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Hydrolyzed Collagen from Salmon Skin Increases the Migration and Filopodia Formation of Skin Keratinocytes by Activation of FAK/Src Pathway

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Previous studies reported hydrolyzed collagen increase cell proliferation and migration involved in the wound repair process. Nevertheless, the knowledge related with wound repair mechanism of hydrolyzed collagen from salmon skin (HCSS) has not been fully elucidated. Therefore, this study aimed to elucidate the effects of HCSS on the migration of keratinocyte HaCaT cells. Additionally, its molecular mechanism through cell division control protein 42 (Cdc42), Ras-related C3 botulinum toxin substrate 1 (Rac1), and Ras homolog family member A (RhoA) *via* focal adhesion kinase (FAK)-steroid receptor coactivator (Src) regulation and keratinocyte stem cells (KSCs) markers were also evaluated. After 24 h of incubation, keratinocyte proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and double stranded DNA (dsDNA) assays, and by determining the total cellular protein content. Keratinocyte migration and filopodia formation were measured by wound healing assay and phalloidin-rhodamine staining, respectively. The migratory related proteins were evaluated by western blot analysis. HCSS had a high content of hydrophobic amino acids and imino acids. HaCaT cell proliferation and migration were significantly increased in response to HCSS at the concentration of 100–1000 μg/mL. The formation of filopodia was subsequently increased in response to HCSS at concentrations of 100–1000 μg/mL. Moreover, HCSS upregulated Cdc42, Rac1, and RhoA protein expression and activated the phosphorylation of FAK and Src pathway. HCSS at the concentration of 100–1000 μg/mL could trigger stemness by increased KSC markers, including keratin 19 and β-catenin expression. This study has demonstrated that HCSS induces proliferation and migration of keratinocytes, subsequently promotes the second phase of wound healing process by FAK-Src activation and also increases the KSC properties.

INTRODUCTION

Human skin, especially the epidermis, is a major barrier against noxious pollutants which could be impaired by various factors including ultraviolet (UV), chemical and mechanical stimuli [Baroni *et al*., 2012]. Furthermore, the renewal of skin in epidermis layer is important for human skin barrier function [Baroni *et al*., 2012; Wickett & Visscher, 2006]. In response to skin damages, the renewal process and cell proliferation were observed in the UV, heat, and wound-induced skin injury [Wikramanayake *et al*., 2014]. Keratinocyte stem cells (KSCs) regulate epidermal renewal and skin homeostasis. In addition, KSCs can produce extracellular matrix (ECM) components, cytokines, and growth factors in both normal function and response to skin injury [Fuchs, 2008; Pincelli & Marconi, 2010; Sotiropoulou & Blanpain, 2012]. The decrease of KSCs numbers and activities leads to the epidermal barrier impairment [Yang *et al*., 2019a]. Besides, keratinocyte proliferation and migration are accepted to play a major role in the re-epithelialization process of skin repair and healing [Abate *et al*., 2021].

Fish skins are a by-product of the food industry and a rich source of collagen. Marine collagen has been noted to promote cell proliferation and migration of skin cells by augmenting various mediators in the stimulation of skin wound repair process [Chotphruethipong *et al*., 2021a; Hu *et al*., 2017; Yang *et al*., 2019b] and KSCs function [Thaweekitphathanaphakdee *et al*., 2019]. To date, bioactive peptides released from marine origin proteins, for example fish collagen have been reported to exhibit many biological effects including anti-inflammatory,

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anti-oxidant, anti-aging, and wound repair activities [Chotphruethipong *et al*., 2021a; Edgar *et al*., 2018; Huang *et al*., 2015]. Protein hydrolysates prepared by enzymatic processes from fish skin cause a reduction of aging and skin damage by photoaging [Chalamaiah *et al*., 2019; Chen *et al*., 2016; Edgar *et al*., 2018]. However, the information on the mechanism of hydrolyzed collagen from salmon skin (HCSS) effect on the proliferation and migration of human keratinocytes HaCaT remains unclear. Therefore, the main purpose of this study was to examine the effects of HCSS on keratinocyte proliferation and migration. The properties of KSC markers were also elucidated in this study.

MATERIALS AND METHODS

Salmon skin preparation

Frozen salmon skins were obtained from King-fisher Holdings Co., Ltd., Songkhla, Thailand. The skins at 3×3 cm^2 were applied with NaOH (100 mM) and subsequently washed until neutral pH was reached [Chotphruethipong *et al*., 2019]. Alkali-treated skins were saturated in 10 volumes of 100 mM citric acid for 2 h, followed by washing with tap water until wash water became neutral [Chotphruethipong *et al*., 2019]. The resulting swollen skins were utilized for hydrolyzed collagen preparation.

