# Cytocompatibility of Lactic Acid-extracted Barramundi Skin Collagen for Cell Culture Applications

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Fish collagen is becoming a potent material in many fields including the biomedical field by substituting for domestic animals that may cause zoonotic diseases. In this study, the skin of farmed barramundi *(Lates calcarifer)* was employed as a collagen material, and the affinity of this collagen for live cells was assessed. Four extraction solutions for the skin collagen were compared : acetic acid with/without pepsin as conventionally used, and alternatively lactic acid with/without pepsin. Among the resulting collagens, lactic acid-extracted collagen (LC) showed a meshwork of relatively fine fibers after lyophilization in scanning electron microscopy and a hydroxyproline content of 7.7 % in amino acid analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that this skin collagen was type I with $\alpha$1 and $\alpha$2 chains, and these chains had relatively close molecular weight as compared with those of porcine collagen. The viability of mouse fibroblasts exposed to LC at various concentrations for 24 hours was maintained at ≥ 93 %, demonstrating its low cytotoxicity. In a wound healing assay using each collagen in the culture medium, LC supported more active motility of mouse fibroblasts than collagens obtained by the other extraction methods. Collectively, these results suggest that barramundi skin collagen extracted by lactic acid has suitable properties in cellular activities, exhibiting advantages over the conventional methods, and is expected to be a useful material for cell culture applications. *Shinshu Med J 72 : 149—157, 2024*

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## I Introduction

Many materials that originate from collagen, such as collagen gels, gelatin, and collagen peptides, are widely used as cell culture substrates in many research fields. Collagen functions as a framework for tissues and plays an important role as a signaling molecule that directly or indirectly controls cell activities, including differentiation, wound healing, and blood clotting[1)-3)]. Such biological activities are mediated by the interaction between collagen and biopolymers such as proteins and polysaccharides. Dozens of collagen-binding proteins have been identified, and the role of collagen as a signaling molecule is becoming evident[4)].

The use of natural collagen is increasing in research in the biomedical field[2)]. Collagen is also marketed as a food additive, health functional food, and functional cosmetic. Most raw materials for collagen production are obtained from the bones or skin of domestic animals, such as bovine and porcine, which have high resources and yields. However, there is a risk of zoo-

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notic diseases, such as bovine spongiform encephalopathy and foot-and-mouth disease, which became a hot topic in 1990s to early 2000s[5)6)]. Additionally, some people have cultural issues that restrict the handling of mammalian products. For these reasons, the search for raw materials to replace mammalian tissues has become a challenge. Therefore, attention has been focused on fish with similar materials and few zoonotic diseases. This replacement has been progressing rapidly in recent years[7)].

In this context, we focused on fish skin. The fish skin is rich in collagen and other major components that comprise the body. In particular, when considering the diversity of their functions, proteins unique to fish skin possibly exist, and they have emerged as attractive materials that warrant marketization and research. In this study, we used skin from barramundi (*Lates calcarifer*). This is a large fish reaching a length of 2 m, inhabiting the tropics of Indian and Pacific Oceans, and is farmed in various places in Southeast Asia for food. Barramundi skin is discarded and produces enormous amounts of waste, and is therefore inexpensive. Moreover, this skin has been scarcely reported[8)], and has not been practically used. Thus, it is expected that new materials will be discovered and applied.

To search for new materials applicable for development in the biomedical field, we evaluated the suitability of barramundi skin collagen for live cells. Among four extraction methods assessed, a method using lactic acid was found to yield collagen in a reasonably natural form. This collagen showed unique features and favorable effects on cell activities in vitro.

## Ⅱ Materials and Methods

### A Materials

Barramundi skin was purchased from Qihai (Zhujiang, China).

