# Isolation and Characterization of Collagen from Marine Fish (Thunnus obesus)

**ARTICLE** *in* BIOTECHNOLOGY AND BIOPROCESS ENGINEERING · NOVEMBER 2013

Impact Factor: 1.11 · DOI: 10.1007/s12257-013-0316-2



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Biotechnology and Bioprocess Engineering 18: 1185-1191 (2013)
DOI 10.1007/s12257-013-0316-2

RESEARCH PAPER

# Isolation and Characterization of Collagen from Marine Fish (*Thunnus obesus*)

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Received: 13 May 2013 / Revised: 16 June 2013 / Accepted: 18 June 2013


**Abstract** In the present study, we isolated collagen from *Thunnus obesus* bone, which was physiochemically characterized. Two different kinds of methods were used to isolate the collagen; they are the Acid Soluble Collagen (ASC) and Acid Soluble Enzyme Collagen (ASEC) methods. The isolated collagen was characterized with Fourier Transform Infrared Spectroscopy (FT-IR), SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Optical Microscopy (OM) and Scanning Electron Microscopy (SEM). FT-IR results revealed the presence of collagen. SEM and OM results depicted that collagen was in the form of fiber sponge-like scaffolds. The isolated collagen scaffold was checked with pre-osteoblast (MC3T3-E1) cell line for biocompatibility. The *in vitro* results revealed that the collagen scaffolds were highly biocompatible and non-toxic in nature. Herewith, we are suggesting that marine fish-derived collagen will be an excellent material for leather, film industry, pharmaceutical, cosmetics, biomedical and food applications.

**Keywords:** marine biomaterials, marine protein, artificial bone, sea processing waste

## 1. Introduction

Collagen is the main component of connective tissue, the most abundant fiber form protein in vertebrates, and constitutes about 30% of vertebrate total proteins. Collagen structure is distinguished by the formation of a right-handed triple superhelical rod, consisting of three almost identical polypeptide chains [1]. The triple helix structure forming collagens are present in several forms: skin, bones, cartilage, teeth, cornea, *etc*. [2]. Twenty-eight different types of collagen composed of at least 46 distinct polypeptide chains have been identified in vertebrates, and many other proteins contain collagenous domains [3,4]. Collagen has a wide range of applications in leather, the film industry, pharmaceutical, cosmetic and biomedical materials, and food [5]. Collagen has been widely used in tissue engineering biomaterials in the last two decades, due to its mimicking properties of human protein, which can be used in the treatment of hypertension, urinary incontinence and pain associated with osteoarthritis, use in tissue engineering for implants in humans, and inhibition of angiogenic diseases, such as diabetes complications, obesity, and arthritis [6].

Several isolation techniques and various kind of source are available for the collagen isolation. Generally, collagen is obtained from animal skin or bone, and is currently used mostly attained from animal skin at 12 g per 1 kg. The process is complex, costly and time-consuming. Non-denatured collagens from these sources find applications in cosmetics, biomedical, and pharmaceutical industries. Denatured collagen, known as gelatin, finds applications in the food and biomedical industries [2]. Reconsideration of industrial use of bovine collagen is mandatory, to avoid the risks of BSE (bovine spongiform encephalopathy), and TSE (transmissible spongiform encephalopathy) [7]. Additionally, the collagen obtained from pigs cannot be used as a component of some foods, for religious reasons [8].

As a consequence, increasing attention has been paid to alternative collagen sources, especially fish skin and bone from seafood processing wastes [9-11]. About 30% of

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these wastes consist of skin and bone, which are very rich in collagen [12]. Annually, over 100 million tons of fish are harvested worldwide, and a third of the total catch is used for fishmeal and animal feed [13]. *Thunnus obesus* (Tuna) is widely eaten in Asian countries, and sliced as raw fresh, canned tuna and steak. A lot of tuna food has been dumped as commercial and domestic waste. Improper disposal of wastes, including the bone, brings about adverse ecological impacts on coastal resources. Hence, the extraction of collagen from fish bone could be a promising means to gain value-added product, and to lower environmental pollution [14]. Recently, many studies have reported the extraction of collagen from marine resource [7,8,14-18]. As per our scientific knowledge, no information regarding collagen isolation from tuna bone has yet been reported. Thus, in the present study, we are attempted to isolate pure collagen. The main objective of the present study is to establish the optimal conditions for the isolation of collagen from tuna bone.