Preparation of hydrolyzed collagen from salmon skin (HCSS) and analysis of amino acid composition

HCSS was prepared using two-step enzymatic hydrolysis. Firstly, the swollen skins were treated with papain (3% of solid content of fish skin) as tailored by Benjakul *et al.* [2018a]. The reaction was performed at 40°C for 3 h and subsequently terminated by heating at 90°C for 15 min. Thereafter, Alcalase (2% of solid content of fish skin) was added into the mixture, followed by hydrolysis at 50°C for 2 h. After hydrolysis, the inactivation was done at 90°C for 15 min. The obtained hydrolyzed collagen was filtered, and the filtrate was concentrated with the solid content of 10% according to the method of Benjakul *et al.* [2018a]. The resulting concentrate was subjected to drying using a spray-dryer [Benjakul *et al.,* 2018a]. Salmon hydrolyzed collagen powder was packed in ziplock bag and stored at -40°C until used for analyses. The content of amino acids in HCSS was determined as detailed by Benjakul *et al*. [2018b]. Briefly, HCSS was hydrolyzed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (*v/v*) 3-(2-aminoethyl)indole at 115°C for 24 h and neutralized with 3.5 M sodium hydroxide. Digest was diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was analyzed using an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

Molecular weight (MW) distribution of HCSS

Size exclusion chromatography was applied to determine MW distribution of HCSS powder by using a 2.5×50 cm Sephadex G-25 gel filtration column (GE Healthcare Bio-Science AB, Uppsala, Sweden) [Chotphruethipong *et al*., 2021c]. The absorbance was detected at 220 and 280 nm. Blue dextran (2,000,000 Da) was used for void volume measurement. The MW standards were insulin chain B (3495.89 Da), vitamin B_{12} (1355.4 Da), glycine-tyrosine (238.25 Da), and tyrosine (181.2 Da). MW of the fraction was estimated from the plot between available partition coefficient (K_{av}) and the logarithm of MW of the standards.

Keratinocyte culture and cell viability assays of HCSS

Human keratinocyte HaCaT cell line was purchased from the Cell Line Service, Heidelberg, Germany and cultured with 5% CO_2 atmosphere at 37°C in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Keratinocyte HaCaT with a density of 1×10^4 cells/well were seeded in 96-well plate. Then, keratinocyte HaCaT were exposed to 0, 1, 5, 10, 25, 50, 75, 100, 250, 500, and 1000 μg/mL of HCSS for 24, 48, and 72 h. After each time of incubation, 500 μg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was applied into each well for 2 h, the insoluble purple formazan was solubilized in dimethyl sulfoxide (DMSO). The cell viability was assessed at 570 nm with a microplate reader (Synergy™ HT, Bio-tek Instruments, Winooski, VT, USA).

Detection of cell proliferation by MTT and double stranded DNA (dsDNA) assay

Keratinocyte HaCaT at 1×10^4 cells/well were cultured into 96-well plate. The cells were incubated with 0, 50, 100, 500, and 1000 μg/mL of HCSS for 24 h. Cell proliferation was detected using a colorimetric MTT assay [Lü *et al*., 2012]. Briefly, 500 μg/mL of MTT was applied into each well for 2 h. The insoluble purple formazan was solubilized in DMSO. The cell viability was assessed at 570 nm with a microplate reader (Synergy™ HT). For dsDNA assay, cells were washed and incubated with 0.1% Triton X-100 for 10 min. Thereafter, the DNA content in keratinocyte HaCaT was measured using a dsDNA assay kit (Invitrogen, Carlsbad, California, USA). The intensity of fluorescence was measured at 485 nm (emission wavelength) and 535 nm (excitation wavelength) using a microplate reader (Synergy™ HT), and the percentage of HaCaT proliferation was calculated using the following formula:

$$\text{Cell proliferation (\%)} = \frac{\text{DNA concentration of cells treated with HCSS}}{\text{DNA concentration of untreated cells}} \times 100 \quad (1)$$

Detection of cell proliferation by total cellular protein content assay

Keratinocyte HaCaT at a density of 1×10^4 cells/well was grown in 96-well plates. Then, cells were incubated with 0, 50, 100, 500, and 1000 μg/mL of HCSS for 24 h. After 24 h of incubation, the cells were mixed with cold trichloroacetic acid (TCA) solution (40%, *w/v*) and incubated for 1 h at 4°C as described by Thaweekitphathanaphakdee *et al*. [2019]. Sulforhodamine B (Sigma-Aldrich, St. Louis, MO, USA) solution (0.04%, *w*/v) was added to each well and incubated for 1 h at room temperature. Thereafter, the cells were washed quickly with acetic acid (1%, *v/v*), and then 0.01 M Tris base was applied to each culture well. The absorbance at 510 nm was measured by a microplate reader (Synergy™ HT)

and the percentage of HaCaT proliferation was calculated using the following formula:

$$\text{Cell proliferation (\%)} = \frac{\text{absorbance of mixture with HCSS}}{\text{absorbance of control}} \times 100 \quad (2)$$

Wound healing assays

The effect of HCSS on the migration of skin keratinocytes was evaluated using an *in vitro* scratch wound healing assay. The HaCaT keratinocytes (3.5×10^5 cells/well) were grown in 6-well plates with DMEM medium containing 0.1% FBS. Prior to HCSS treatment (0–1000 μg/mL), a sterile P200 micropipette tip was used to make a wound space. The wound areas were captured on the image field at 3 points per line at 0 and 24 h using a phase-contrast microscope (Olympus IX70 Inverted Microscope, Olympus Corporation, Tokyo, Japan). The percentage of wound area was calculated as described by Singkhorn *et al*. [2018].

