Collagen analysis in mouse tissues using various assays

Application note

QuickZyme Biosciences

Introduction

Collagens are the most abundant proteins in the vertebrate body. Accurate analysis of collagen is important both for research in diseases where collagen plays an important role and in the application of purified collagens in biomedical, cosmetic or nutriceutical industries.
Although in the human body collagen is a major component, it is a molecule that is difficult to purify and quantitate. This is partly due to the extensive network that is formed by collagen molecules via different types of crosslinking, which makes the collagen molecules insoluble and difficult to extract.
The number and type of commercially available collagen assays is limited. Some assays for soluble collagen exist, based on precipitation of collagen molecules with the dye Sirius Red. However, this assay seems less applicable for the analysis of collagen in tissues. The most widely used collagen assay is based on hydrolysis of collagen to free amino acids, followed by measuring collagen specific hydroxyproline, with either HPLC or a colorimetric method. However, these assays are laborious, time consuming and require special equipment. To overcome these disadvantages, we recently developed the QuickZyme Total Collagen assay, based on the same proven principles of acid hydrolysis and colorimetric detection, but much faster and without the requirement of special equipment.

In this application note we have compared the different assays for their ability to measure collagen in a variety of mouse tissues. We used the QuickZyme Total Collagen Assay (acid hydrolysis, colorimetric detection of hydroxyproline), the HPLC ‘golden standard’ method (acid hydrolysis, HPLC detection of hydroxyproline) and the QuickZyme soluble collagen assay (extraction by acid/pepsin, Sirius red binding, colorimetric detection).

Methods

Healthy mice (C57Bl6, aged 8-10 weeks) were sacrificed and the following tissues were isolated: lung, liver, kidney, spleen, heart and dermis. In addition we used bovine tendon as a sample. The tissues / organs were divided in three parts, of which one part was used for wet weight determination, dry weight determination (after freeze drying the tissue), acid hydrolysis in 6 M HCl (10 mg wet tissue per 100 µl 6M HCl, with a minimal volume of 200 µl) and quantification of hydroxyproline either by HPLC, or using the colorimetric QuickZyme Total Collagen assay. Another part was used for wet weight determination directly followed by acid hydrolysis and hydroxyproline quantification using the QuickZyme Total Collagen assay. The third part was used for wet weight determination, collagen extraction by overnight incubation with 0.5 M acetic acid/pepsin followed by centrifugation and quantification using the Sirius Red based Soluble Collagen assay. See Fig.1 for assay principle of the various assays.

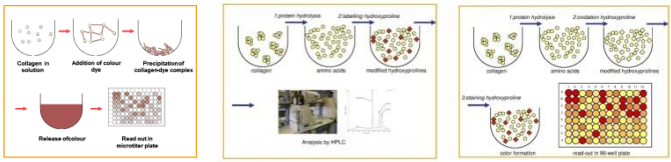

Fig 1. Assay principle of soluble collagen assay (left), total collagen assay by HPLC (middle) and colorimetric total collagen assay (right)

Results

- The quantification of collagen by acid hydrolysis, followed by either HPLC, or the QuickZyme colorimetric assay correlates very good and yields similar values

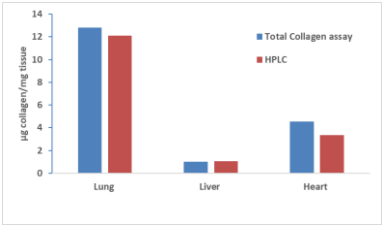

Figure 1. Comparison of HPLC and colorimetric hydroxyproline analysis. Values represent µg collagen/mg wet tissue

- Hydrolysis can either be performed with wet tissue or dry tissue with similar results.

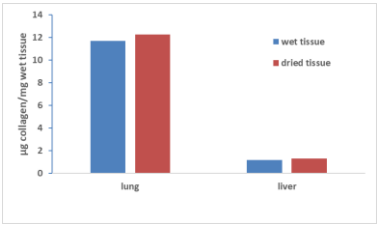

Figure 2. Comparison of hydroxyproline analysis in wet and dried tissue. Values represent µg collagen/mg tissue

- The analysis of solubilized collagen in the various tissues with the Sirius Red based Soluble collagen assay, gave varying results, the values were much lower than obtained for total collagen and for many tissues even near the limit of detection. Compared to the QuickZyme total collagen assay much lower levels were obtained by the soluble collagen analysis, e.g. lung 2.5%, kidney 10%, spleen 8%, dermis 9%, tendon 3%. The low values are probably due to poor extraction of mature collagen from most of the tissues.

- The amounts of collagen in the various tissues measured by the different methods are shown in Table 1. It is clearly shown that the levels of collagen vary per organ, with tendon having the highest collagen content followed by skin, lung, spleen, kidney, heart and liver.

Organ	lung	kidney	spleen	heart	skin	liver	tendon
Collagen µg/mg wet weight tissue	10	5	5	4	150	1	300

Table 1. Approximate amounts of collagen in various tissues (µg collagen per mg wet weight tissue), analyzed by acid hydrolysis followed by either HPLC or QuickZyme colorimetric assay.

- Tissue was hydrolysed at 100 mg/ml (wet weight, see Table 3 for ratio wet/dry weight) in 6 M HCl (minimum volume of HCl 200 µl), the hydrolysate was diluted with 4M HCl as indicated in Table 2 and 35 µl of the diluted hydrolysate was used in the total collagen assay (according to protocol in assay manual). The optimal dilution factor for analysis using the QuickZyme total collagen assay differed per tissue as shown in Table 2. This dilution factor is determined such that no matrix effect is present (occurring if sample is not diluted enough) and the value fits within the range of the standard line.

