

Modified Lowry's Method for Acid and Pepsin Soluble Collagen Measurement from *Clarias* Species Muscles

Peck Loo Kiew and Mashitah Mat Don*

School of Chemical Engineering, Universiti Sains Malaysia, 14300 Nibong Tebal, Seberang Perai South, Penang, Malaysia

Abstract

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the muscles of cultured *Clarias* species (hybrid of *Clarias gariepinus* × *C. macrocephalus*), a freshwater catfish that is widely consumed in Malaysia. The concentration of extracted collagen was determined using Lowry's method with slight modification. Results showed that such modification was possible for measurement of collagen concentration from the extracting medium at any time directly. The yield of PSC was 3.8-fold higher as compared to ASC though no significant increase in the yields of both collagens was achieved after 12 hr of the extraction period. At the end of extraction process, the yields of ASC and PSC were 97.523 mg/g and 368.360 mg/g, based on the dry weight of muscles, respectively. This showed that measurement of collagen yield by modified Lowry's method was appropriate to govern and monitor the extraction of collagen from any sources.

Keywords: Catfish; Muscles; Collagen; Modified lowry's method; Extraction

Introduction

Collagen is the most abundant animal protein polymer, accounting for almost 25 to 30% of total protein in animal body [1,2]. Being one of the extracellular matrix constituents of multi-cellular animals [3], it can be found in connective tissues and served as major component of bones, cartilage, skin, tendons, ligaments, blood vessels, muscles, teeth and other organs of vertebrate [4,5]. Studies on extraction of both mammalian and aquatic collagens have been extensively carried out recently due to its broad applications in cosmetic, biomedical and pharmaceutical industries [6]. Widespread utilization of collagen as a biomaterial is urging the needs of developing a reliable and effective routine collagen measurement method. This ensures better control of the collagen extraction process by rapid determination of collagen concentration in the extraction medium from time to time.

Conventionally, quantification of collagen extracted from various sources is done either by determining content of one of the imino acids [7] known as hydroxyproline [8-10] or yield by dry weight fraction resulting from weight of lyophilised collagen over dry weight of the raw material used [11-13]. Even though these methods are reported as reliable and sensitive, they are inappropriate for routine measurements since hydrolysis and drying of collagen sample are required [14]. Moreover, hydroxyproline is correlated to collagen content by a particular value of conversion factor which differs for each source. The value of conversion factor 7.7 is used for the amount of collagen extracted from bigeye snapper [9], 14.7 for cod [8], 7.46 for bovine tendon [15] and 7.25 for porcine [16], respectively. It is worth to note that not every collagen sources have the established conversion factor, with exception to those being reported in literatures. Moreover, these would be difficult to correlate hydroxyproline content to the amount of collagen for any new sources due to limited information.

According to few researchers, photometry is another typical routine method to determine concentration of collagen in a substance [14,17]. Measurement of collagen UV absorbance was normally done between 230 to 280 nm [14] due to presence of phenylalanine and tyrosine which absorbed ultra-violet light at 251 and 283 nm respectively [18]. In order to determine an increase of collagen concentration in extracting medium, quantitative measurements of bovine tendon collagen were conducted at the wavelength of 280 nm [19]. According to [14], this

method is pH dependent and is not appropriate for opalescent, turbid and coloured solution. Presence of interfering substances such as urea, glycerol and glycine lead to inaccuracy in results as well. Photometric determination of collagen concentration using 2, 3, 5, 6-tetrachloro-p-benzoquinone (chloranil) as the reagent on the other hand depends upon reaction between collagen with chloranil in water and hydrophilic organic solvents [17]. This makes it less applicable to measure collagen concentration in extracting medium which is normally made up of 0.5 M acetic acid.

The objective of this study was to describe on the routine measurement in determining acid soluble and pepsin soluble collagen concentration profile from muscles of freshwater fish, particularly catfish which is abundant in the Malaysian market. So far, the literature available on this topic is very limited. There is no information concerning direct measurement of collagen concentration from the extracting medium, even though a lot of alternative sources of collagen have been extensively studied. Less attention was paid to quantitative study of collagen extraction in contrast to physiochemical and biochemical properties of extracted collagen. Therefore, modification of Lowry's method as reported by Komsa-Penkova et al. [14] was exploited to study on changes of catfish's muscles collagen concentration in extracting medium at any time along the extraction process.