Filopodia formation determination by phalloidin--rhodamine staining

After HCSS treatment at the concentrations of 0, 50, 100, 500, and 1000 μg/mL for 24 h, filopodia formation was determined as described previously [Singkhorn *et al*., 2018]. Briefly, the cells were fixed with paraformaldehyde (4%, *w/v*), and the Triton-X100 (0.1%) was added for cell permeabilization. After blocking with 2% bovine serum albumin (BSA) for 1 h, keratinocyte HaCaT was incubated with phalloidin--rhodamine (10 μg/mL) and Hoechst 33342 (10 μg/mL) for 30 min. The images were taken with a fluorescence microscope (Olympus IX70 with DP50), and the percentage of filopodia formation was determined using the following formula:

$$\text{Filopodia formation (\%)} = \frac{\text{number of filopodia}}{\text{number of cells}} \times 100 \quad (3)$$

Western blot analysis

Keratinocyte HaCaT were exposed to HCSS at the concentrations of 0, 50, 100, 500, and 1000 μg/mL for 24 h. The treated cells were then lysed in the lysis buffer containing protease and phosphatase inhibitor for 30 min at 4°C. The lysed cells were collected and centrifuged at 14,024×*g* for 15 min. Protein of the samples (75 μg) was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and moved onto nitrocellulose membranes. The non-specific protein of membranes was blocked using 5% non-fat dry milk or 3% BSA in Tris-buffered saline containing 0.1% Tween 20 for 2 h. The primary antibodies to FAK (Santa Cruz Biotechnology, Dallas, TX, USA; 1:1000), pFAK (Santa Cruz Biotechnology; 1:1000), Src (Abcam, Cambridge, UK; 1:1000), pSrc (Abcam; 1:1000), Akt (Santa Cruz Biotechnology; 1:1000), pAkt (Santa Cruz Biotechnology; 1:1000), RhoA (Abcam; 1:1000), Rac1 (Abcam; 1:1000), Cdc42 (Abcam; 1:1000), keratin 19 (Abcam; 1:1000), β-catenin (Abcam; 1:1000), and β-actin (Thermo Scientific, Waltham, MA, USA; 1:1,000) were incubated at 4°C overnight. The membranes were washed and incubated with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h. The protein bands were exposed using an enhanced chemiluminescence (ECL) western blotting detection reagent (Merck Millipore, Burlington, MA, USA) and analyzed by ImageJ software (Image Processing and Analysis in Java, National Institutes of Health, http://rsbweb.nih.gov/ij/).

Statistical analysis

The data were described as a mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way analysis of variance and Tukey post hoc test. Differences were considered significant when p values were below 0.05 ($p<0.05$).

RESULTS AND DISCUSSION

Amino acid composition of hydrolyzed collagen from salmon skin (HCSS)

As shown in Table 1, Gly was found as the dominant amino acid (19.66 g/100 g), followed by Pro (10.95 g/100 g), Gln/Glu (9.24 g/100 g), Ala (7.64 g/100 g), and Asn/Asp (7.49 g/100 g). Moreover, HCSS contained imino acids (Hyp and Pro) (16.20 g/100 g). Gly is located at every third position in the collagen polypeptide chains in the presence of imino acids (Pro

TABLE 1. Relative amino acid composition of hydrolyzed collagen from salmon skins (HCSS).

Amino acid	Content (g/100 g)
Alanine (Ala)	7.64
Arginine (Arg)	7.00
Asparagine/ Asparatic acid (Asn/Asp)	7.49
Cysteine (Cys)	0.04
Glutamine/Glutamic acid (Gln/Glu)	9.24
Glycine (Gly)	19.66
Histidine (His)	ND
Isoleucine (Ile)	1.63
Leucine (Leu)	4.22
Lysine (Lys)	5.25
Hydroxylysine (Hyl)	0.76
Methionine (Met)	2.74
Phenylalanine (Phe)	3.08
Hydroxyproline (Hyp)	5.25
Proline (Pro)	10.95
Serine (Ser)	5.13
Threonine (Thr)	3.42
Tyrosine (Tyr)	3.50
Valine (Val)	2.89
Tryptophan (Trp)	0.11
Total	100.00

ND: Not detected

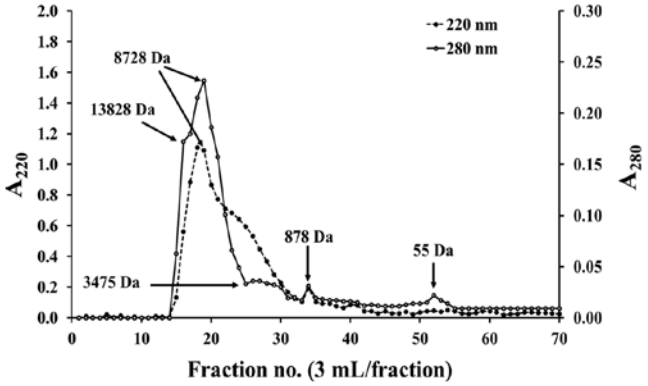

FIGURE 1. Elution profile by Sephadex G-25 size exclusion chromatography of hydrolyzed collagen powder from salmon skin.

and Hyp) as Gly-Pro-Hyp [Benjakul *et al*., 2018b]. A high content of hydrophobic amino acids, constituting approximately 50.07 g/100 g of total amino acids, was also noted (Table 1). These amino acids played an essential role in the proliferation of skin cells [Chotphruethipong *et al*., 2021a,b]. Additionally, bioactive peptides from fish skin rich in Pro, Hyp, and Gly promoted the wound healing process [Chotphruethipong *et al*., 2021a]. Previous reports have shown that marine collagen from the skin of Nile tilapia has a high content of Gly-Pro-Hyp [Hu *et al*., 2017; Yang *et al*., 2019b].

