CHAPTER V
DISCUSSION

Isolation of collagen extracted from skin and scale of giant gouramy collagen determination collagen

In this study, collagen extracted from skin and scale of giant gouramy gave 82.15% and 24.78% yield respectively (lyophilized dry weight). The percent extraction of collagen from fish skin was higher than that from fish scale due to high content of collagen in the skin (Lee et al., 2001). The difference in percent yield may result from discrepancy of glycosylation level in its molecule (especially the number of glycosylgalactosyl hydroxylysine residues). Solubility characteristics of type I collagen in diluted acid is decreased in high level glycosylation collagen (Purna Sai and Babu, 2001). Moreover, the type of crosslink is another factor relating to its solubility. The weak crosslink like aldimine crosslink is found in the skin whereas the calcified tissue usually consisted of the high level of keto-amine crosslink is more resistant to the acid and enzyme activities (Friess, 1998).

Sadowska and co-workers (2003) reported that yield of collagen could be better when the ratio of acetic acid in Baltic cod skin increased. However, the technique was not practical because collagen would be more diluted and more chemical was spent in order to precipitate collagen. In addition, the extracted collagen could be tainted with the pungent acetic acid odor.

The yield of giant gouramy skin in this study was higher than collagen yield obtained from skin of the other fish spp; Nile perch (Muyonga et al., 2004), Japanese flounder (Nishimoto et al., 2005), Bigeye snapper (Kittiphattanabawon et al., 2005) Red eye snapper (Jongjareonrak et al., 2005) and marine organism such as cuttlefish (Nagai et al.,2001), puffer fish (Nagai et al., 2002), paper nautilus (Nagai and Suzuki., 2002) and skate (Hwang et al., 2007).

Apart from the advantage of enzymatic activity of pepsin in the present study, we take advantage from the activity of pepsin in that this enzyme activity can be reduced by limited low temperature and short time exposure but it still cut the telopeptides at the ends of collagen molecule. There was a research reported about effect of temperature and time to pepsin digestion in bird feet collagen. They indicated that optimum condition for digestion of bird feet collagen was 12°C for 24 hours (Lin and Liu, 2006). However, in present study, the extraction was done at 4°C

instead of 12°C because fish collagen is usually easy to degrade than mammalian collagen (Nagai, 2002). Purna Sai and Babu (2001) studied about the difference between ASC and PSC isolated from frog skin collagen. They demonstrated that the re-dissolution of acid treated collagen was less than that of pepsin treated collagen because ASC has more stability than PSC. The stability results from non-helical ends, which are still intact, is found only in ASC. The lysine derived aldehydes crosslink is able to be synthesized by aldehydes groups in this part. The crosslink has an impact on promoting fibril formation as well as stabilizing the fibril thus formed. For this reason, fibril formation between enzyme treated collagen and acid one is different. There was study about the intermolecular interaction on native and enzyme-treated acid soluble collagen. They observed that the molecular length after aggregating of enzyme-treated collagen was smaller than of that of acid treated collagen and its quantity of aggregation was also lower than non-enzyme treated collagen (Bernengo et al., 1978). PSC fibril was much shorter, distorted and less banded than ASC counterpart. Moreover, only lateral assembly was reversed while the linear assembly could not be reversed on cooling. The difference results in their different strength and stability. This finding indicated that the telopeptide regions played an essential role for the kinetics of normal fibrillogenesis. (Purna Sai and Babu, 2001). However, these drawbacks of PSC can be corrected by chemical agent in order to synthesize the crosslink (Usha, 2000; Angele et al., 2004; Song et al., 2006) or stabilize the collagen molecule (Penkova et al., 1999; Fathima et al., 2006).

In addition, the difference of percent extractability between each collagen source may be due to the method of protein determination. It is hard to find the standard procedure to determine collagen concentration to accommodate the helical structure and specific amino acid contents. Moreover, various substances used in the extraction process might interfere with the interaction of reagents such as buffer and salt (Komsa-Penkova et al., 1996)

Generally, collagen concentration can be determined by its hydroxyproline content. The method depends on the oxidation of hydroxyproline and the concomitant chromophore formation with p-dimethylaminobenzaldehyde. This method is sensitive and reliable. However, it is time consuming and required the sophisticated equipments. Therefore, the method is not suitable for a routine measurement. The absorbance at OD 230 nm is another method for measurement collagen concentration but the method is non-reliable with low sensitivity using the

opalescent, turbid and colored solution characteristic of collagen. The method is also interfered by substances such as urea and glycerol. Furthermore, the result depends largely on the pH of collagen sample. Modifications of Biuret and Bradford methods are also applied for routine collagen concentration measurement, but the method is not sensitive enough (~ 0.1 absorbance units/ 1 mg/ml collagen concentration). On the other hand, modified Lowry's method used for the determination of collagen quantities is simple and sensitive when compared with the other methods (Table 5.1) (Komsa-Penkova et al., 1996).