Materials and Methods

Materials

Cultured catfish (hybrid of *Clarias gariepinus* × *C. macrocephalus*) were purchased from a local wet market in Parit Buntar, Perak. Upon arrival at the laboratory, the fishes were killed, dissected, deboned and

*Corresponding author: Mashitah Mat Don, Associate Professor, School of Chemical Engineering, Universiti Sains Malaysia, 14300 Nibong Tebal, Seberang Perai South, Penang, Malaysia, Tel: 604-5996468; Fax: 604-5941013; E-mail: chmashitah@eng.usm.my, ita56@hotmail.com

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the muscles were cleaned of adhering tissues before being cut into small pieces. Skin was manually removed by using a sharp knife. The muscles were then washed with distilled water and kept frozen at -20°C prior to collagen extraction.

Isolation of muscle type I collagen

Extraction of acid soluble collagen (ASC): All procedures were performed as previously described by Wang et al. and Kimura et al. [11,20] with slight modifications. Whole extraction processes were carried out at 4°C. To remove non-collagenous proteins, the muscles were mixed with 10 volumes (v/w) of 0.1 M NaOH and stirred for 5 to 6 hours. The sample was then washed thoroughly with excessive distilled water until the pH was neutral or slightly basic. The residue was filtered using cheese cloth and actively stirred in 5 volumes (v/w) of 0.5 M acetic acid for 20 hours to extract acid soluble collagen. 8 ml of extracting medium was extracted every hour in the first 4 hours of extraction and every 4 hours subsequently to determine the increase of acid soluble collagen concentration in the medium versus time. After centrifugation at 3,840×g for 15 minutes, soluble collagen solution was obtained from the supernatant. The collagen was precipitated by adding NaCl to a final concentration of 2.0 M in the presence of 0.05 M Tris-HCl buffer (pH 7.2). Resulting sediment was collected by centrifuging at 3,840×g for 20 minutes. The purified collagen was redissolved in minimal amount of 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, followed by distilled water and lyophilized. The freeze-dried product was designated as acid soluble collagen (ACS).

Extraction of pepsin soluble collagen (PSC): The PSC was obtained by the same method as the ASC with the exception that alkali-treated muscles were continuously stirred in 5 volumes (v/w) of 0.5 M acetic acid containing pepsin with an enzyme/substrate ratio of 1:40 (w/w) for 20 hours. 8 ml of extracting medium was extracted every hour in the first 4 hours of extraction and every 4 hours subsequently to determine the increase of pepsin soluble collagen concentration in the medium versus time.

Collagen concentration measurement by Lowry's modification method

Reagent used: Solutions and reagents used included Folin-Ciocalteu reagent, reagent A and reagent B were prepared as described by Komsa-Penkova et al. [14]. Briefly, reagent A was a solution of 0.4% potassium-sodium tartrate, 10% Na₂CO₃ and 0.5 M NaOH. Reagent B consisted of 2% potassium-sodium tartrate, 3% CuSO₄·5H₂O and 0.1 M NaOH. The Folin-Ciocalteu reagent was diluted with distilled water at the ratio of 1:15.

Concentration measurement of acid soluble collagen: Extracting mediums extracted at timed intervals were centrifuged at 3,840×g for 15 minutes and the supernatant were collected, known as collagen solutions. 0.80 ml of the collagen solution was incubated with 0.72 ml of reagent A and 0.08 ml of reagent B at 50°C for 20 minutes. This was followed by addition of 2.40 ml of Folin-Ciocalteu reagent to the samples after cooling to room temperature. The solution was shaken vigorously immediately after the addition and incubated at 50°C for another 10 minutes. After cooling the sample to room temperature, absorbance was read at 650 nm against the reference. A calibration curve was established using different dilutions of calf skin Type I collagen in 0.05 M acetic acid prior to the measurement to obtain linear correlation between concentration and absorbance value of the collagen solutions. Proper dilutions (with dilution factor 5 and 8) were done to samples to obtain absorbance readings within linear range of

the calibration curve. The testing for each set of dilution was performed in quintuplicate.