Size distribution of HCSS

HCSS contained peptides with various molecular weights (MW) (Figure 1), with those having MWs of 8728, 878, and 55 Da being dominant. Peptides with high MW (>8 kDa) were also found (Figure 1). In general, the size of peptides is a vital factor affecting bioactivity of hydrolyzed collagen. The smaller size peptides exhibited higher bioactivities, especially cell proliferation activity [Chotphruethipong *et al*., 2021c,d]. Also, short chain peptides could be rapidly digested and absorbed in the human body [Morgan & Breen, 2021].

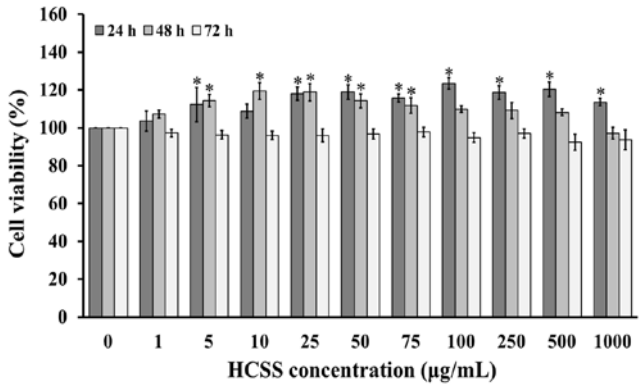

FIGURE 2. The viability of HaCaT keratinocytes after treatment with hydrolyzed collagen from salmon skin (HCSS) at concentrations of 0–1000 μg/mL for 24, 48, and 72 h.

Results are presented as mean ± standard error of the mean of four independent experiments (n=4). The asterisk above bars indicates a significant difference between the HCSS treatment and the corresponding control without HCSS ($p<0.05$).

Effect of HCSS on cell viability of keratinocyte HaCaT

Human keratinocyte cell lines are used as *in vitro* models to study the biological activities of molecules linked with dermatological conditions such as wound healing, contact dermatitis, psoriasis, or skin cancer. In our study, first, the effect of various concentrations of HCSS on keratinocyte HaCaT cell viability was assessed using the MTT assay to determine the cytotoxic effect and ensure the safe use of HCSS. As shown in Figure 2, exposure of the HaCaT cells to HCSS (5–1000 μg/mL) for 24 h, and HCSS (5–75 μg/mL) for 48 h caused a significant ($p<0.05$) increase in cell viability. However, HCSS at concentrations of 1–1000 μg/mL had no effect on the cell viability of keratinocyte HaCaT after 72 h of treatment. These findings proved the safety of all concentrations of HCSS as they did not cause the loss of cell viability even at the highest concentrations. Altogether, we could explain that the increment of cell viability may result from the high content of hydrophobic amino acids in HCSS (Table 1). Our findings were similar to the previous studies reporting that the hydrolyzed collagen from seabass skin significantly enhanced fibroblast and keratinocyte viability [Chotphruethipong *et al*., 2021a,b]. Research conducted by Yang *et al.* [2019b] also showed that peptides from *Nibea japonica* skin collagen increased cell viability of NIH-3T3 fibroblasts. Additionally, abalone collagen was observed to increase keratinocyte viability [Thaweekitphathanaphakdee *et al*., 2019]. Based on the results, we selected the HCSS concentrations of 50, 100, 500, and 1000 μg/mL for further experiments.

Proliferative effects of HCSS

Cell proliferation involves crucial events at a cellular level in the second phase of wound repair process and also important for epidermis renewal [Martin & Nunan, 2015; Yang *et al*., 2019a]. Therefore, HCSS at 50, 100, 500, and 1000 μg/mL was further investigated for the proliferative activity in skin keratinocyte HaCaT cells. The cells were maintained in the growth medium (1% FBS) in the presence or absence of HCSS at 50, 100, 500, and 1000 μg/mL for 1 day. The MTT assay revealed that HCSS at 100, 500, and 1000 μg/mL significantly ($p<0.05$) increased HaCaT proliferation after 1 day of cultivation in keratinocyte HaCaT (Figure 3A). Moreover, the proliferative activity of HCSS at 50, 100, 500, and 1000 μg/mL was confirmed using the dsDNA assay and total cellular protein content determination because the MTT assay has some limitations in cell proliferation measurement [Van Tonder *et al*., 2015]. HCSS at 100, 500, and 1000 μg/mL induced a significant ($p<0.05$) increase of cell proliferation in both assays (Figure 3B and Figure 3C), and the effect of HCSS on HaCaT cells was concentration dependent. Results from these three assays demonstrated that HCSS had a proliferative effect in keratinocytes. Our findings were in a good agreement with several previous studies in which fibroblast [Benjakul *et al*. 2018b; Chotphruethipong *et al*., 2021a; Yang *et al*., 2019b] and keratinocyte proliferation [Chotphruethipong *et al*., 2021b; Thaweekitphathanaphakdee *et al*., 2019] was promoted in response to peptides with hydrophobic amino acids (AAs) treatment. Sánchez & Vázquez [2017] reported that the size of peptides, their AAs composition and sequence affect their cell proliferation potential. In addition,

several studies reported that peptides of hydrolyzed collagen rich in Gly, Pro, and Ala affected the proliferation of fibroblast L929, MRC5, and bone marrow-mesenchymal stem (BMMS) cells [Benjakul *et al*. 2018b; Chotphruethipong *et al*., 2021a; Elango *et al*., 2019]. After 24 h of oral prolyl-hydroxyproline (a collagen-derived bioactive peptide) administration in rats, the radioactive (^{14}C) dipeptides of prolyl-hydroxyproline were observed in rats' osteoblasts, osteoclasts, dermal fibroblasts, epidermal cells, synovial cells, and chondrocytes [Kawaguchi *et al*., 2012]. Previous studies demonstrated also that Pro-Hyp affected the fibroblasts [Shigemura *et al*., 2009] and osteoblast [Kimira *et al*., 2017] proliferation. In this study, we found higher levels of Gly, Pro, and Ala in HCSS consistent with the proliferation of skin keratinocyte HaCaT cells. Therefore, it can be assumed that Pro and Hyp of HCSS peptides may regulate keratinocyte proliferation.