## 2. Materials and Methods

### 2.1. Preparation of collagen from fish bone

The bones were broken by hammer, and extracted with 0.1 N NaOH for 3 days, to remove non-collagenous proteins. This process was repeated for twice a day. Then the residue was washed with distilled water and lyophilized. The insoluble bones were decalcified with ethylenediaminetetraacetic acid (EDTA) solution of 5% (w/v) (pH 7.4) for 5 days, by changing this solution once a day. After fully washing the residue with distilled water, fat was removed with 10% butyl alcohol for 3 days, by changing this solution once a day. The residue was fully washed with distilled water, and lyophilized.

Collagen was then extracted using acetic acid, following two different methods. In Method 1, acid soluble collagen (ASC) was extracted with 0.5 M acetic acid, at sample/acid of 2% (w/v) for 4 days. In Method 2, acid enzyme soluble collagen (ASEC) was extracted by the addition of 0.1% (w/v) pepsin (1:10,000) to 0.5 M of acetic acid, at sample/acid of 2% (w/v) for 4 days. After each extraction, the solution was centrifuged at 3,000 g for 15 min. The residue was re-extracted with the same solution for 5 days, and the extract was centrifuged under the same conditions. The procedures for extraction and centrifugation were repeated 4 times. Each viscous solution was mixed, and salted out by adding NaCl to a final concentration of 0.7 M in 0.5 M acetic acid, and followed by precipitation of the collagen by the addition of NaCl, to a final concentration of 2.5 M in Tris-HCl (pH 7.5). After 1 day, each resultant precipitate was obtained by centrifugation at 7,000 g for 10 min, dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water, and then lyophilized (Fig. 1).

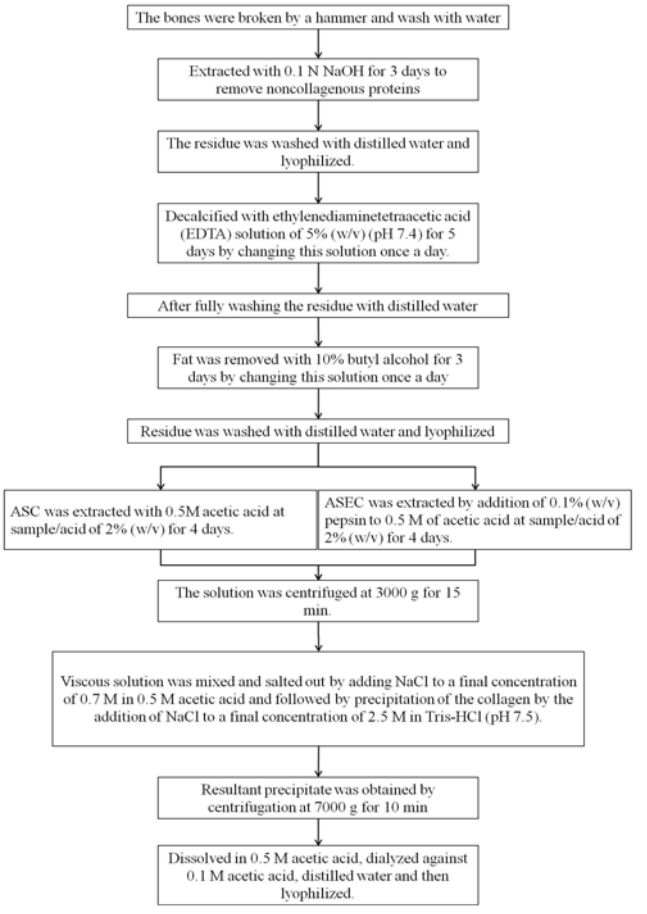


**Fig. 1.** Isolation of collagen-flow chart.

### 2.2. Fourier transform infrared spectroscopy

The collagen samples were prepared in the form of potassium bromide (KBr) disk, and the films were studied. The collagen samples were mechanically blended with KBr. The mixture was compacted using an infrared (IR) spectroscopy hydraulic press, at a pressure of 8 tons for 60 sec. The stretching frequencies of collagens were examined by Fourier transform infrared spectroscopy (FT-IR) (Perkin Elmer Inc., USA) and spectrum GX spectrometry, within the range of 400 ~ 4,000/cm.

