Collagen analysis in fish tissue using the total collagen assay

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QuickZyme Biosciences

Summary

The QuickZyme Total Collagen assay provides an easy and fast method to determine collagen quantities in biological samples. This application note provides guidance for the application of this assay with fish tissue and shows a comparison with HPLC.

Introduction

Collagens are the most abundant proteins in the vertebrate body. In fish (teleostei) the collagen content is approximately 3% of total protein, considerably lower than in mammals (17%).[1] The collagen content is responsible for the integrity and texture of the fillets.
The hydroxyproline content of fish tissue is related to the habitat. Collagens of fish species living in warm water have higher hydroxyproline levels than that of cold water species and hydroxyproline levels in mammals are even higher. These hydroxyproline levels correlate with the melting temperature of the collagens of the species.[2]
Fish collagens are increasingly used to replace traditional animal collagens in the preparation of gelatin for food, cosmetic or pharmaceutical applications. Fish collagens do not have religious objections and no problems related to BSE.
Accurate analysis of collagen in fish tissue is important both as quality control in consumption fish and in optimizing conditions in fish aquaculture. Although collagen is a major component in fish tissue 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 through 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, these assays seem 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, using 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 faster, easier and without the requirement of special equipment.

In this application note we show the optimization of this assay for its use ability to measure collagen in fish tissue.

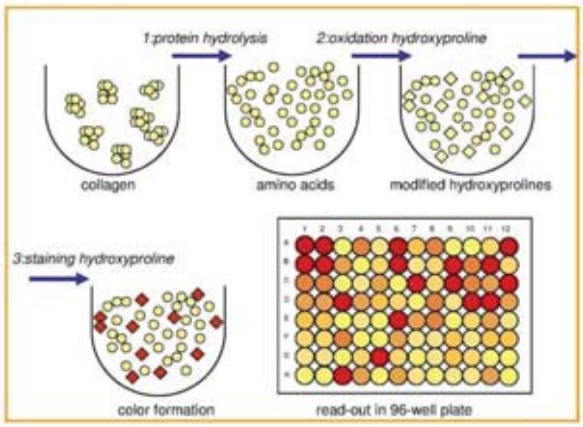


Fig 1. Assay principle of colorimetric Total collagen assay

[1] FAO. © 2008-2013. Fisheries and Aquaculture topics. Proteins from fish and fish products. Topics Fact Sheets. Text by Lahsen Ababouch. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 27 May 2005. [Cited 22 February 2013]. http://www.fao.org/fishery/topic/14869/en
[2] Sikorski et al. Crit Rev Food Sci. and Nutr 2009 (20) 301-343

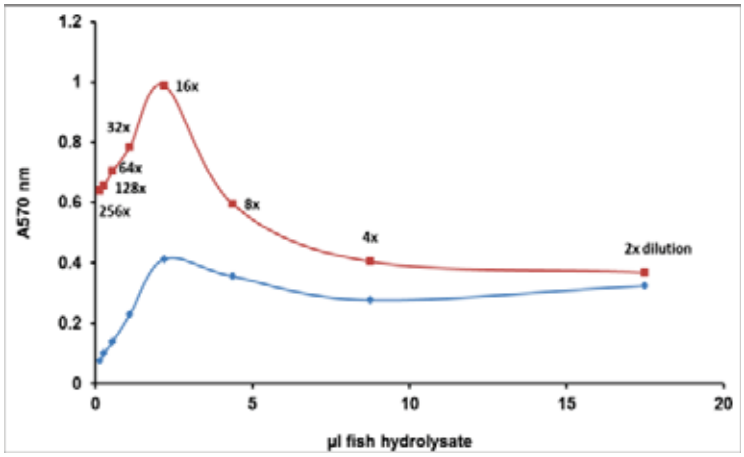
Methods

Frozen Tilapia fillet was obtained in a local supermarket and stored frozen at -20º C. Immediately before use the fish fillets were thawed and pieces of about 300 mg of tissue were weighed in the hydrolysis tubes provided with the QuickZyme Total Collagen assay kit.