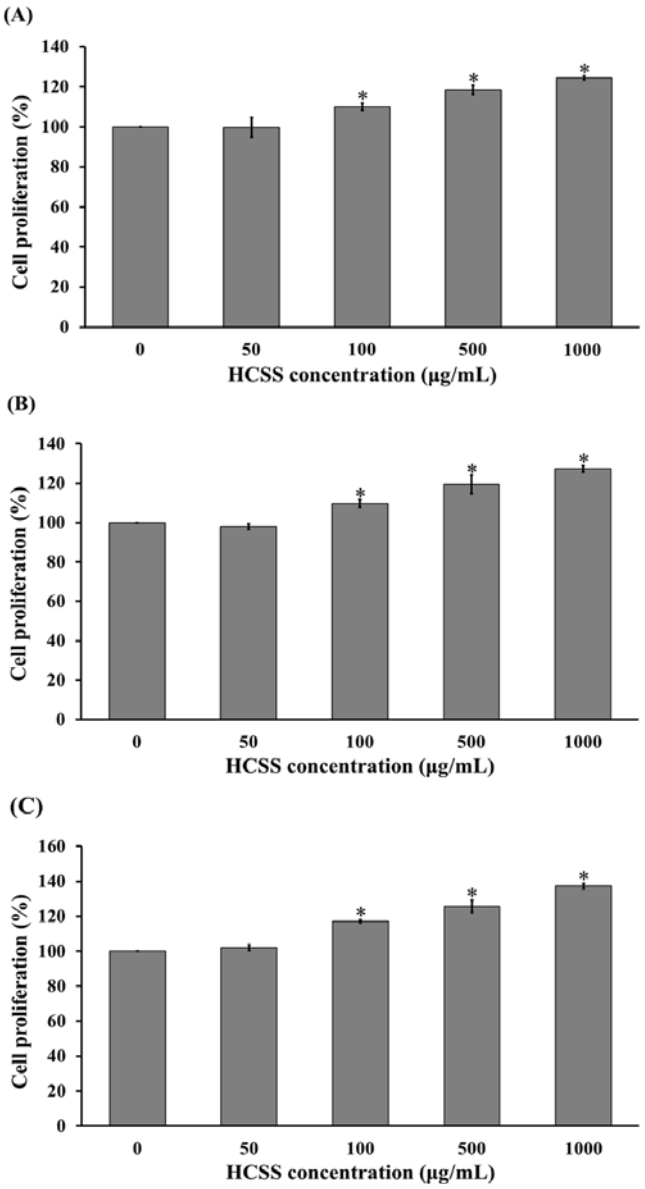

FIGURE 3. The proliferation of skin HaCaT keratinocytes after treatment with hydrolyzed collagen from salmon skin (HCSS) at concentrations of 0, 50, 100, 500 and 1000 μg/mL for 24 h. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, (B) double stranded DNA (dsDNA) assay and (C) total cellular protein assay.

Results are presented as mean ± standard error of the mean of four independent experiments (n=4). The asterisk above bars indicates a significant difference between the HCSS treatment and the corresponding control without HCSS ($p<0.05$).

HCSS increases the migration and filopodia formation in keratinocytes HaCaT

Besides cell proliferation, an appropriate keratinocyte migration is required for minor, superficial, and basic skin lesions for the second phase of wound healing. Keratinocytes are also involved in more complex pathological states, for example ulcers or pressure sores [Horikoshi *et al*., 2018]. Therefore, a wounded area was further determined to assess the potential effect of HCSS at 50, 100, 500, and 1000 μg/mL on the migratory activity of HaCaT cells. Based on the wound-healing assay, HCSS at 50, 100, 500, and 1000 μg/mL significantly decreased the wound area in a concentration dependent manner with 20.57±5.58, 8.31±2.79, 0.00±0.00, and 0.00±0.00%, respectively (Figure 4A and Figure 4B). A low concentration of HCSS supported the efficiency in wound closure by 70–80% as compared to the control. The wound area was completely closed after 24 h when HCSS was used at concentrations of 500 and 1000 μg/mL. This result implied that HCSS could stimulate keratinocyte migration, especially at the high concentration. In general, collagen had a crucial role in wound repair by promoting the endothelial cells mobility to develop new blood vessels [Martin & Nunan, 2015]. Thus, the granulation tissue development was increased, and wound area was declined [Chotphruethipong *et al*., 2021a]. Hyp, a specific component of collagen, is an important indicator to determine collagen deposition during wound healing process [Huang *et al*., 2015]. Chen *et al*. [2019] documented that fish collagen rich in Hyp could accelerate wound healing process of Sprague Dawley rats. Similarly, an earlier report claimed that collagen hydrolysates rich in Gly, Pro, and Ala had a potential activity in wound closure [Chotphruethipong *et al*., 2021a]. Thus, amino acids, especially Gly and Pro, found in HCSS might promote keratinocyte migration as ascertained by the decreased wound gap. Thus, it can be postulated that an increase in the rate of keratinocytes proliferation and migration undoubtedly leads to fast wound healing.

