Video Article

An Improved Method for the Preparation of Type I Collagen From Skin

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Abstract

Soluble type 1 collagen (COL1) is used extensively as an adhesive substrate for cell cultures and as a cellular scaffold for regenerative applications. Clinically, this protein is widely used for cosmetic surgery, dermal injections, bone grafting, and reconstructive surgery. The sources of COL1 for these procedures are commonly nonhuman, which increases the potential for inflammation and rejection as well as xenobiotic disease transmission. In view of this, a method to efficiently and quickly purify COL1 from limited quantities of autologously-derived tissues would circumvent many of these issues; however, standard isolation protocols are lengthy and often require large quantities of collagenous tissues. Here, we demonstrate an efficient COL1 extraction method that reduces the time needed to isolate and purify this protein from about 10 days to less than 3 hr. We chose the dermis as our tissue source because of its availability during many surgical procedures. This method uses traditional extraction buffers combined with forceful agitation and centrifugal filtration to obtain highly-pure, soluble COL1 from small amounts of corium. Briefly, dermal biopsies are washed thoroughly in ice-cold dH_2O after removing fat, connective tissue, and hair. The skin samples are stripped of noncollagenous proteins and polysaccharides using 0.5 M sodium acetate and a high speed bench-top homogenizer. Collagen from residual solids is subsequently extracted with a 0.075 M sodium citrate buffer using the homogenizer. These extracts are purified using 100,000 MW cut-off centrifugal filters that yield COL1 preparations of comparable or superior quality to commercial products or those obtained using traditional procedures. We anticipate this method will facilitate the utilization of autologously-derived COL1 for a multitude of research and clinical applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/51011/

Introduction

For decades, researchers and commercial vendors have isolated solubilized COL1 from an assortment of tissue sources including skin and tendon using some variation of a simple acid extraction protocol followed by neutralization, which results in a resuspension of a matrix of organized COL1 fibrils that can be used for a multitude of biomedical applications [1-4]. While there are many examples of clinical applications for COL1, few of these employ autologously-derived COL1 because preparation of this protein requires lengthy extractions taking days or weeks to perform[5-7]. As a result, research investigators and physicians generally use expensive, commercial preparations of COL1 that are prepared using nonhuman tissues such as rat, bovine, or porcine corium or using skin removed from cadavers or following male circumcision. For regenerative applications, many of these products exhibit variability with respect to their ability to form solid matrices or tissue constructs capable of supporting cell attachment and growth. Consequently, there is a distinct need for a new standardized method designed to quickly extract COL1 from accessible and plentiful autologous tissue sources.

Such a method would save both time and money in the research setting where COL1 could be extracted from the animal model of interest and used for preclinical testing of engineered tissues assembled on collagen-based scaffolds. At the same time, having the option of using a rapidly-isolated, autologously-derived COL1 in the clinic would increase the overall safety for patients that would otherwise receive allogeneic or xenogeneic preparations. For patients receiving an autogeneic preparation, this streamlined method would greatly reduce the interval between biopsy collection and collagen application. For these reasons, we sought to improve upon a long-established COL1 isolation and purification method by using high-speed agitation and size-exclusion centrifugation. This method is simple to perform using standard buffers and equipment found in many laboratories. By employing high-speed agitation, our protocol reduces the time necessary to isolate soluble, dermal COL1 from approximately 10 days to less than 3 hr[8]. Importantly, this method can be easily performed in the clinical setting in order to prepare autologously-derived COL1 for use in patients during a single set of procedures.

Protocol

Here we will demonstrate the isolation of COL1 from lamb skin. As written, this protocol can also be used to successfully isolate COL1 from rabbit and human skin.

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1. Prepare Dermal Sample

1. Equilibrate all reagents to 4 °C prior to use.
2. Rinse dermal sample (25-50 g) in ice-cold dH_2O and remove any wool, fur, or hair with depilatory cream.
3. Use a single-edge razor blade to scrape the sample clean of connective tissue and fat.
4. Rinse the sample in ice-cold dH_2O.
5. Slice the skin sample into 1 cm x 1 cm pieces with a single-edge razor blade.

2. Remove Noncollagenous Solubilized Material

1. Weigh out 5 g of sample per 50 ml conical tube and add 30 ml of ice-cold 0.5 M sodium acetate.
2. Mix tubes for 1 min at the 6 m/sec setting using a 50 ml tube adaptor in the bench-top homogenizer.
3. Discard supernatant and repeat for a total of 7 sodium acetate wash cycles.
4. Rinse the sample in ice-cold dH_2O and mix once to remove residual sodium acetate.
5. Use a spatula to compress the sample against the side of the tube to remove excess liquid and then transfer to a fresh 50 ml conical tube.