1.0 ml of 6 M HCl per 300 mg of tissue was added to the tubes and the tubes were firmly closed. Thereafter the tissue was hydrolyzed overnight at 95º C in a temperature controlled heat block. The hydrolysate becomes very dark and contains small black particles. After cooling to room temperature the hydrolysis tubes were centrifuged for 10 min at 13000xg. Part of the particles could not be precipitated. An aliquot of the supernatant was carefully removed, avoiding as much as possible to transfer the particles. Serial dilutions of the hydrolysates in 4 M HCl were made from 2- to 256-fold dilution. 35 microliter samples of the several hydrolysate dilutions were used in the total collagen assay according to the manual.
The hydrolysates were also analysed by HPLC according to Bank et al.[3]

Results

The dilution curve of the hydrolyzed fish tissue is highly non-linear at low dilutions and only from 16-fold dilution or more a linear relation of response with dilution is found (Fig 2). This suggest that the hydrolysate contains material that at high concentrations interferes with the hydroxyproline color reaction. This interference disappears on considerable dilution of the sample. To study assay recovery a fixed amount of hydroxyproline was spiked to the various hydrolysate dilutions. The recovery of hydroxyproline was very poor at low dilutions (10% at 2-fold dilution) but from dilutions of 16-fold or more a linear relationship with dilution was found. This is in excellent agreement with the results from the fish hydrolysates. The recovery of hydroxyproline at 16-fold or higher dilution was approximately 120%.



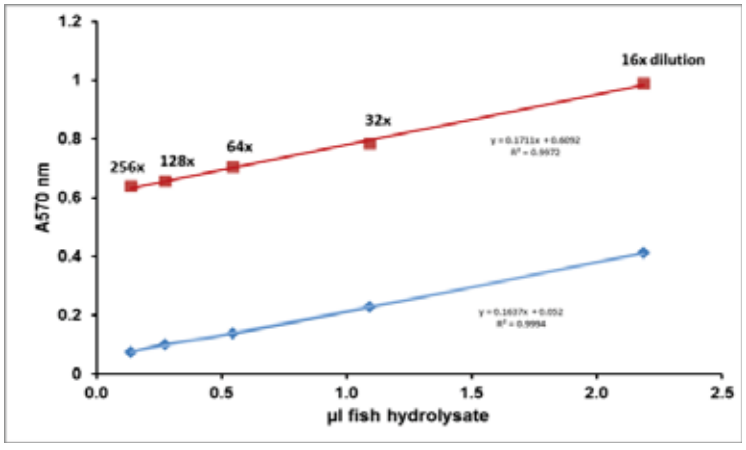


Fig. 2 Effect of dilution of fish hydrolysate on response of total collagen assay. Blue line: fish hydrolysate, red line: fish hydrolysate spiked with 150 µM hydroxyproline (Hyp). Upper panel 2- to 256-fold dilution, lower panel 16- to 256-fold dilution. From 16- fold dilution downwards a linear relationship with dilution is observed

[3] Bank et al. Matrix Biology 1997 (16) 233-243

Comparison of QuickZyme Total Collagen assay with HPLC

The same hydrolysates used for the colorimetric total collagen assay were also analysed by HPLC according to the method of Bank et al [4]. In short: hydrolysates were dried by evaporation of the HCl in a speedVac vacuum centrifuge. Amino acids in the samples were derivatized with OPA, excess of OPA was removed and iminoacids (Pro and Hyp) were derivatized with FMOC and several dilutions were brought on a HPLC reverse phase column. Collagen amount was determined by comparison of the hydroxyproline peak area with that of a standard collagen preparation hydrolysed and derivatized in the same way. Results were expressed in mg of collagen per gram wet Tilapia tissue.

Also with the HPLC method the response was non-linear with dilution at low dilutions. Upon sufficient dilution a linear response with dilution was observed. These dilutions were used for determination of collagen in fish tissue.

With the QuickZyme Total collagen assay 4.5 mg of collagen per g of wet Tilapia tissue was found, in good agreement with the 5.1 mg of collagen per g of wet tissue found with the HPLC method.

Conclusions

- Fish hydrolysate contains factors that disturb the QuickZyme Total collagen assay, since a strongly non-linear relationship with dilution is observed at low dilution factors, both for the fish hydrolysate itself and the spiked hydroxyproline.
- Upon dilution of 16-fold or more a linear relationship of response with dilution is observed both for fish hydrolysate and spiked hydroxyproline
- The parallel lines of hydrolysate and hydroxyproline spiked hydrolysate point to good recovery and similar behavior of the two samples.
- Also with the HPLC method a non-linear relationship between response and dilution was observed at low dilutions, and a dilution of at least 64-fold was required.
- It is advisable to determine the optimal dilution for a the particular species in a pilot experiment. The data presented here can be a guidance to design the pilot.
- The QuickZyme Total collagen assay is well suited for collagen analysis in fish tissues when sufficiently diluted hydrolysates are used. Results are well comparable with the standard HPLC method.

Collagen assays available

- *QuickZyme Soluble collagen assay*
- *QuickZyme Total collagen assay*
- *QuickZyme Hydroxyproline assay*

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