The cytoskeleton is one of essential constituents in wound healing requiring the shrinkage of actomyosin, cell migration and enlistment of repair systems [Abreu-Blanco *et al*., 2012]. The migration of cell involves the formation of cell protrusion, for example lamellipodia and filopodia [Singkhorn *et al*., 2018]. This is the first study to elucidate the protrusion of cell that facilitated cell migration in response to HCSS treatment in keratinocytes. Keratinocyte HaCaT cells were exposed to HCSS (50–1000 μg/mL) for 24 h. Our results showed that 100, 500, and 1000 μg/mL HCSS treatment significantly enhanced the number of filopodia per cells as compared with the control (Figure 4C and Figure 4D). The highest activity was presented in cells treated with HCSS at 1000 μg/mL. Taken together, our results revealed the migratory activities of HCSS in keratinocytes. This result was consistent with wound area (Figure 4A and Figure 4B) as evidenced by the increased formation of filopodia, particularly at the HCSS concentrations

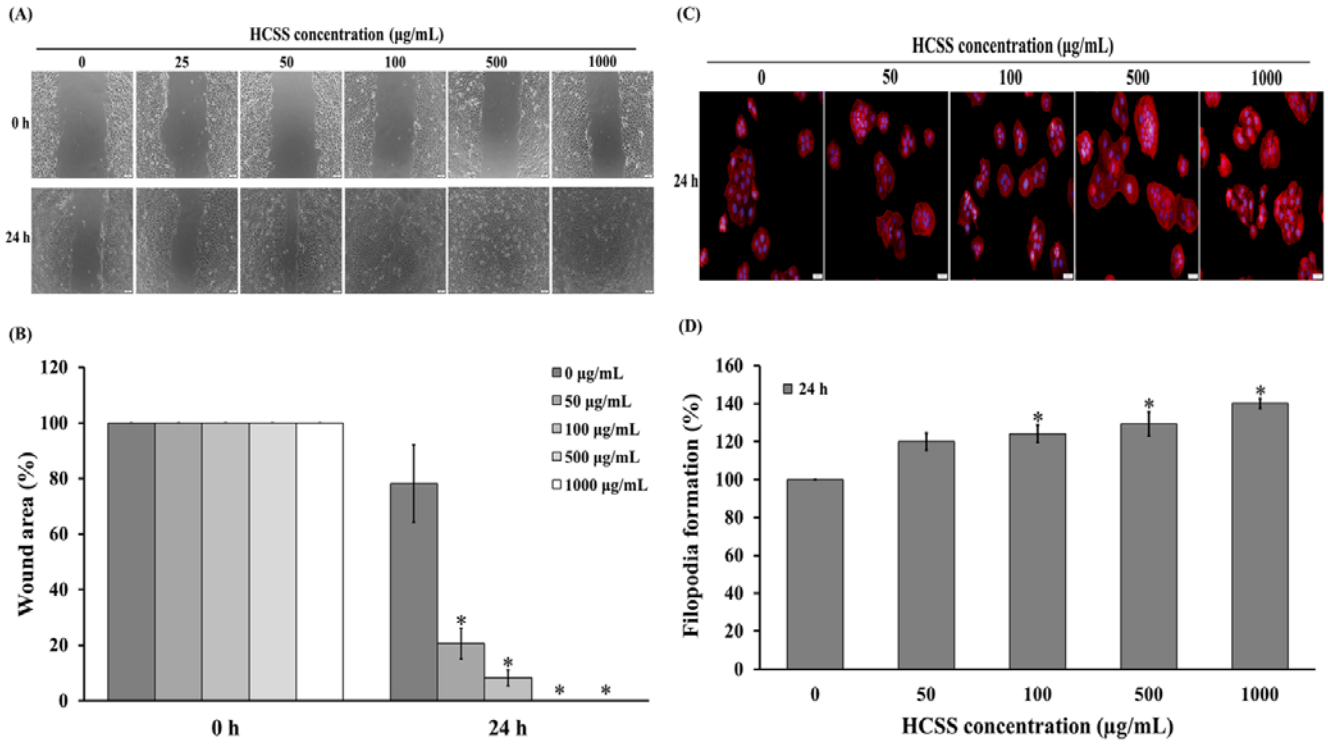

FIGURE 4. The migration activities and filopodia formation of skin HaCaT keratinocytes after treatment with hydrolyzed collagen from salmon skin (HCSS) at concentrations of 0, 50, 100, 500 and 1000 μg/mL for 24 h. (A) wound healing assay, (B) the percentage of wound area, (C) phalloidin--rhodamine stained for filopodia (scale bar = 50 μm), and (D) percentage of filopodia formation.

Results are presented as mean ± standard error of the mean of four independent experiments (n=4). The asterisk above bars indicates a significant difference between the HCSS treatment and the corresponding control without HCSS ($p<0.05$).

of 100, 500, and 1000 μg/mL. Our findings agree with results of previous studies which have shown that hydrolyzed collagen from fish skin increased the formation of lamellipodia in fibroblasts [Chotphruethipong *et al*., 2021a]. Altogether, we could explain that the upregulation of filopodia formation in this study may be due to the AAs content in hydrolyzed collagen and may subsequently contribute to the increased keratinocyte migration in the wound healing process.

