Aspects of Collagen Isolation Procedure

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Abstract

Because of the extreme diversity of tissues and types of collagen' it is difficult to develop a standard method of extraction for all types of collagen from different tissues. Two procedures based on acid- and enzymatic-soluble collagen isolation were combined and offer some advantages and disadvantages to methods used in the present. Our results have demonstrated relatively low concentrations of collagen in the final solutions. There is 4.7 mg/ml from theoretically 10 mg/ml of acid-soluble fraction of collagen. This article describes some advantages and disadvantages of this isolation method. Due to the importance of biocompatible matrices for biomedical tissue engineering, the availability of native collagen should be studied by refining the extraction procedure of collagens.

Key words: collagen type I, methods of isolation and purification, tissue engineering.

Аспекты методики выделения коллагена

По причине чрезвычайного разнообразия тканей и типов коллагена содержащиеся в них, сложно разработать унифицированный метод получения коллагена. Были объединены две процедуры выделения коллагена, основанных на кислотном и ферментном растворении коллагена животного происхождения. Наши результаты показали относительно невысокие концентрации коллагена в конечном растворе, что составляет 4,7 мг/мл кислото-растворимого коллагена из теоретически возможных 10 мг/мл. В статье описаны некоторые преимущества и недостатки существующих методов. Разработка методов выделения коллагена первого типа важна по причине его использования для получения биосовместимых матриц и их применения в тканеинженерных конструкциях.

Ключевые слова: коллаген I типа, методы выделения и очистки, тканевая инженерия.

Introduction

Collagen is a fibrillar protein composing different forms of the conjunctive tissue: bone, cartilage, tendon and skin. More and more studies have shown that, in addition to the structural function, collagens can induce or regulate many cellular functions and processes such as cellular differentiation, motion, communication and apoptosis [1, 9]. But its main function is the formation of insoluble fibrils with high strength characteristics.

Collagen is the major component of the extracellular matrix, and more than 27 genetic isoforms have been identified. Collagens type I, II and III are the most abundant and well investigated for biomedical applications. These are widely used as a plastic material in different domains of medicine and cosmetology, and also in the pharmaceutical industry as compounds that prolongs the action of drugs [1, 6]. Type I collagen has been described as a natural scaffold and a potential candidate for tissue engineering and reconstructive medicine [4].

Such diverse functions are due to physical and chemical properties of collagen protein. Collagen type I usually forms fibrils with a length of 300 nm and a fibrillar diameter of up to 1000 nm. Type I collagen is trimeric [$(\alpha 1)_2\beta 2$] and exists as triple helix. The helices have the typical repeats for collagen Gly-X-Y (where X and Y mainly Pro and Hyp). Thus, proline and hydroxyproline constitute about 23% of the total protein sequence and the structure Gly-Pro-Hyp is the most common form often founded [3]. The hydroxiproline content of collagen extracted from different mammals had been found to be 13.4 ± 0.24 per cent [10].

Through the extreme diversity of tissues and types of collagen it is difficult to develop a standard method of extraction for all types of collagen. The number of the covalent intermolecular interactions in collagen structure increases in time and frequently determines almost full insolubility in utilized solvents for proteins [1, 9].

The main task of our study was to analyze the known methods of collagen isolation and purification, evaluate their efficiency and adjust them to our conditions and necessities.

Material and methods

Sources for collagen. Type I collagen was isolated from steer (3 years old) flexor bovine tendon. Tendons were suspended in cold distilled water at 4^0 C for three days. The water was changed two times per day. A fixed mass of tendon was defrosted and washed with cold water several times. Collagen fibers were cut into small pieces (1 cm in length) and pulverized in a mill after that. Pieces were dried for 24 hours at 40-45^0 C.

Collagen type I isolation – several methods of collagen isolation were used – extraction with neutral salt, acid and enzymatic solutions [5, 8, 9, 10].

Salt precipitation method

One of the procedures of salt extractions based on the treatment of tendon pieces with neutral salt solutions, as 0.05 M Na_2HPO_4, pH = 8.7-9.1. Afterwards collagen was isolated by the gradual increase of the sodium chloride concentration by the addition of 4M NaCl, 5^0C. The supernatant, contained the salt-soluble fraction of collagen, was dialyzed vs 0.01M Na_2HPO_4 [8].

Method of acid isolation

The procedure is based on the extraction of tendon pieces with organic acid, with 0.5M CH_3COOH in the presence of

5mM EDTA, pH = 2.5-3 for 48-96 h at 4^{0}C. The supernatant, that contained acid-soluble fraction of collagen, was dialyzed vs 0.05M Na_2HPO_4 [1, 9].