### 2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The collagen samples were dissolved in 0.1 M acetic acid containing 5% SDS, and the mixtures were centrifuged at 8,500 g for 5 min at room temperature, to remove undissolved debris. Solubilised samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS and 20% glycerol). Samples were loaded onto polyacrylamide gels consisting of a 6% running gel and a

5% stacking gel, and subjected to electrophoresis at a constant current of 20 mA/gel, using the manufacturer's instructions (BioRad Laboratories, Hercules, CA). After electrophoresis, the gel was then stained for 3 h with 0.025% (w/v) Coomassie blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was finally destained for 1 day with 40% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight markers (Fermentas cat #SM0671) were used to estimate the molecular weight of proteins.

### 2.4. Microscopic analyses

The surface microstructures of collagens were analyzed by scanning electron microscope (JEOL JSM-6490LV) and optical microscopy, to evaluate the structure of isolated collagen. The surface of collagens was sputtered with gold, and was photographed.

### 2.5. Cell proliferation and cytotoxicity

The cell proliferation and cytotoxicity with the prepared collagens were studied by MTT assay [19], using pre-osteoblast MC3T3-E1 cells. In order to measure the cytotoxicity, 100 μL of $1 \times 10^3$ cells were seeded in 96-well culture plate. After 24 h, collagens (10, 50, 100, and 200 μg/mL) were treated and incubated at 37°C in a humidified 5% $CO_2$ atmosphere, until 1 and 3 days, respectively. The media were removed, and incubated with fresh culture medium containing 100 μL of MTT for 4 h in darkness. Then, the unreacted dye was removed; finally, 100 μL dimethyl sulfoxide was added to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). The relative cell viability was determined by the amount of MTT converted into formazan salt. Cell viability and cell proliferation of cells were quantified as a percentage, compared to that of the blank.

### 2.6. Statistical analysis

All the data are expressed as means ± standard deviation of a minimum of three replicates in each experiment, using Sigma Plot 10.0.

## 3. Result and Discussions

### 3.1. General observation

In the present study, collagen was extracted by acid-soluble extraction (ASC), and acid enzyme-soluble extraction (ASEC) methods. The ASEC method produces greater yield collagen, as compared to the ASC method. The ASC results might be attributed to the low solubility of cross-links formed via the reaction of aldehyde with lysine and hydroxylysine at telopeptide helical sites [20]. With further limited pepsin digestion, the cross-linked molecules at the telopeptide region were most likely cleaved, resulting in the increased collagen extraction efficacy [21]. Pepsin was able to cleave specifically at the telopeptide region of collagen from snakehead fish scale [17]. This might be the main reason the ASEC method produces a higher amount of collagen.

### 3.2. Fourier infrared spectroscopy analysis

FT-IR spectra of ASC and ASEC are exhibited in Fig. 2 and Table 1. The IR spectra of ASC and ASEC were similar. They showed the characteristic peak of Amide I, II, III, as

**Table 1.** Important stretching FT-IR frequencies of isolated collagen

| Region | Peak wavenumber (/cm) | | Assignment |
|---|---|---|---|
| | ASEC | ASC | |
| Amide A | 3446 | 3352 | NH stretch coupled with hydrogen bond |
| Amide B | 2926 | 2927 | $CH_2$ asymmetrical stretch |
| - | 2860 | 2880 | $CH_2$ symmetrical stretch |
| - | 2066 | 2082 | |
| Amide I | 1645 | 1646 | C=O stretch/hydrogen bond coupled with COO- |
| Amide II | 1554 | 1558 | NH bend coupled with CN stretch |
| - | 1494 | / | |
| - | 1452 | / | $CH_2$ bend |
| - | 1406 | 1404 | COO- symmetrical stretch |
| - | 1385 | 1385 | |
| Amide III | 1263 | 1262 | NH bend coupled with CN stretch |
| - | 1050 | 1051 | C-O stretch |
| - | 876 | 876 | |
| - | 824 | 825 | |
| | 605 | 606 | Skeletal stretch |