HCSS increases cell migration and filopodia formation *via* focal adhesion kinase (FAK)/steroid receptor coactivator (Src) activation

In the present study, the migratory activity of skin keratinocytes was induced by HCSS administration. Several signaling molecules have been identified and found to be necessary in the control of cell migration, extension, and cytoskeleton contraction, for example, FAK, Src, protein kinase B (Akt), Ras-related C3 botulinum toxin substrate 1 (Rac1), Ras homolog family member A (RhoA), and cell division control protein 42 (Cdc42) [Masraksa *et al*., 2020; Ritto *et al*., 2017; Singkhorn *et al*., 2018]. The upstream regulatory cell signals of cell migration controllers, such as FAK, Src, and Akt, were further analyzed. The results revealed that HCSS administration increased the expression of pFAK (phosphorylated at Tyr397) and pSrc (phosphorylated at Tyr418), in keratinocyte HaCaT at 100, 500, and 1000 μg/mL (Figure 5A and Figure 5B). However, the pAkt (phosphorylated at Ser473) was not affected by the HCSS treatment. FAK and Src are important for reepithelialization during the wound repair process [Seo *et al*., 2018; Singkhorn *et al*., 2018]. In addition, stimulation of FAK and Src complex activates keratinocytes migration in epidermal wound healing [Petpiroon *et al*., 2015; Seo *et al*., 2018; Singkhorn *et al*., 2018]. Nevertheless, this is the first study to examine the mechanism of HCSS induced keratinocyte migration through the FAK-Src complex pathway activation. Few studies reported that the amino acid domain containing Asn, Gly, Gln, and Ala in collagen can interact with $\alpha_2\beta_1$ integrin on the cell membrane that is involved in the activation of the FAK-c-Jun N-terminal kinase (JNK) pathway [Chiu *et al*., 2014]. Thus, we could explain that the activation of FAK-Src by HCSS may result from the presence of Asn, Gly, Gln, and Ala in hydrolyzed collagen which was found in the high level in our study.

Our previous reports demonstrated that Cdc42, Rac1, and RhoA proteins were involved in the filopodia formation and migration of skin keratinocytes [Ritto *et al*., 2017; Singkhorn *et al*., 2018]. In order to authenticate the mechanism of HCSS effect on the migration stimulation in keratinocyte, we used western blot to examine the important downstream Cdc42, Rac1, and RhoA proteins expression involved in the migration process. In this study, Cdc42, Rac1, and RhoA proteins expression was established to be raised in response to HCSS treatment at the concentrations 100, 500, and 1000 μg/mL (Figure 5C and Figure 5D). To our knowledge, this is the first study that described the downstream Cdc42, Rac1 and RhoA proteins activation by HCSS. Thus, we suggest that HCSS

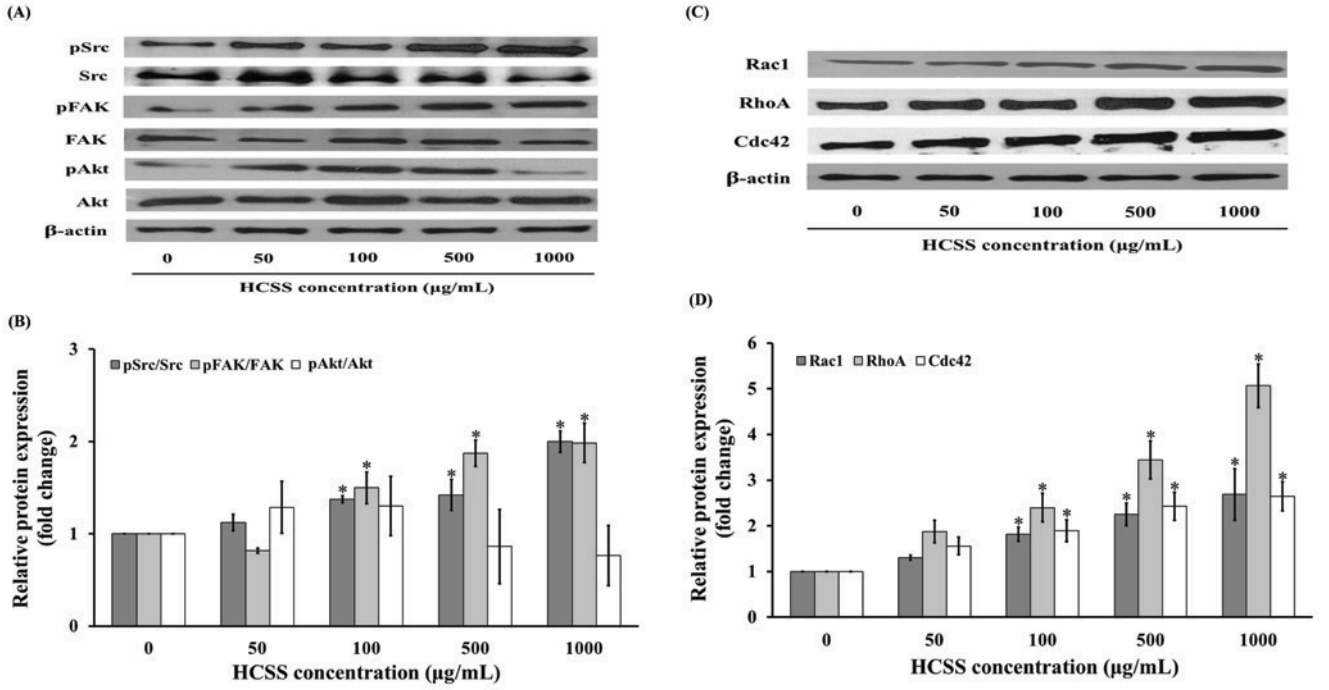

FIGURE 5. The migration protein expression of skin HaCaT keratinocytes after treatment with hydrolyzed collagen from salmon skin (HCSS) at concentrations of 0, 50, 100, 500 and 1000 μg/mL for 24 h. (A) western blotting indicating the expression of pSrc, Src, pFAK, FAK, pAkt, and Akt, (B) the relative expression of pSrc/Src, pFAK/FAK, and pAkt/Akt, (C) western blotting indicating the expression of Rac1, RhoA, and Cdc42, and (D) the relative expression of Rac1, Cdc42, and RhoA.