### B Collagen extraction from fish skin

Collagen was extracted from barramundi skin using a previously established method[9)-11)] with some modifications. Briefly, the fish skin was cut into small pieces measuring 0.5 $cm^2$ using scissors. Subsequently, the pieces were immersed in a 0.1 M NaOH solution [1 : 20 (m/v)] for 36 hours to remove impurities and pigments. The alkali-treated fish skin was rinsed with deionized water to achieve neutrality and then soaked in four acidic solutions [0.5 M lactic acid, 0.5 M acetic acid, 0.5 M lactic acid containing 1 g/100 mL pepsin (Fujifilm Wako, Osaka, Japan), and 0.5 M acetic acid containing 1 g/100 mL pepsin] while stirred in a stirrer (300 rpm) for 24 hours to extract collagen. The resulting solution was centrifuged at 10,000×g for 20 minutes at 4 ℃. The supernatant was collected and thoroughly precipitated with salt to a final concentration of 0.9 M so that remnant pepsin was removed. The sample after salting out was centrifuged at 10,000×g, for 15 minutes at 4 ℃, and the precipitate was collected. The collected precipitate was completely dissolved in a 0.5 M lactic acid or acetic acid solution, followed by dialysis for 24 hours in a 0.1 M lactic acid or acetic acid solution [1 : 50 (v/v)], and then dialyzed in deionized water of the same volume for 72 hours. The dialyzed sample was freeze dried to obtain the extracted collagen. All operations were carried out at 4 ℃. The four kinds of collagen extracted by the various acidic solutions, lactic acid-extracted collagen (LC), acetic acid-extracted collagen (AC), lactic acid-pepsin-extracted collagen (LPC), and acetic acid-pepsin-extracted collagen (APC), were subjected to analyses.

### C SEM Observation

The morphology of freeze-dried collagen was observed by scanning electron microscopy (SEM ; JSM-7600F, JEOL, Tokyo, Japan). Collagen samples were attached to an adhesive carbon stub and coated with gold. A SEM was used for imaging at a voltage of 15 kV.

### D SDS-PAGE analysis

The chain compositions of collagens were analyzed by 8 % SDS-PAGE under reducing condition. Each collagen sample was dissolved in diluted HCl (pH 3) at a concentration of 3 mg/mL. A 21 μl collagen solution was mixed with 7 μl of 4×Laemmli sample buffer containing 2-mercaptoethanol (Bio-Rad, CA, USA). The mixed solution was boiled for 5 min and then loaded onto the gel. Electrophoresis was performed at 80 V for 90 min. After electrophoresis, the

gel was stained with Coomassie brilliant blue, followed by de-staining in a solution of 10 % (v/v) methanol and 10 % (v/v) acetic acid. The stained gel was imaged by a scanner.

**E Amino acid composition analysis of collagen**

After hydrolysis in hydrochloric acid, the collagen sample was sealed in a digestion tube and subjected to vacuum treatment at 110 ℃. Subsequently, high-performance liquid chromatography was employed for analysis at The Food Technology Department of the Nagano Prefectural Industrial Technology Center (Nagano, Japan). The results are expressed as the number of specific amino acid residues per 1,000 amino acid residues.

**F Cytotoxicity assay**

A cytotoxicity assay of the extracted collagens was performed by evaluating the cell viability of mouse fibroblasts (SNL ; ATCC, USA) after exposure to each collagen at 0.5–2.0 mg/mL for 24 hours. SNL cells were seeded in 96-well plates at a density of $1 \times 10^5$ cells/mL and cultured in high glucose Dulbecco's Modified Eagle Medium (Fujifilm Wako) containing 10 % fetal bovine serum (FBS ; Gibco, MO, USA). After 24 hours of culture, a collagen solution (0.5–2.0 mg/mL) was applied to the culture for an additional 24 hours. Cell viability was assayed using alamarBlue® reagent (Bio-Rad).

**G Wound healing assay**

SNL cells were cultured in a 12-well plate ($1.5 \times 10^5$ cells/well) to confluency (24 h). This single layer of cells was scratched with a 200 μL pipette tip. The medium was aspirated, and the cells were washed with Dulbecco's phosphate-buffered saline (DPBS ; Fujifilm Wako). Then, medium with 2 % FBS and 1.5 mg/mL collagen extract was applied to the cells. This concentration of collagen was determined according to preliminary study that examined various concentrations based on the results of cytotoxicity assay. The appearance of cells migrating and filling the defect was observed under a phase-contrast microscope (BZ-X710 ; KEYENCE, Osaka, Japan) immediately after the collagen had been applied and then 24 hours later.

**H Statistical analysis**

Statistical analysis of cytotoxicity was performed using Dunnett's multiple comparisons test. $P < 0.05$ was considered significant.

## Ⅲ Results

**A Light and electron microscopic observations of collagens**

When barramundi skin collagens extracted by the four methods were lyophilized, they had a white cotton-like appearance under macroscopic observation **(Fig. 1A–D)**. These collagens exhibited a complex meshwork consisting of filamentous and film-like fibers in light microscopy **(Fig. 1E–H)**. In SEM, the meshwork fibers had different shapes, pore sizes, and arrangements **(Fig. 2)**. LC had more fine and regular filaments than AC **(Fig. 2A, B)**. Addition of pepsin to each acid extraction formed fibers enriched with film-like structures **(Fig. 2C, D)**.