Organ	lung	kidney	spleen	heart	skin	liver	tendon
Dilution factor	10-50	10-50	10-50	5-25	100-1000	5-10	100-1000

Table 2. Dilution factor range for collagen analysis using the QuickZyme total collagen assay, based on hydrolysates of 100 mg/ml wet weight.

- The ratio of wet weight versus dry weight of the various tissue ranged between 5.0 (lung) and 3.5 (tendon and skin). Analysis of collagen in either wet or dried tissue gave similar results (data not shown).

- In liver tissue hydrolysate the dilution factor is 5-10, due to matrix effect occurring in less diluted samples. This results in low OD values (around or below lowest concentration in standard line). For this type of tissue we therefore developed a modified collagen assay in which no matrix effect is observed, and thus no dilution of the hydrolysate is required (see description page 7, the QuickZyme Sensitive Tissue Collagen assay).

Conclusions

- The amount of collagen differs per tissue: tendon > skin >> lung > kidney > spleen > heart > liver
- Sirius Red based collagen assays are less suited for collagen analysis in tissues, probably due to limited extraction of collagen from tissues
- The best method for quantification of collagen in tissues is full hydrolysis of the tissue followed by analysis of hydroxyproline either by HPLC or by a colorimetric assay
- The QuickZyme total collagen assay is well suited for collagen analysis in tissues, gives similar results as compared with HPLC, but is fast, easy and does not require special equipment.
- For tissue samples with low collagen content and plagued by large matrix effects we developed the QuickZyme Sensitive Tissue Collagen assay.

QuickZyme Biosciences has developed a range of assays for the analysis of collagen from any species, each with a different application area

Collagen assays available at QuickZyme Biosciences:

- *QuickZyme Soluble Collagen assay*
- *QuickZyme Total Collagen assay*
- *QuickZyme Hydroxyproline assay*
- *QuickZyme Sensitive Tissue Collagen assay*
- *QuickZyme Sensitive Tissue Hydroxyproline assay*

QuickZyme Soluble Collagen assay

This assay recognizes soluble or (acid/pepsin) solubilized collagen. The assay is colorimetric, has a 96-well plate format and is based on precipitation of collagen with Sirius-Red, an anionic dye with sulphonic acid groups. This dye can bind the side-chain groups of basic amino acid residues. The dye is released from the precipitated complex at high pH followed by colorimetric detection. The assay is optimized such that other proteins (like albumin) do not interfere. Gelatin (unfolded collagen) is not recognized by this assay.

Application: The assay is used for the measurement of soluble collagen in e.g. cell culture conditioned culture media, and (acid or acid/pepsin) solubilized collagens e.g. from cellular extracts. This assay is less suited for measuring collagen in tissue extracts since extraction efficiency of mature collagens in tissues is very low.

QuickZyme Total Collagen assay

This assay recognizes all types of collagen irrespective of its form (mature, immature, procollagen, degraded collagen, crosslinked collagen, collagen from various sources).

The assay is colorimetric, has a 96-well plate format, and is based on the quantification of hydroxyproline, an amino acid exclusively occurring in collagen. Hydroxyproline is released from collagen upon acid hydrolysis of the collagen containing sample. Hydrolysis is carried out at 95^0C, and the product can directly be used for hydroxyproline analysis, without washing or drying steps. This analysis is based on established Chloramine T/DMBA methodology.

Application: The assay is used for the measurement of total collagen. This includes all collagen types and forms, such as: procollagen, unfolded collagen, mature collagen as well as collagen degradation products of all collagen types present in the sample. Since the first step is complete hydrolysis of the sample, difficulty in extraction of collagen plays no role. The assay is applicable for various types of samples, including tissues.

QuickZyme Sensitive Tissue Collagen assay

A common complication in measuring collagen with hydroxyproline based colorimetric assays applied to tissue samples is the occurrence of so called matrix effects, caused by non-identified components in the sample. The matrix effect can result in erroneous high or low values. Matrix effects can be prevented by dilution of the sample. This dilution,

however, results in lower effective sensitivity for the sample, which is particularly problematic for small samples with low collagen content. In contrast to other hydroxyproline-based assays, the QuickZyme Sensitive Tissue Collagen assay has no matrix effect and no need for dilution of the hydrolysate enabling measuring in small samples with low collagen concentrations. The assay is simple and doesn't need the drying step following acid hydrolysis for which often special equipment is required.
Application: The assay is used for the measurement of collagen in tissue samples and particularly tissues with low collagen content such as liver.

QuickZyme Hydroxyproline assay
This assay is similar to the total collagen assay, with the difference that no protocols and materials are included for collagen hydrolysis and no collagen standard, but a hydroxyproline standard is provided.
Application: This assay has the same application area as the total collagen assay, but is intended for customers who have their own hydrolysis method, or have a collection of hydrolyzed samples to be analyzed.

QuickZyme Sensitive Tissue Hydroxyproline assay
This assay is similar to the Sensitive Tissue collagen assay, with the difference that no protocols and materials are included for collagen hydrolysis and no collagen standard, but a hydroxyproline standard is provided.
Application: This assay has the same application area as the Sensitive Tissue collagen assay, but is intended for customers who have their own hydrolysis method, or have a collection of hydrolyzed samples to be analyzed.

For more detailed information
(including other application notes, product sheets, assay manuals and FAQs) please visit the product and support sections on our web site:

https://www.quickzyme.com/products/

https://www.quickzyme.com/support/

QuickZyme Biosciences B.V.

Sylviusweg 71
2333 BE Leiden
The Netherlands

www.quickzyme.com
info@quickzyme.com