Concentration measurement of pepsin soluble collagen: Extracting mediums extracted at timed intervals were tested. Higher dilutions (e.g., 15, 20 and 30) were done to the samples and much higher absorbances were recorded as compared to acid soluble collagen.

Collagen yield measurement

Yield of collagen from catfish muscle was calculated as proposed by Li et al. [19]:

$$Y = (V \times C) / W \quad (1)$$

where Y is the yield of collagen in mg/g, V is the volume of extracted collagen solution in ml, C is the concentration of the same solution measured using spectrophotometer in mg/ml, and W is the lyophilized weight of catfish muscle in g.

Results and Discussion

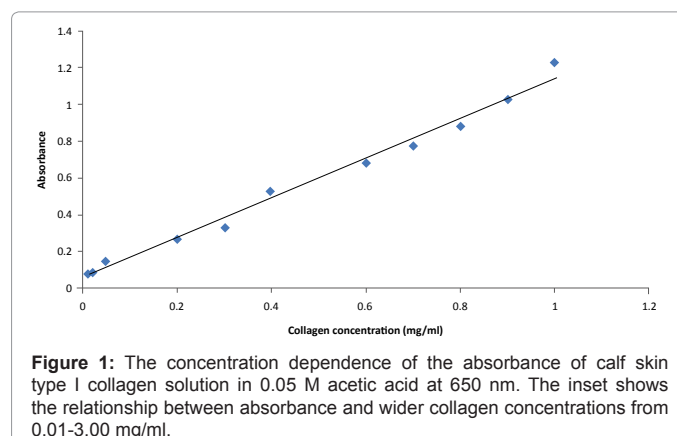
Correlation between collagen concentration and absorbance using Lowry's modified method

The inset in figure 1 shows the relationship between absorbance and concentration of calf skin Type I collagen from 0.01-3.00 g/ml. The basic law of light absorption or Beer-Lambert law is obeyed over the collagen concentration range 0.01-1.00 g/ml in the photometrically measured solution. A linear relationship between absorbance and collagen concentration with a high coefficient of determination (R²=0.99) was found and is shown in figure 1. This is in accordance with the findings by [14,19] that good linear relationship can be achieved only within certain range of collagen concentrations. The calibration curve can be used to monitor concentration of extracted collagen at any time along the extraction process. It is described by the straight line equation:

$$A = 1.0876c + 0.0561 \quad (2)$$

where A is the absorbance and c is the concentration of collagen in mg/ml in the photometrically measured solution.

The Lowry's method is a well-known and widely used protein estimation procedure which the sensitivity is claimed to be moderately constant from protein to protein [21], relying on two different reactions. It starts with formation of copper ion complex with peptide bonds, being stabilized by tartrate under alkaline conditions, known as Biuret chromophore. Colour develops in the second reaction when reduction of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by the reduced copper-peptide bond complex as



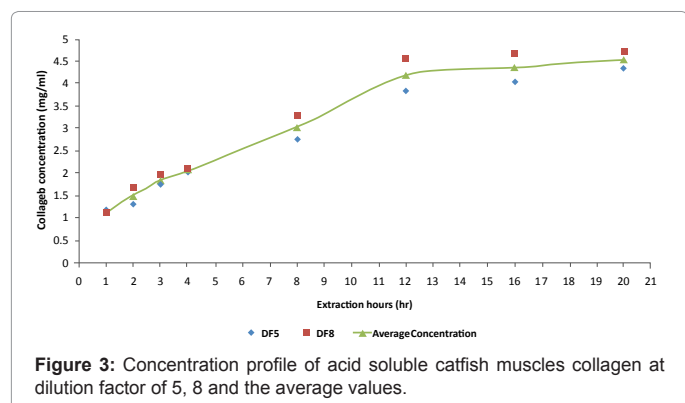
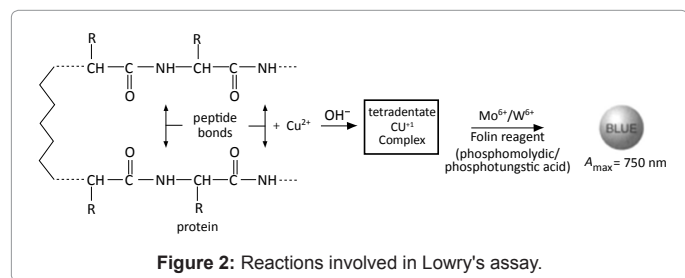
well as by tyrosine and tryptophan residues takes place [22,23]. The reduced Folin-Ciocalteu reagent is blue colour and intensity of colour produced is proportional to protein concentration.