**B Amino acid composition analysis**

**Table 1** shows the results of amino acid analysis of LC and AC. In terms of the contents of the three amino acids that characterize collagen molecules, LC was 34.4 % glycine, 11.7 % proline, and 7.7 % hydroxyproline. These values were close to those of AC. Because the glycine content was almost one third of the total amino acids, and hydroxyproline that nearly exclusively exists in collagen was detected to a certain extent, the extraction was successful and nearly pure collagen was extracted.

**C SDS-PAGE analysis of collagens**

The type of barramundi skin collagen was examined by SDS-PAGE **(Fig. 3)**. Two bands around 120 kDa indicating α chains were most discernible in LC. This suggested that the barramundi skin collagen was mainly type I with a heterotrimeric structure of α1 and α2 chains, which constituted the control of porcine skin collagen. The molecular weight difference between α1 and α2 was less than 5 kDa in LC, which was smaller than that in porcine type I collagen with a difference of ≥ 10 kDa. The band around 120 kDa appeared to be nearly single for AC, LPC, and APC, which may be due to the proximity and amount of the two α chains.

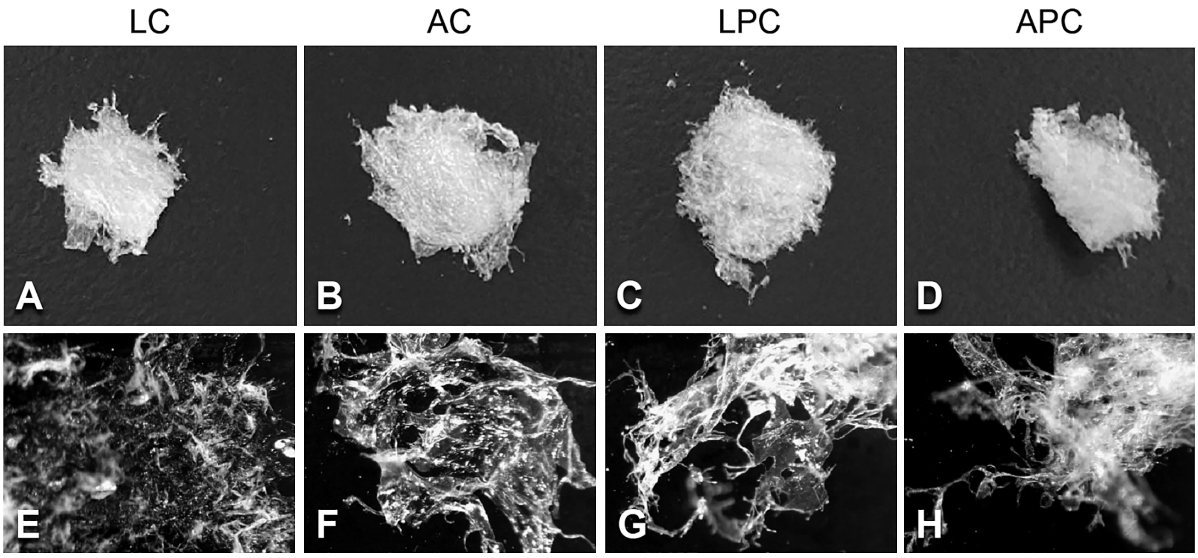


Fig. 1 Macroscopic (A–D) and light microscopic (E–H) observations of lyophilized collagens extracted from barramundi skin by four methods : LC (A, E), AC (B, F), LPC (C, G), and APC (D, H). Each collagen exhibited a meshwork of fibers with various appearances. E–H : ×80.