Method of enzyme isolation

The procedure is based on the extraction of tendon pieces in organic acid (0.5M CH_3COOH) in the presence of 5mM EDTA and pepsin with concentration 0.05 g per 100g of tissue, pH = 2.5-3.0 for 48-96 h at 4^{0}C.

The complete description of the proposed collagen isolation procedure included the next steps:

1. Non-collagen proteins extraction, with 0.05 M Na_2HPO_4, pH = 8.7-9.1 at 4^{0}C.

2. Gradual dissolution in acidic media (0.5M CH_3COOH) with 5mM EDTA. Pepsin was added directly into the solution of collagen in acetic acid at a ratio of 50 mg of pepsin per 1 g of tendons in two doses during 24 hours at 4^{0}C each. This procedure was repeated 3-4 times.

3. The supernatants of the extracted solutions were collected after centrifugation at 3000 g for 15 min and salted out with 4M NaCl.

4. The obtained precipitate contained pure collagen that was dissolved in 1M acetic acid.

5. Then the solution was dialyzed against 0.02M Na_2HPO_4 for 24 hours at 4^{0}C.

The general scheme of collagen isolation and purification is presented in fig. 1.

The collagen amount determination

Collagen's content was determined indirectly from the hydroxyproline content which was evaluated according to the Sharaev's spectrophotometric method after complete hydrolysis in acidic media [10].

The amount of collagen in % in a tissue sample may be calculated by the relation:

m (colagen) = m(Hyp)/W(Hyp) × 100%

where m (collagen) is amount of collagen in mg, m (Hyp) is amount of hydroxiproline, W(Hyp) is 13% of hydroxiproline in collagen [10].

Results and discussion

After testing several of the collagen isolation' procedures [2, 5, 6, 9] we have chose the method based on acetic acid dissolution of collagen fibers with some modifications. One of them is the extraction in neutral salt or low ionic strength acidic solutions.

Collagen can also be obtained by gradually increasing the sodium chloride concentration by the addition of 4M NaCl in neutral salt solutions, e.g. 0.05 M Na_2HPO_4, pH = 8.7-9.1, stored at 5^{0}C. The efficiency of this extraction is low, and we got 0.6 mg/ml of soluble fraction of collagen. The higher capacity of solubilization could be obtained by the increase of the salt concentration, which will increase the ionic power in the solution. However, in normal tissues the proportion of neutral salt-soluble collagen is very small so that the final yield is very low.

Another method that was used is based on the solubilization with diluted organic acid e.g. 0.5 M acetic or citric acid, pH = 3. We used as a solvent the acetic acid, diluted to 1M, pH = 2.5 in the presence of EDTA that effectively inhibits degradation of tissues. We have received 1.4 mg/ml of soluble fraction of collagen from bovine tendon. Clearly, in comparison, this method has a higher capacity to solubilize collagen than neutral salt extraction but is still limited to young non-cross-linked collagen.