Modification of conventional Lowry's method has successfully adapted this method in determining collagen concentration as well. According to Komsa-Penkova et al. [14], presence of imino acids in collagen which ensure the rigidity of collagen polypeptide backbones, resulted in inaccessibility of peptides to Cu^{2+} ions and finally interfered the formation of copper complexes, causing conventional Lowry's method to be inappropriate to measure collagen concentration. Therefore, incubation of collagen samples at high temperature (50°C) beyond the denature temperature ($40\text{--}43^\circ\text{C}$) is suggested to unfold the triple helix, leading to destruction of rigidity of the polypeptide chains, thus increase the ability to form complexes with Cu^{2+} ions [14]. Reaction schemes for the Lowry's assay are illustrated in figure 2 which makes it measurable at the wavelength of 500-750 nm using a spectrophotometer [22].

Concentration of acid soluble collagen

Direct measurement of acid soluble collagen concentrations without any dilution resulted in high value of absorbance readings which exceeded linear range of the calibration curve. This was in line with the report by Wrolstad et al. and Curtis et al. [24,25] that in many applications, limited linear range of calibration curve is often not sufficient to estimate actual concentration of unknown samples. Some samples would contribute responses beyond the upper end of the established calibration curve. Therefore, samples analysed with dilutions are required to obtain results within linear range of the curve. Figure 3 shows the concentration profile of acid soluble collagen from catfish muscles estimated by dilution factor of 5 and 8.

From figure 3, the collagen concentration for each dilution is determined from the average of its quintuplicate. According to [24], if multiple dilutions of sample have been done, the results for all dilutions which fall within linear portion of working range are averaged. Therefore, final collagen concentrations were calculated from the



average of both 5 and 8 folds dilutions. A positive relation was found between collagen concentration and the extension of time, especially when the time was at the range of 4-12 hours. Increase in collagen concentration was no longer significant when the time was longer than 12 hours. Maximum concentration of collagen extracted in 0.5 M acetic acid was found to be approximately 4.5 mg/ml.

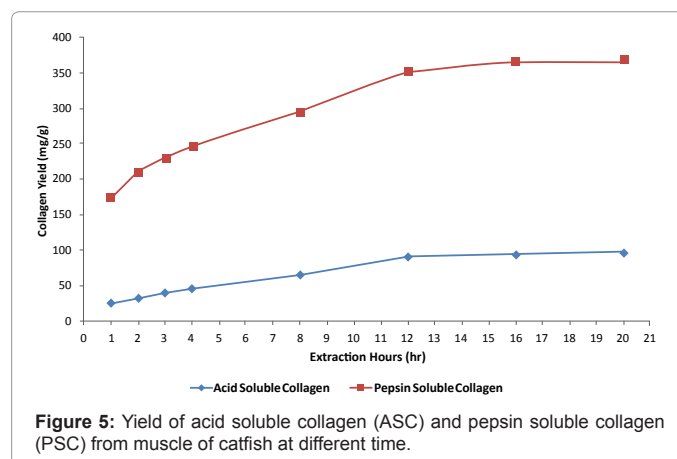
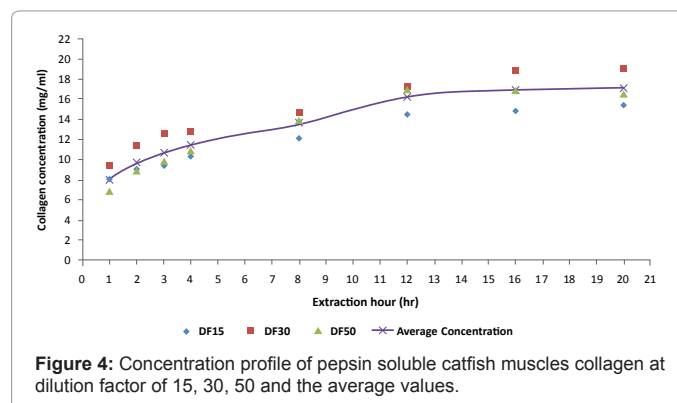
Concentration of pepsin soluble collagen

Higher dilution factors were required to analyse concentration of pepsin soluble collagen in contrast to acid soluble. However, similar pattern of the concentration profile was obtained for both acid and pepsin soluble collagen as illustrated in figure 4. Rapid increase in pepsin soluble collagen concentration was noticed in the extracting medium from 1-12 hours of extraction. The increment was no longer obvious after reaching approximately 17 mg/ml after 12 hours. This was similar to that of acid soluble collagen.