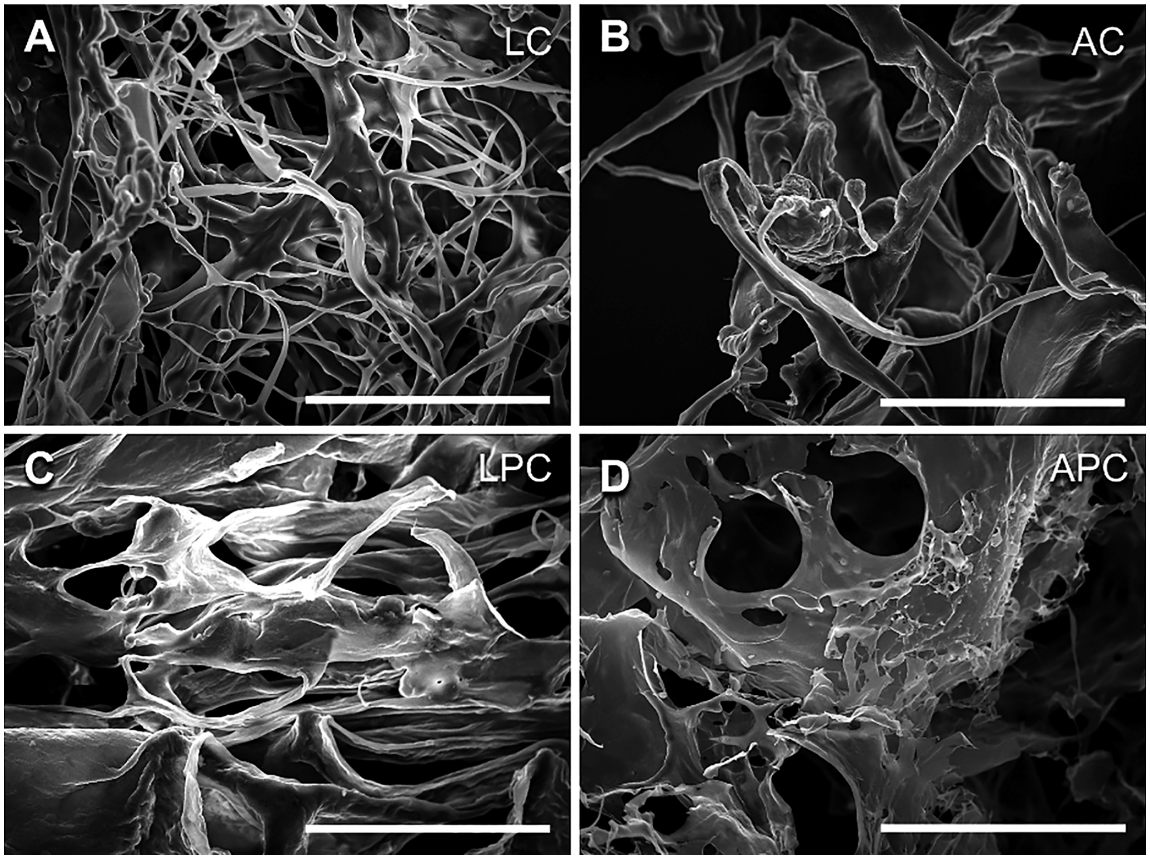


Fig. 2 SEM observation of the meshwork of LC (A), AC (B), LPC (C), and APC (D). Note the relatively fine and regular fibers in LC (A) compared with AC (B). Film-like strands were abundant in LPC and APC (C, D). Bars = 100 μm.

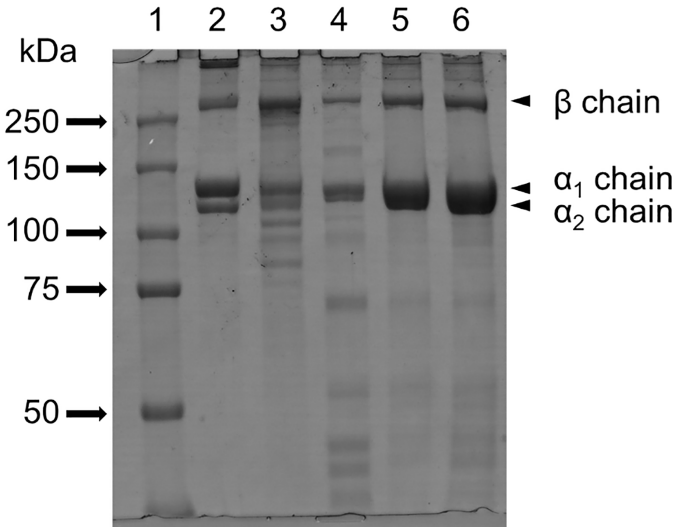


Fig. 3 SDS-PAGE analysis of extracted collagens

Lane 1 : molecular weight marker. Lane 2 : control (porcine skin collagen). Lane 3 : LC, Lane 4 : AC, Lane 5 : LPC, and Lane 6 : APC. LC showed a split band or close bands of $\alpha$1 and $\alpha$2 chains. The molecular weight difference between the two $\alpha$ chains was relatively small. The difference in porcine skin collagen was approximately ≥ 10 kDa. The $\beta$ chain around 240 kDa was two $\alpha$ chains connected by crosslinking.