Results are presented as mean ± standard error of the mean of four independent experiments (n=4). The asterisk above bars indicates a significant difference between the HCSS treatment and the corresponding control without HCSS ($p<0.05$).

regulated cell migration and cytoplasmic protrusions formation of skin by activating FAK/Src upstream pathways, and Rac1, Cdc42, and RhoA downstream pathway.

HCSS increases the expression of stem cell markers

Keratinocyte stem cells (KSCs) are accountable for sustaining epidermal homeostasis and healing the tissue damages [Fuchs, 2008; Pincelli & Marconi, 2010; Sotiropoulou & Blanpain, 2012]. Several evidence indicate that KSCs contain various proteins which demonstrated the powerful function in maintaining stem cell-like phenotypes, for example keratin 19, β-catenin, and others [Abbas *et al*., 2011; Leng *et al*., 2020]. The present study showed that HCSS treatment at the concentrations 100, 500, and 1000 μg/mL significantly enhanced keratin 19 and β-catenin protein expression in keratinocyte HaCaT cells when compared to the untreated control (Figure 6A and Figure 6B). Our results were similar to those from the previous studies reporting that the collagen extract from abalone caused an increase in KSCs marker expression, such as ALDH1A1, keratin 19, and β-catenin in keratinocyte HaCaT cells [Thaweekitphathanaphakdee *et al*., 2019]. In addition, collagen type I enhanced the properties of stem cell-like phenotype by the $\alpha_2\beta_1$-integrin activation [Kirkland, 2009]. Integrin is a cell surface receptor that plays a crucial role in maintaining several signaling cascades such as Akt [Desgrosellier & Cheresh, 2010] and Wnt/β-catenin [Crampton *et al*., 2009; Leng *et al*., 2020]; thereby mediates cell survival and proliferation, and stimulates stemness of cells [Leng *et al*., 2020]. It is possible that HCSS may activate stemness

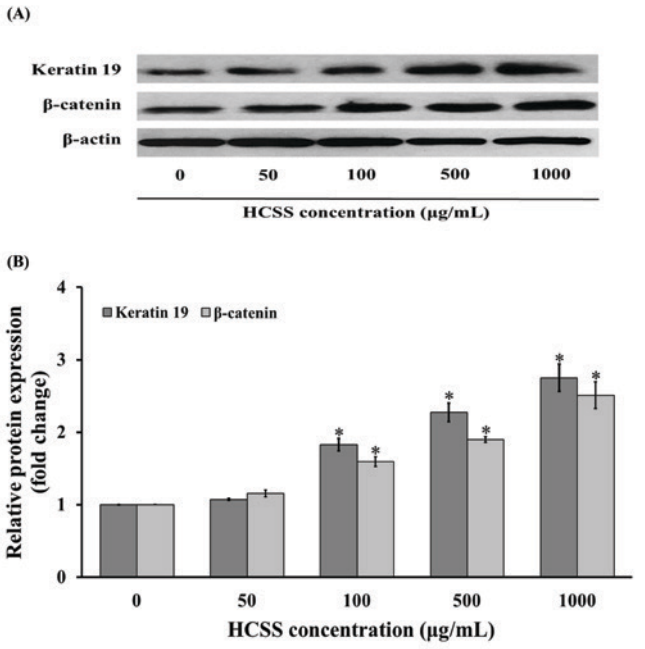

FIGURE 6. The expression of stem cell markers in skin HaCaT keratinocytes after treatment with hydrolyzed collagen from salmon skin (HCSS) at concentrations of 0, 50, 100, 500 and 1000 μg/mL for 24 h. (A) western blotting indicating the expression of keratin 19 and β-catenin, and (B) the relative expression of keratin 19 and β-catenin.

Results are presented as mean ± standard error of the mean of four independent experiments (n=4). The asterisk above bars indicates a significant difference between the HCSS treatment and the corresponding control without HCSS ($p<0.05$).

of keratinocytes by β-catenin-dependent mechanism. Our results add to the existing knowledge that HCSS improves the keratinocytes stemness properties, which are essential for epidermal homeostasis and skin barrier function [Fuchs, 2008; Pincelli & Marconi, 2010; Sotiropoulou & Blanpain, 2012].

CONCLUSIONS

Fish skins, being by-products of the food industry, represent a viable material for producing collagen hydrolysates. A hydrolyzed collagen from salmon skins (HCSS) significantly activated the migration of keratinocytes, a predominant cell type in the epidermis, by the activation FAK-Src upstream pathway and Rac1, RhoA, and Cdc42 downstream pathway. In addition, HCSS significantly increased the expression levels of stem cell markers, which are crucial factors for keratinocyte stem cell's function. Taken together, HCSS has been highlighted to elicit the beneficial effect which may have a promising utilization for wound healing, skin repair, and skin barrier function.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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