3. Type I Collagen Extraction

1. Wash the sample twice in 2 ml/g 0.075 M sodium citrate buffer for 1 min at 6 m/sec in the bench-top homogenizer compressing the sample and discarding the supernatant after each wash.
2. Add a fresh 2 ml/g aliquot of 0.075 M sodium citrate buffer to the sample.
3. Perform 6 sequential 1 min, 6 m/sec bench-top homogenizer mix cycles of agitation without removing the buffer between each cycle.
4. Transfer the thick, clear supernatant to a collection tube.
5. Add an additional 1 ml/g 0.075 M sodium citrate buffer to the sample and perform one final bench-top homogenizer agitation cycle.
6. Add this final supernatant to a collection tube.
7. Centrifuge the collection tube at 3,200 x g for 10 min at 4 °C.
8. Transfer the supernatant to the top compartment of a 100,000 molecular weight cut off centrifugal filter device.
9. Centrifuge at 3,200 x g for 30 min at 4 °C.
10. Transfer the purified COL1 from the upper compartment to a clean conical tube and store at 4 °C.

Representative Results

This COL1 isolation protocol requires about 2 hr and 15 min to complete. Figure 1 shows a schematic diagram outlining the major steps in this procedure. Preparing the sample and performing the 7 cycles of high-speed agitations and rinses with sodium acetate takes approximately 35 min **(Figures 1A-C)**. Performing one agitation and rinse cycle with dH_2O takes approximately 5 min **(Figures 1D** and **1E)**. Adding sodium citrate and subjecting the sample to one agitation and rinse cycle followed by 6 cycles of agitation takes approximately 45 min **(Figures 1F** and **1G)**. Centrifuging the remaining solids from the liquid extract takes 15 min **(Figure 1H)**. Finally, transferring the sample to a filter device and performing another centrifugation step to concentrate the purified, solubilized COL1 and remove small proteins takes 30 min **(Figures 1I** and **1J)**. The final product is a concentrated, highly-pure preparation of soluble COL1 that can be further polymerized by adding Na_2HCO_3 and incubating at 37°C. Phase contrast microscopy images of this solidified product reveal COL1 fibrils that could serve as a substrate for cell attachment to tissue culture plates or be used as a scaffold for 3-D tissue engineering applications **(Figures 2A** and **2B)**.

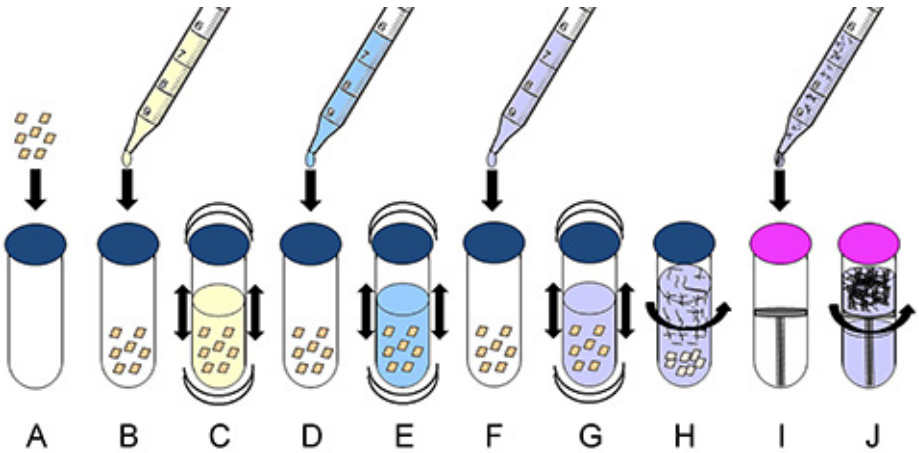

Figure 1. Schematic overview of the improved COL1 isolation method. A) Sliced dermis is added to a 50 ml conical tube. **B**) 0.5 M sodium acetate is added to the sample. **C**) The sample is subjected to 7 cycles of agitation with sodium acetate buffer replaced after each cycle. **D**) dH_2O is added to the skin sample. **E**) The preparation is subjected to 1 cycle of agitation and dH_2O is poured off. **F**) 0.075 M sodium citrate is added to the sample. **G**) The sample is subjected to 6 cycles of agitation. **H**) The sample is centrifuged to remove large solids. **I**) The supernatant is transferred to a 100,000 molecular weight cut off centrifugal filter unit in a conical tube. **J**) This unit is centrifuged and the viscous liquid that remains in the top compartment is collected.