**D Cytotoxicity assay of the collagens in mouse fibroblasts**

In the cytotoxicity assay, mouse fibroblasts were exposed to each collagen at concentrations of 0.5–2.0 mg/mL for 24 hours, and then cell viability was evaluated **(Fig. 4)**. LC exposure resulted in no significant reduction in viability at all concentrations, which was maintained at ≥ 93 % **(Fig. 4A)**. A slight but significant reduction in viability was evident after AC exposure **(Fig. 4B)**. LPC and APC also significantly reduced cell viability to approximately 80 % **(Fig. 4C, D)**. These results showed that LC did not remarkably affect cell viability and had low cytotoxicity in mouse fibroblasts.

**E Wound healing assay of mouse fibroblasts**

The motility of mouse fibroblasts was assessed by a wound healing assay in medium containing each collagen at 1.5 mg/mL **(Fig. 5)**. LC induced more active cell migration than AC, and the cell defect area was completely filled after 24 hours **(Fig. 5A–D)**. Also, the defect area was favorably filled after exposure to LPC, but not APC **(Fig. 5E–H)**. LC and even LPC were demonstrated to promote active motility of mouse fibroblasts.

Table 1 Amino acid analysis

| Amino acid | Number of residues (/1000 residues) | |
|---|---|---|
| | LC | AC |
| Asp | 46 | 46 |
| Thr | 23 | 23 |
| Ser | 26 | 26 |
| Glu | 73 | 72 |
| Gly | 344 | 347 |
| Ala | 137 | 137 |
| Val | 21 | 21 |
| Ile | 10 | 9 |
| Leu | 20 | 19 |
| Tyr | 3 | 2 |
| Phe | 17 | 15 |
| Lys | 27 | 27 |
| His | 6 | 6 |
| Arg | 53 | 53 |
| Hyp | 77 | 83 |
| Pro | 117 | 115 |
| total | 1000 | 1000 |

Amino acid analysis of LC and AC revealed a high content of hydroxyproline (Hyp), which almost exclusively exists in collagens among proteins.

## Ⅳ Discussion

Collagen has, at least partially, a helical structure composed of three $\alpha$ chains. The single $\alpha$ chain with a molecular weight of approximately 100,000 is characterized by a repeated amino acid triplet, glycine-X-Y, in which X and Y are frequently proline and hydroxyproline, respectively. The $\alpha$ chain is subclassified into $\alpha$1 and $\alpha$2 chains by slight differences in the amino acid composition, and they maintain the triple helix

