the commercial collagen gel is pulled apart, the individual strands of collagen can be shown more readily. The collagen strands were arranged in semi-uniform manner while the sample was intact, but the strand orientation became more chaotic when the sample was stretched and distorted. Once the gel's shape was distorted, it could not return to its original shape by itself. The commercial collagen gel had to be handled with gloves to prevent contamination and the loss of water-soluble collagen from contact with human skin.

Microscopy of Water-soluble Collagen Matrix

The water-soluble collagen matrix was a white cotton-like mesh material, with fibrous strands that held the sample to the wall and/or bottom of the vial after it the water was removed. The samples did not hold their form under physical stimuli, such as shaking the vial or trying to remove the sample with a metal instrument. Upon contact with the metal instrument to loosen the sample from the vial, the collagen sample shrank slightly. The sample was similar to the commercial collagen gel in that it could be flattened and distorted, but would not return to its original shape by itself. This material acted more like clay. This cotton-like material could be flattened and remain flattened. The sample could not come in contact with human skin because the water-soluble collagen would dissolve in water and shrink in size upon contact. Adding distilled water to the sample caused the entire sample to dissolve into the water. This result supports that the material was still water-soluble after exposing it to freezing and vacuum drying. The result also verified that the sample was not contaminated with insoluble collagen that may have gotten into the sample during the water mixture layer extraction step following centrifugation. During the vacuum drying step, the water-soluble collagen strands formed a structure in a chaotic, non-uniformed manner. The water-soluble collagen resembles the commercial collagen gel when it is pulled apart, where the collagen strands became oriented in a chaotic fashion within the sample. The main difference being that the water-soluble collagen never had a uniform pattern within the sample during its formation.

SEM — Water-soluble Collagen Matrix

Water-soluble collagen matrices were imaged using a Scanning Electron Microscope (SEM) at the United States Department of Agriculture. SEM images of the water-soluble collagen matrices at four different magnifications are shown in Figures 2 through 5. The images indicate that the water-soluble collagen matrix was reconstituted into a structure of a non-uniform manner. The collagen strands made connections to each other at random angles and orientations, creating a chaotic structure. The images indicate a 3-dimensional structure, which is difficult to capture in its entirety because of the SEM's inability to simultaneously focus on every depth of the material. Based on the relative diameters of the water-soluble collagen strands in the matrix, which ranged from around 4 to 10 microns, the strands are classified as collagen fibers.

CONCLUSIONS

Collagen Composition of Commercial Collagen Gel

The experimental data for the collagen composition of the commercial collagen gel was determined to be approximately 7% collagen in the material. This was a little higher when compared to the 6% collagen composition that was expected from the manufacturer. The variation between the compositions of collagen in the gel was small, but it affected the remaining of the mass fraction data for the water-soluble collagen matrices. Using a factor of 7% instead of 6% gave slightly higher calculated mass fractions. The variation could have been cause by a number of factors, including the variation in the initial composition from the manufacturing process. The composition of the collagen within the commercial collagen gel may vary slightly between samples, because the gel is not a solid, pure material, which has a uniform consistency.