Yield of acid soluble collagen (ASC) and pepsin soluble collagen (PSC)

The effect of time on the yield of both acid soluble and pepsin soluble collagen is shown in figure 5. Influence of pepsin addition into the extracting medium was obvious in which the yield of collagen increased significantly even at the first hour of extraction. With the aid of pepsin digestion, yield of muscle collagen increased from 97.523 mg/g to 368.360 mg/g at the end of extraction, which were about 3.8-fold higher. This finding pointed out that yield of PSC from balloon fish skin was 5-fold higher than the ASC [26].

Catfish muscle was not completely solubilised by 0.5 M acetic



acid, as shown by the much lower yield of ASC. It was in agreement with Singh et al. and Muralidharan et al. [27,28] who reported that incomplete solubilisation of striped catfish skin and trash fish in 0.5 M acetic acid. In fact, such increase in collagen solubility regardless of the sources after pepsin treatment was also reported by many authors [1,9,26,29]. Enzymatic treatment of muscles with pepsin assisted in removing only the non-helical ends (telopeptides) of the collagen which act as the inter-molecular crosslink [30]. The difference in yields of ASC and PSC from muscle of catfish might be suggesting the existence of inter-molecular crosslink at the telopeptides region of the collagen that resulted in low solubility in acid. Following pepsin digestion, crosslinked regions at the telopeptides were cleaved without damaging the integrity of the triple helix. This led to higher solubility of collagen in acid [31,32]. Therefore, [33] proposed applying enzymatic pre-treatment of connective tissue by proteolytic enzymes non-specific for collagen included pepsin, trypsin, pancreatin, ficin, bromelain and papain as one of the alternatives to increase yields in collagen extraction.

As presented in figure 5, further prolongation of the extraction time after 12 hours did not increase the collagen solubility in extracting medium for both ASC and PSC. According to [11], mass transfer of analyte from the matrix is the key role in determining efficiency of extraction. In our study, it was directly controlled by the rate of diffusion of collagen from the matrices of muscles into the bulk extracting medium (0.5 M acetic acid) which was time-concerned. Therefore, recovery of collagen was expected to keep increasing with the extension of time which explained the present pattern of the concentration profile for both ASC and PSC up to 12 hours in this work. Insignificant increase in yield for ASC after 12 hours was contributed by the limited solubility of collagen in 0.5 M acetic acid as described earlier. Likewise, the positive effect of pepsin digestion on collagen yield of PSC was more pronounced during the first 12 hours. No improvement in the yield was attained after that [34] suggested that larger solvent to material ratio could favour the extraction by increasing the concentration gradient and diffusion rate of analyte into the solvent. Thus, it was assumed that equilibrium collagen concentration was achieved during that period of time. No more collagen would be diffused into the bulk acid medium due to absence of concentration gradient.

Conclusion

Modified Lowry's method has been proven to be a simple way that was suitable for routine measurement of collagen concentration in any collagen extraction processes. In present study, acid soluble and pepsin soluble collagen concentration from muscles of cultured catfish (hybrid of *Clarias gariepinus* × *C. macrocephalus*) were studied. Effect of pepsin treatment showed a significant role in the extraction, rendered much higher collagen yield of PSC as compared to ASC. The results of this work were helpful for the development of collagen from cultured catfish, a commonly consumed freshwater fish in Malaysia. However, further research on the effect of variables such as acetic acid concentration, extraction temperature and solvent to material ratio is important in order to obtain the optimum yield of collagen from the fish muscles. Recovery and isolation of collagen from freshwater fish muscles would be able to boost up the commercial values of these cheap and under-utilized protein sources while assisting in conservation of these natural resources.

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