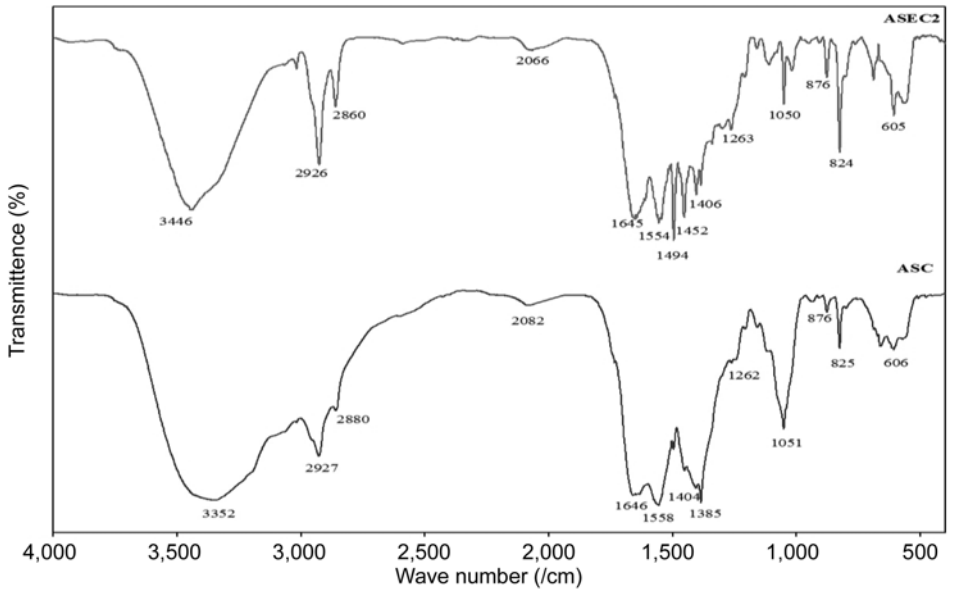


**Fig. 2.** FT-IR of acid soluble collagen and acid soluble enzyme collagen from tuna bone.

well as amide A and B. The absorption characteristics of Amide A, commonly associated with N-H stretching vibration, occurs in the wave number range 3400 ~ 3440/cm [22]. The absorption peaks of ASC and ASEC were found at 3352 and 3446/cm, respectively. When the N-H group of a peptide is involved in a hydrogen bond, the position starts to shift to lower frequencies. Amide B peaks of ASC and ASEC were found at 2927 and 2926/cm, representing asymmetrical stretch of $CH_2$ [23]. The wave number of characteristic absorption in Amide I bond is usually in the range of 1600 ~ 1700/cm, which is generated by stretching vibration of C=O in polypeptide backbone of protein. This is the sensitive area of changes of protein secondary structure, and often used for protein secondary structure analysis. The absorption peaks of ASC and ASEC were found at 1646 and 1645/cm, respectively. The Amide II peak usually takes responsibility for the combination of the N-H in-plane bend and the CN stretching vibration [24]. The amide II bands position of ASC and ASEC were detected 1558 and 1554/cm, respectively. Amide II of ASEC was found at lower wave number, compared with ASC. Therefore, Amide I and II bands of ASEC shifted to lower wave number, as compared to those of ASC, suggesting that there was more and/or a stronger hydrogen bond in ASEC [14]. This result was in accordance with the Amide A band. Amide III bands were found at wave number of 1262 and 1263/cm for ASC and ASEC, respectively. The Amide III peak is complex, with intermolecular interactions in collagen, consisting of components from C-N stretching and N-H in plane bending from amide linkages, as well as absorptions arising from wagging vibrations from $CH_2$ groups, from the glycine backbone and proline side-chains [25]. The IR spectra of ASC and ASEC had similarity to each other, which suggested their structures were quite similar [15].

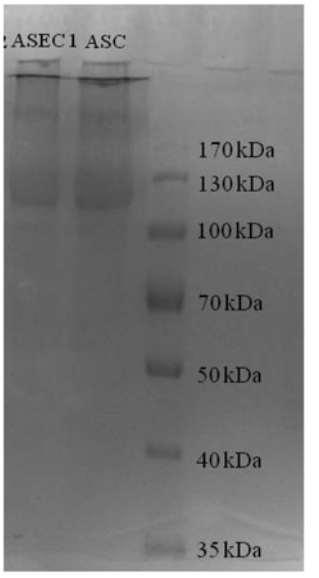


**Fig. 3.** SDS-PAGE pattern of collagens from tuna bone.

### 3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To identify each of the α chains, they were analyzed by SDS-PAGE (Fig. 3). ASC and ASEC had at least two different α chains, $\alpha_1$ and $\alpha_2$, and their cross-linked chains can also be observed. In both ASC and ASEC, subunits were about 130 kDa for $\alpha_1$, and 115 kDa for $\alpha_2$. These