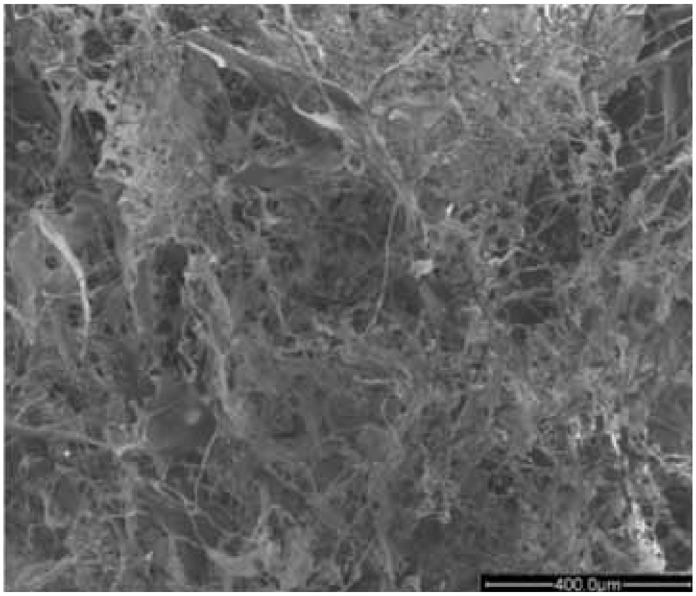

Figure 2. SEM — Water Soluble Collagen Matrix at 100x Magnification

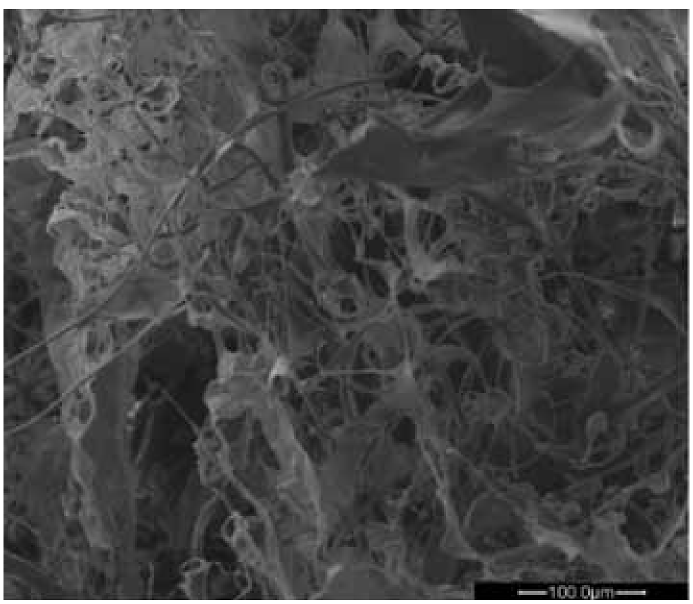

Figure 3. SEM — Water Soluble Collagen Matrix at 250x Magnification

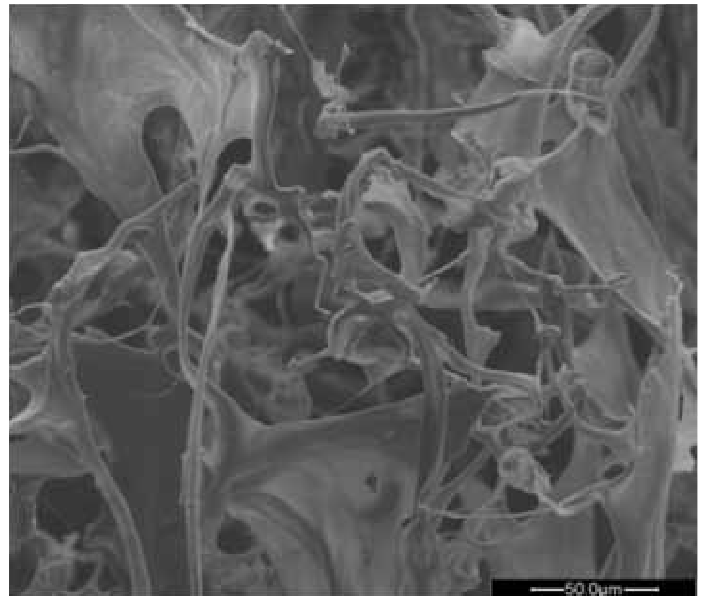

Figure 4. SEM — Water Soluble Collagen Matrix at 500x Magnification

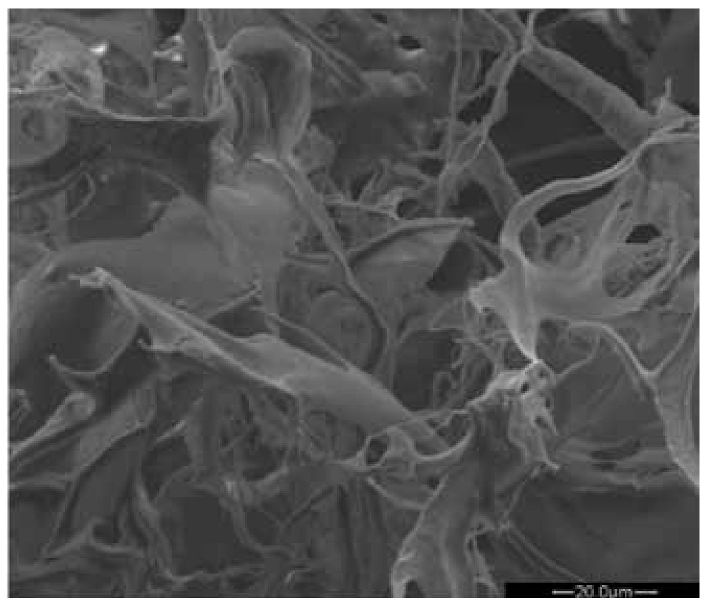

Figure 5. SEM — Water Soluble Collagen Matrix at 1000x Magnification

Mass Fraction of Water-soluble Collagen Matrices

The experimental data for the mass fraction of water-soluble collagen extracted was scattered, based on the standard deviation for each commercial collagen gel to water ratios. The scattering of the mass faction data could have been a result of the ability to effectively remove the water mixture layer from the samples after the centrifugation step. Removing the water mixture layer proved to be difficult sometimes, based on how the collagen was compacted from centrifugation. If the collagen was compacted at the bottom of the vial, the water could be removed easily, but if the collagen was not compacted, there was the chance that less water could be removed, leading to less mass and ultimately less of a mass fraction extracted. The amount of water-soluble collagen extracted depended on the amount of commercial collagen gel used. The data showed that more water-soluble collagen could be extracted by using smaller amounts of the commercial collagen gel. When lesser amounts of commercial collagen gel were used, more water was available to dissolve more water-soluble collagen from within the sample. Each sample contained a certain amount of water-soluble collagen in it, and water only had the ability to dissolve a certain amount of water-soluble collagen in it. Therefore, if more water was added, more water-soluble collagen could be extracted from the commercial collagen gel.

Microscopy of Commercial Collagen Gel