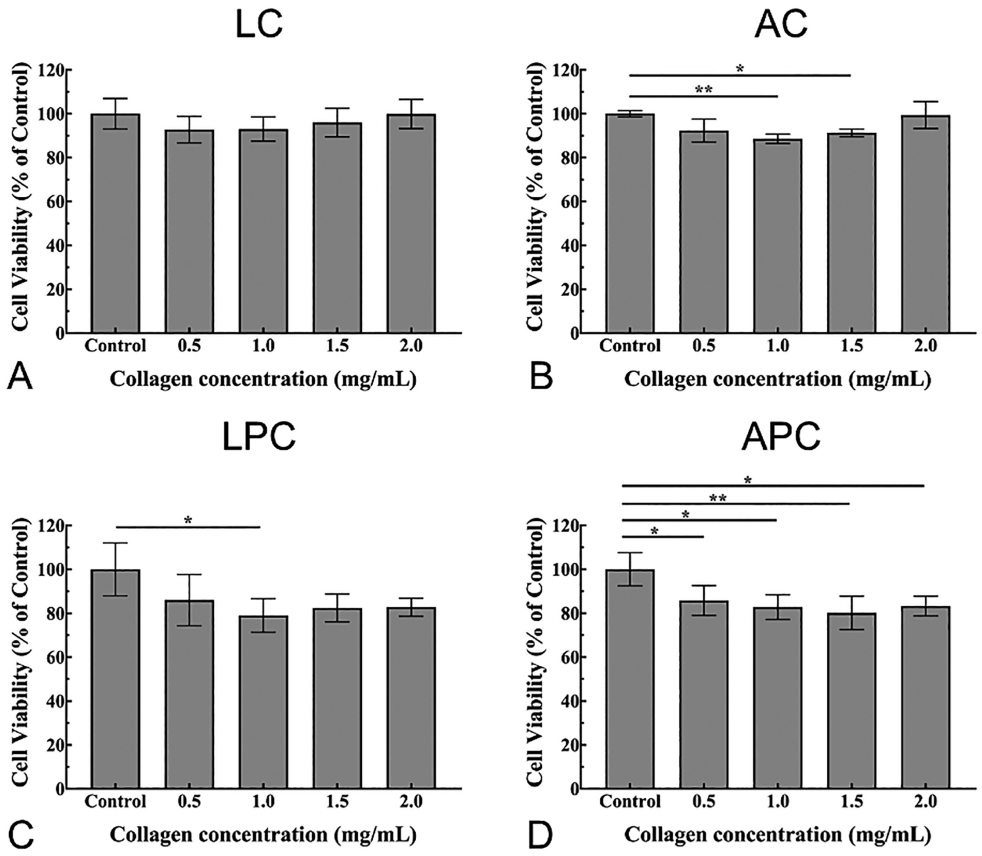


Fig. 4　Viability of cells exposed to the extracted collagens

Mouse fibroblasts were exposed to each extracted collagen at concentrations of 0.5–2.0 mg/mL for 24 hours, and then cell viability was evaluated by alamarBlue® reagent. (A) LC maintained cells viability at ≥ 93 % without a significant decline compared with the control. (B) Cell viability under AC exposure was similar to that under LC exposure, but the decline was statistically significant at concentrations of 1.0 and 1.5 mg/mL. (C, D) Cell viability was significantly reduced by LPC and APC to approximately 80 %. Control : no collagen exposure. Data are expressed as the mean ± SD (n = 4) ; *p < 0.05, **p < 0.01.

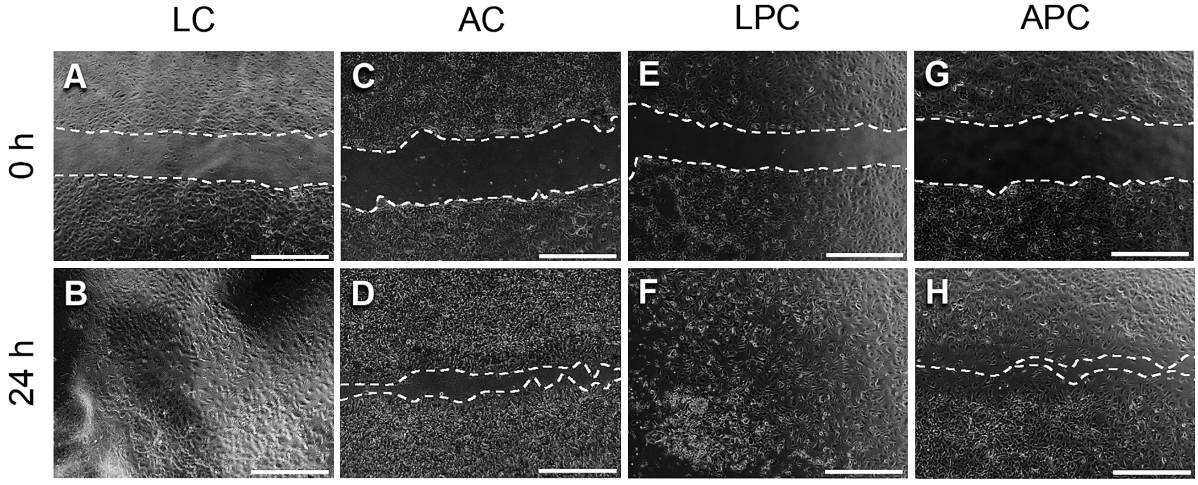


Fig. 5　Effect of the barramundi skin collagens on wound healing of mouse fibroblasts

Confluent fibroblasts with a linear scratch defect were cultured for 24 hours in medium containing 1.5 mg/mL of LC (A, B), AC (C, D), LPC (E, F), or APC (G, H). The defect area was fully covered by migrated cells after 24 hours of LC exposure (A, B), but remained after AC exposure (C, D). Similar results were observed for LPC (E, F) and APC (G, H). Dotted lines indicate the edge of cell layers or the front line of migrating cells. Bars = 1 mm.

structure unique to collagen[12]. Twenty-nine types of collagen molecules have been reported, depending on the structure[13]. Most collagen is type I with a heterotrimer of two α1 chains and a single α2 chain (α1×2, α2×1), but homotrimer collagens composed of three α1 chains (α1×3) exist, such as type II and III collagens[14]. Homotrimeric type III collagen promotes self-assembly to fibrils compared with heterotrimeric type I collagen[15]. Among the collagens, types I–IV are mainly used as biomaterials[13].