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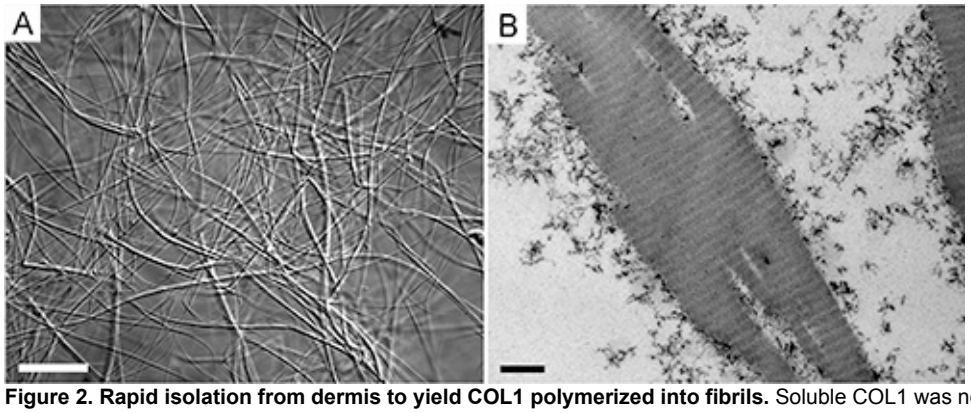

Figure 2. Rapid isolation from dermis to yield COL1 polymerized into fibrils. Soluble COL1 was neutralized by adding Na_2HCO_3 and then incubated at 37 °C. A phase contrast image (**A**) and transmission electron micrograph (showing the preserved d-banding) (**B**) of COL1 fibrils generated using our rapid isolation method. Scale bars = 10 µm and 500 nm (respectively). Click here to view larger image.

Discussion

A crucial component to this method is the repeated efficient compression of the dermal sample to remove excess liquid. It is critical to remove as much sodium acetate as possible in step 2.5 by compressing the sample. We use a spatula to compact the skin against the side of the tube; however, cheesecloth could also be used, although this would necessitate removal of the sample from the tube and increase the time required to perform these steps. Likewise, it is important to compress the sample in step 3.1 so that the volume of sodium citrate is accurate in the subsequent extraction steps. Failure to adequately remove liquid from the sample in any of these steps will have a detrimental effect on the final COL1 yield. Another key component to this protocol is to maintain all reagents at 4 °C. This ensures the efficient extraction of acid solubilized COL1 and prevents solidification of the collagen. Additionally, the procedure could be performed using all sterile reagents in a sterile environment. This would be of particular importance for those wishing to employ this method to isolate COL1 for use in humans. Many commercial sources of COL1 have been effectively sterilized by gamma-irradiation. Unfortunately, this practice greatly reduces its ability to form gels due to protein fragmentation and denaturation[9,10].

This procedure represents the optimal conditions for COL1 extraction as determined from many experiments performed to establish the ideal proportion of dermal sample to buffer volume. The addition of more tubes is suggested when the investigator wishes to employ this procedure for larger skin samples. Based on our experience, it is not advisable to add more sample or buffer than indicated for each tube. It is also worth noting that beads composed of silica, ceramic, garnet, aluminum oxide, silicon carbide, or zirconium oxide, which are commonly used in conjunction with bench-top homogenizers, are omitted from our procedure. The 6 sequential 1 min agitation cycles described in step 3.3 are necessary due to a limitation imposed by our agitation equipment. We can only agitate the sample for a total of 60 sec at a time, after which the machine requires a stop. If one has equipment that can be set for 6 min straight, it would likely be fine to switch to one, six-minute cycle. We do, however, suggest strict adherence to the sample weight and buffer volumes described.

We have found this preparation method efficiently isolates soluble COL1 from lamb, rabbit, and human dermal samples[8]. On the other hand, this protocol was not successful at purifying COL1 from porcine skin samples. Even with extensive modifications to the method, porcine dermis produced an exorbitant amount of froth because of residual lipids and fat resulting in inefficient COL1 extraction. Conversely, COL1 can be purified from whole rat tails or isolated rat tail tendons using a slightly modified preparation that includes lysing matrix beads. This has been previously described in detail[8].

For research scientists, the potential uses of the final acid-solubilized COL1 product obtained using this method include: a) providing an adhesive substrate for culture plates to support cell attachment and proliferation, b) micro-patterning of surfaces for 2- or 3-dimensional (2- or 3-D) cell culture, and c) creation of 3-D scaffolds for tissue engineering applications. While some small amount of lot-to-lot variability is to be expected from any purification method, we have found that these liquid COL1 preparations are consistently able to be combined with various cell populations and cast into molds. After neutralizing the pH and warming, the result is a solidified engineered tissue construct with spatially-oriented cells distributed evenly throughout the matrix. Depending on the shape of the mold, these constructs can be made in virtually any shape and size[3,4,11-14].

In summary, it is our opinion that the most compelling reason to utilize this method is that it dramatically reduces the time and effort required to isolate highly-pure, soluble COL1 from an autologous tissue source such as small skin biopsies. The method is based on long-established procedures and produces a product that meets or exceeds the quality of expensive commercial preparations with respect to COL1 purity and the ability to form stable 3-D tissues. At the same time, this simplified method allows the efficient and rapid isolation of autologously-derived COL1 that can be safely used for a multitude of clinical applications.

Disclosures

The authors have no competing financial interests to disclose.

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