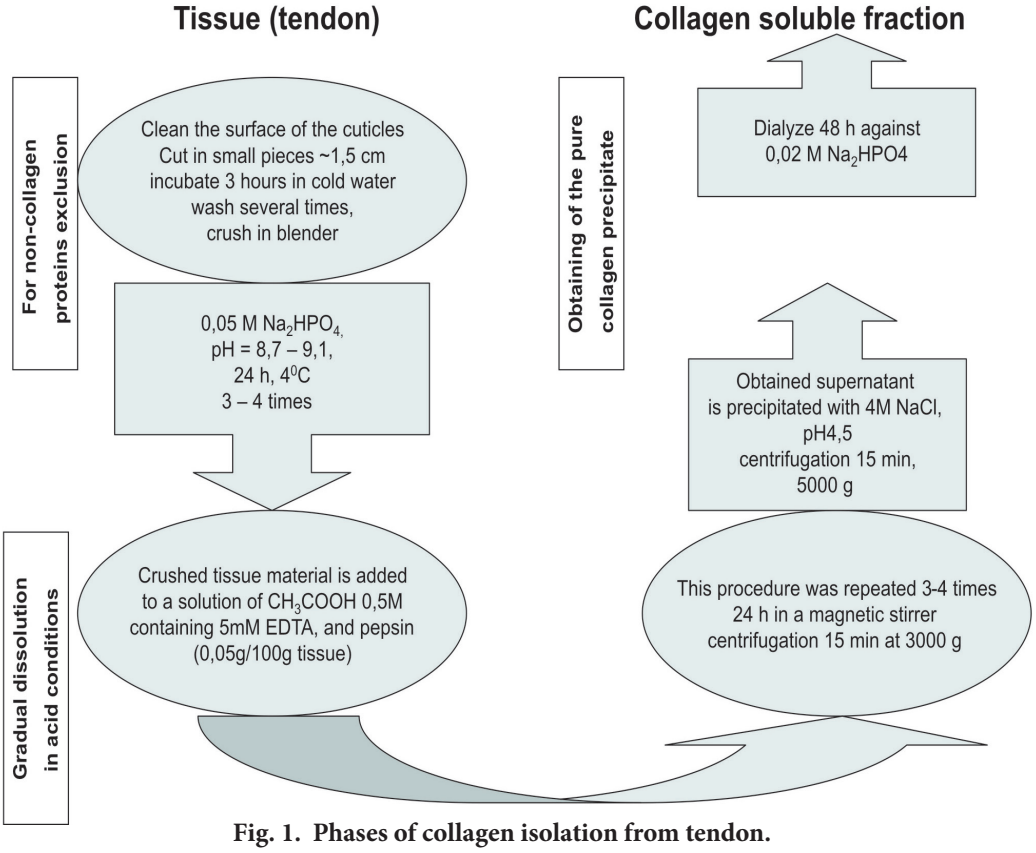

Fig. 1. Phases of collagen isolation from tendon.

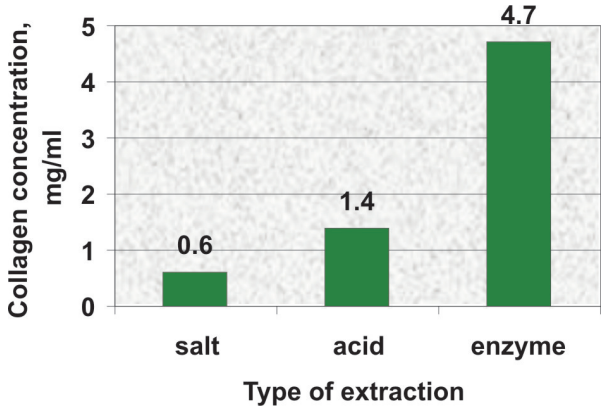

Fig. 2. Comparative data of the extracted soluble collagen fractions.

We have combined these two procedures based on acid- and neutral-salt extraction with enzymatic collagen isolation. The procedure was described in the compartment "Materials and Methods". There are several types of soluble collagen that can be extracted: neutral salt-soluble collagen, acid-soluble collagen and enzymatic-soluble collagen.

Our results have demonstrated relatively low concentrations of collagen in the final solutions – there is 4.7 mg/ml.

Thus a combined approach was developed which contains pepsin digestion in acidic solution. The necessary amount of pepsin for collagen solubilization is tissue dependent, and also the pepsin can cause collagen cleavage [9].

Nevertheless, the method using acetic acid and pepsin for collagen extraction from tissues is well established and is the most widely used in research and in industrial production of collagen. Although this extraction was standardized more than 40 years ago, it still has two major problems.

First, the collagen solubility is still ill-defined due to cross-link mediated aggregation, so that the reproducibility of the collagen preparations is poor. Secondly, the collagen peptides, especially the short non-helical regions of collagen, are susceptible to proteolysis/hydrolysis during the isolation [7, 9].

In addition to these two problems, the time required to solubilize collagen from tissues is usually between 1-3 weeks, with high protein loss and partial degradation of the collagen peptides [9]. For this reason the utility of the acidic-extracted collagen is limited, as the isolated material must be stored in cold acetic acid solution or dried. The maximal concentration of collagen obtainable is also limited to 10 mg/ml [7, 9] as estimated by wet weight and also by amino acid composition.

To overcome these disadvantages of partial degradation methods of collagen isolation, purification and determination should be modified using new, stronger detergents for deeper dilution of collagen fibers.

During collagen purification it is required to eliminate the antigenic components of the protein, represented by the telopeptide fragments regions of collagen type I. Such purification that is more efficient after treatment with pepsin. However, collagen extracted from animal sources presents only a small degree of antigenity, and is therefore considered acceptable for tissue engineering in humans [9].

Conclusions

Recent studies in cell biology, nanotechnology, and computation gave more new insights regarding the physical properties that in combination with chemical ones can together regulate cell signaling and gene expression. Due to the importance of biocompatible matrices for tissue engineering and their application in medical technology, the availability of native collagen should be studied by refining the collagens extraction procedure.

One of the goals was to reexamine the quality of Type I collagen after acid-extraction combined with pepsin extraction. It is very important to elaborate methods of collagen isolation that give us fully or partially soluble collagen. Further, the soluble collagen can be used in manufacturing of tissue engineering products matrices, powder, sponge, fibers or filaments.

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