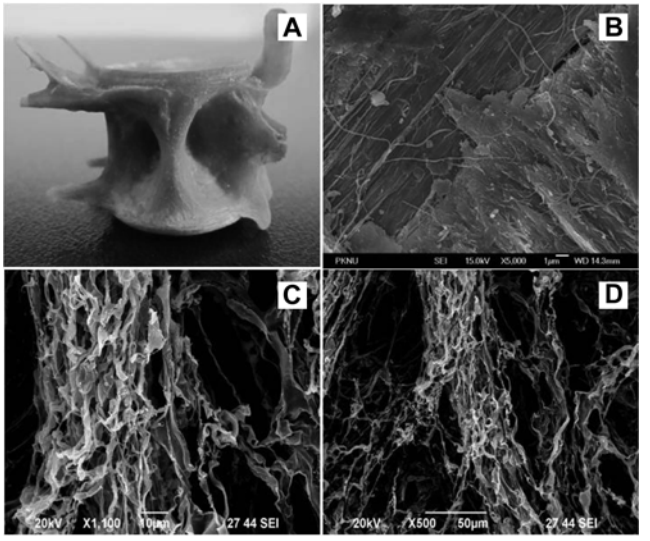


**Fig. 4.** (A) Unwashed Raw bone, and Scanning Electron Microscopy images of (B) Raw bone, (C) ASC Collagen, and (D) ASEC Collagen.

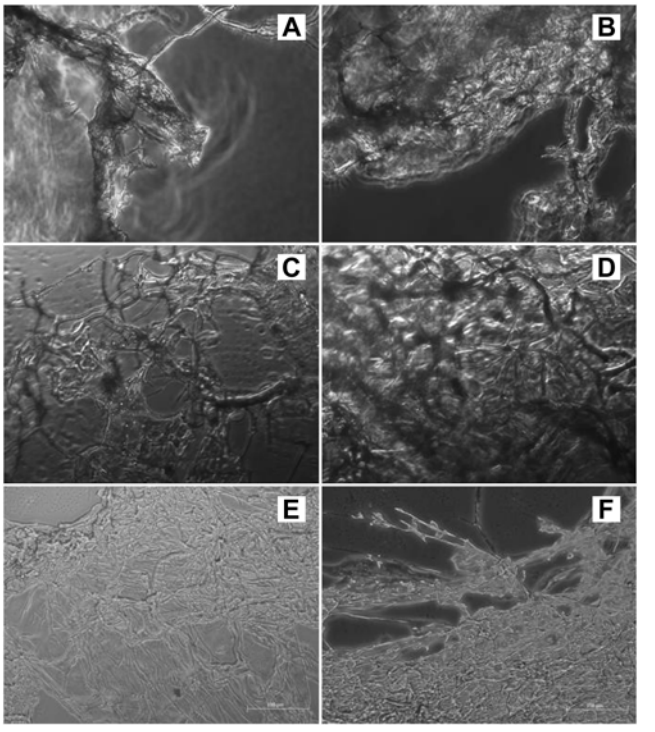


**Fig. 5.** Optical microscopy images of collagen in (A) and (B) dry condition, (C) and (D) with water, and (E) and (F) in ethanol.

values were similar to collagens from Nile perch, black drum, sheeps head sea bream, bigeye snapper, Brown stripe red snapper, *etc*. [16,18,26]. Additionally, both collagens found cross-linked components, β and γ components. The intra- and intermolecular cross-linking of collagen, β and γ components, were richer in ASC, than in ASEC. This greater cross-linking was explained by conversions of some β- and γ-chains in fish bone collagen matrix to α-chains, by the action of pepsin [1]. Pepsin removes the cross-link-containing telopeptide, and concomitantly the one β-chain is converted to two α-chains [27]. The $\alpha_3$ chain could not be observed under the electrophoretic conditions employed, because $\alpha_3$ migrates electrophoretically to the same position as $\alpha_1$ [28-30]. Based on electrophoretic mobility and subunit composition, it was suggested that the collagens from bone was type I collagens.