The commercial collagen gel was a white, flexible, fibrous material, nothing at all like the water-soluble collagen matrices extracted. This commercial collagen gel could be formed into different shapes much easier because of its gel-like properties. The commercial collagen gel consisted of a hair-like structure, where the collagen strands are oriented in a uniform manner within a water-like gel. As the commercial collagen gel was pulled apart, the individual strands of collagen become more chaotic as the sample was stretched and distorted.

Microscopy of Water-soluble Collagen Matrices

Under microscope as well as the naked eye, the structure of the water-soluble collagen matrices had a hair-like structure, similar to that of cotton, as well as nearly identical structures at the various 40x, 100x, 400x, and 1000x magnifications. The orientation of the collagen strands were very chaotic and did not form any type of pattern within the matrices. This could have been because the samples were reconstituted from water. The water-soluble collagen matrix could be flattened easily, and would retain that new distorted shape. It also had the ability to re-dissolve in water easily, which indicated that a good separation had taken place after centrifugation.

SEM — Water-soluble Collagen Matrices

The images indicate that the water-soluble collagen matrix was reconstituted into a structure of a non-uniform manner. The collagen strands made connections to each other at random angles and orientations, creating a chaotic structure. The approximate diameters of the water-soluble collagen strands ranged from around 4 to 10 microns, which is on the level of the collagen fiber. The level of the collagen fibers is the largest diameter strand of collagen. This would make sense, considering that the commercial collagen gel was not milled, before the water-soluble material was extracted. Milled collagen, produced at Widener University, had diameters around the level of the collagen fibril, which is a sub-set of the collagen fiber.

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