Barramundi skin collagen as LC had both α1 and α2 chains, which suggested type I collagen with the α1×2 and α2×1 structure **(Fig. 3)**. The difference in the molecular weights of α1 and α2 chains was smaller than that of porcine type I collagen. The amino acid composition, particularly the hydroxyproline content, determines the denaturation temperature of collagen, at which the triple helix dissociates to the monomer and changes to gelatin[13]. Thus, the higher the content, the more thermally stable collagen gel is formed when applied to a cell culture environment. In the present study, the hydroxyproline content of barramundi skin collagen was 7.7 % in LC **(Table 1)**. In previous reports on collagens from various barramundi tissues, the denaturation temperature and hydroxyproline content (in parentheses) were 36.4 ℃ (8.4 %) in scales[16], 35.0 ℃ (8.3 %) in the swim bladder, and 33.3 ℃ (7.9 %) in skin[8], which are at the boundary with terrestrial animals that have been used so far and close to human body temperature[17]. LC from barramundi skin in our study was supposed to have a similar denaturation temperature to these reports as judged from the hydroxyproline content, though the content will differ depending on extraction condition. It may have good affinity for human cells.

In conventional collagen purification methods, an enzyme such as pepsin is added to hydrolyze collagen for a long time, combining acid solubilization if necessary[10][11]. The enzyme treatment reduces the antigenicity of collagen by deleting telopeptides at both ends of the triple helix and is efficient to obtain collagen applicable to humans. However, since treatment with a protease inhibitor may be employed to inactivate the enzyme, conventional methods are generally time-consuming and costly[11]. The extraction method using lactic acid alone in this study has advantages over the conventional techniques because of simplicity and low cost.

The lyophilized collagens from barramundi skin showed variable aspects under four extraction conditions **(Fig. 1, 2)**. LC had a relatively fine meshwork compared with AC, while LPC and APC showed film-like fibers. A previous study reported that collagen entanglement depends strongly on the type of acid used, and the telopeptides regulate the properties of collagen molecules[18]. Native collagen with the telopeptides showed finely meshed amorphous structures, whereas telopeptide-poor collagen formed crystalline-like and well-ordered structures[18]. In addition, the viscosity of collagen solution seems to influence the meshwork structure[18]. The viscosity of collagen solution in lactic acid was reported to be lower than that in acetic acid at 25 ℃[19]. In our study, there may have been some difference in the viscosity of the collagen solution even after dialysis. Therefore, it is supposed that the deletion of telopeptide by pepsin, as well as the differential effects of lactic acid and acetic acid on collagen solutions, alters the physicochemical properties of collagen and explains the structural variation associated with lyophilization.

Because LC exhibited better results in cell viability and motility assays than AC **(Fig. 4, 5)**, LC seems to be useful when applied to cell culture. When application to living body is considered, however, LPC may have some benefit due to the absence of telopeptides. Although the cell viability was inferior to LC, LPC showed favorable result next to LC in the wound healing assay. Anyway, the issue of antigenicity of acid-extracted collagen still remains and should be evaluated and resolved.

The limitations of this study can be considered as follows. Collagen coating was not employed for the wound healing assay. The collagens were directly added into the culture medium to confirm their effect on cell motility at the same concentration of 1.5 mg/mL used in the cytotoxicity assay. Moreover, since these experiments were conducted using mouse fibroblasts, they cannot be applied directly to human

cells. Considering the potential application to human cells and bodies, the effect of barramundi skin collagens on human fibroblasts should be further confirmed by assessing cell viability and cell motility on collagen-coated surface in further studies.

Our method for collagen extraction from barramundi skin converts unused waste into valuable raw materials with a reasonable price and quality. This has an advantage in the development and manufacture of collagen raw materials and products applicable to cells, being suitable for a recycling-oriented society. Barramundi skin collagen can be used for various purposes, such as cell culture additives, 3D culture substrates, and scaffolding materials. This study contributes to the practical application of fish collagen in the biomedical field.

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