### 3.4. Scanning electron microscopy and optical microscopy

Scanning Electron Microscopy and Optical Microscopy images of isolated collagen are shown in Figs. 4 and 5. Fig. 4A, a normal photograph of tuna bone, shows it in the form of yellow color, consisting of hydroxyapatite and fibril collagen. Fig. 4B is the scanning electron microscopy images of collagen; it confirms the presence of fibril collagen

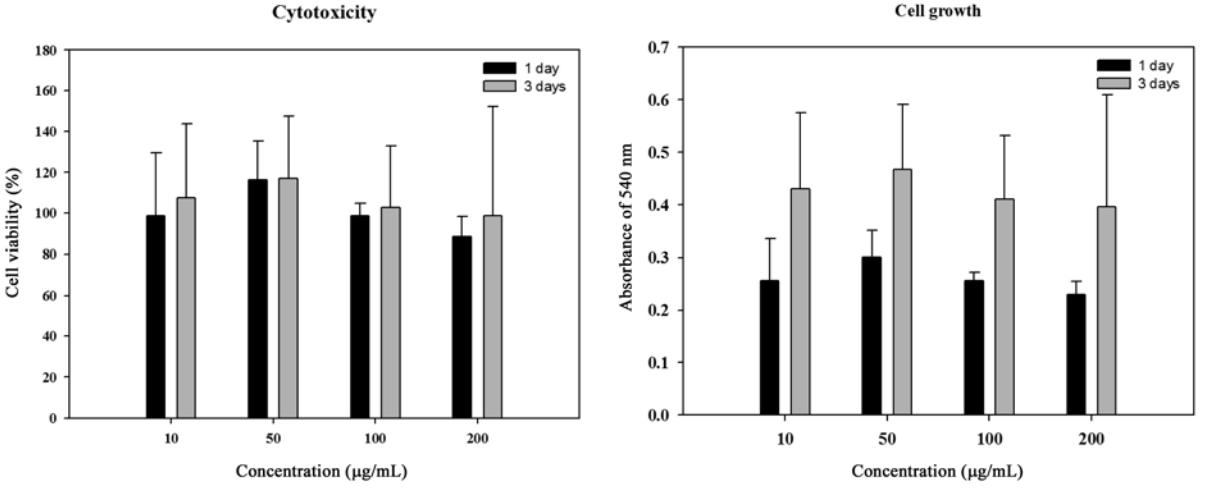


**Fig. 6.** Cell proliferation of collagen derived by the ASEC method: (A) cytotoxicity and (B) cell growth.

in the raw fish bone. In the present experiment, we used two different kind of isolation methods, ASC and ASEC, and the obtained SEM images of collagen shown in Figs. 4C and 4D depict that presence of fibril collagen.

For more confirmation, we checked the isolated collagen with optical microscopy analysis. Through an optical microscope, dry collagen, water wet collagen and ethanol wet collagen were observed. The optical microscopy result infers the presence of fibrils structure of collagen (Fig. 5).

### 3.5. *In vitro* analysis

The *in vitro* cytotoxicity of collagen was investigated using preosteoblast-like MC3T3-E1 cells by MTT assay, which relies on the mitochondrial activity of vital cells, and represents a parameter for their metabolic activity [31,32]. The cytotoxicity results of isolate collagen are shown in Fig. 6. This cytotoxicity was compared with that of collagen in different concentrations, of 10, 50, 100, and 200 μg/mL. Cell viability in high concentration collagen was observed in good conditions, as well as low concentration collagen. Cell growth was measured in the same way. The 3rd day of cell growth showed significant increased growth of MC3T3-E1 cells over the 1st day. This result means that extracted collagen will show no cytotoxicity, and will be biocompatible with bone-derived cells.

## 4. Conclusion

Collagen was successfully isolated from marine fish bone with two different methodologies, and confirmed by different kind of analytical techniques. Collagen is confirmed as type I, and of fibril structure. The results suggest that collagen could be obtained effectively from processing the waste of tuna bone by the ASEC method, as an alternative to animal collagen. Further, *in vitro* cell culture results confirm that the isolated collagen scaffolds are biocompatible, and nontoxic in nature.

## Acknowledgement

This research was supported by a grant from the Marine Bioprocess Research Center of the Marine Biotechnology Program, funded by the Ministry of Oceans and Fisheries, Republic of Korea.

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