Table 5.1 Comparison between reference methods and modified Lowry's method (modified from Komsa-Penkova et al., 1996)

Method	Slope absorbance units/mg ml^{-1}	Minimal detectable concentration (μg/ml)	Linear range(μg/ml)	Color stability (%/10 min)	pH limitation
hydroxyproline by Stegemann	2.5	22 (collagen) 2 (4-OH-pro)	22-440	2.5	independent on pH
UV absorbance at 230 nm	1.5	20	20-700		dependent on pH
Modified Lowry's method (standard protocol)	1.52	20	20-500	0.85	independent on pH

Modified Lowry's method is the technique applied from Lowry's method by changing the incubation temperature of collagen samples in alkali solution resulting in reducing of rigidity of the collagen backbones and enhancing their ability to form complex with copper as a result in decreasing concentration of reagents used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The SDS-PAGE patterns of giant gouramy skin and scale collagen were shown in Figure 4.2. There was no difference in molecular weight (MW) between both of the fish collagens. The electrophoretic profiles of the fish collagen (skin and scale) were also similar to the pattern of porcine tendon type I collagen. The result suggested that PSC isolated from skin and scale of giant gouramy is consisted of type I collagen as a major composition.

Muyonga and co-workers (2004) reviewed mobility difference between globular protein and collagenous protein. They suggested that in the electrophoresis field, although these proteins had the same molecular mass, the migration of α chain was slower than the globular protein. The result is due to differences in amino acid composition in collagen like glycine, proline and alanine. The molecular mass of α1 in this study was approximately 120 kDa instead of 130 kDa.

From the present result, it was observed that the density of α1 chain was approximately two-fold higher than that of α2 chain. Normally, type I collagen is composed of two α1 chains and one α2 chain [$(\alpha1)_2$ α2] as the major form. Nishimoto and co-workers (2004) reported that this structure is recovered more than 90% of total type I collagen. Furthermore, type I collagen also has distinct α3 chain as α1α2α3 which cannot be found in the mammalian collagen. Since α3 has the same molecular weight as α1, it could be that the presence of α3 is obscured by α1 chain under electrophoretic condition.

Moreover, the skin and scale collagen contained high level of intermolecular component namely ß-chain whereas another component (γ) was analysed in a limited amount. Despite the fact that high molecular weight crosslink is generally related to animal age. The crosslinking rate of fish collagen is extremely slow because most connective tissue in fish is renewed annually (Kittiphattanabawon et al., 2005).

Subunit composition

The subunit composition of denatured giant gouramy skin and scale PSCs were analyzed using HiTrap CM FF column chromatography. Several fractions were collected and indicated as fraction numbers. The chromatographic of eluted fractions was analysed by 6% SDS-PAGE. The electrophoretic pattern of skin PSC was quite similar to that of the scale PSC, suggesting that the composition of skin type I collagen was not different from that of scale. Because of the identical mobility in electrophoretic field between α1 and α3, these two α chains could not be differentiated in the SDS-PAGE condition. However, their molecular charges were different. Generally, charge on protein molecule can change according to the pH, in acidic environment protein becomes positive charge (pH 4.8). In this experiment, the denatured PSC samples were prepared in the acid condition. Then, the denatured protein was applied to the carboxymethycellulose column chromatography, a cation column exchanger. Each of the denatured collagen molecule would bind to the carboxymethycellulose with different affinity. The fractions were eluted by gradually increasing NaCl concentration. As the Na concentration increased, Na in an ionic form competes with the collagen molecule and then different collagen subunits were eluted at different retention time.

In this study, partial separation between two major elution peaks was found in chromatogram of skin and scale collagen (Figure 4.5a, 4.6a). The fist peak had two protein bands about 200 kDa and 130 kDa whereas the second peak contained three protein bands approximately 200 kDa, 130 kDa and 116 kDa (Figure 4.5b, 4.6b). The protein band of molecular weight 200 kDa, 130 kDa and 116 kDa were indicated as β, α1 and α2 chains respectively. Appearance of α1 and α2 in the second peak indicated that there was co-elution between these two α–components. However, it might be suggested that collagen extracted from giant gouramy skin and scale was a heterotrimer consisting of two α1 and one α2 [$(\alpha1)_2\alpha2$]. There are two reasons supporting this theory. Although there was difference in band intensity between molecular weight at 130 kDa and 116 kDa in the second peak, if the band at 130 kDa were α3, the intensity of band should be gradually increase and finally disappear. However, such an electrophoretic pattern was not found. Therefore, the protein with 130 kDa in the second peak is likely to be α1 rather than α3. The pH buffer might be one of the factors that had an impact on co-elution condition. Matsui

and co-wokers (1991) have demonstrated that α2 and α3 of chum salmon were co-eluted in the conventional method (pH 4.8). After changing the pH buffer to 5.6 and they were able to complete the separation of these α chains. α1 and α2 in this study could be more clearly identified by adjusting the pH of buffer.

Besides the α chains, there was another high molecular weight protein having molecular weight at approximately 200 kDa. This protein was an intramolecular crosslink called β chain. The β component co-eluted in the primary and subsequent peak was identified as β11 and β12 respectively (Figure 4.5b, 4.6b). Hwang and co-workers (2007) suggested that a faint band of the α2 chain and a dense band of the β component might dimerize into the β component and form β12 dimer.

Kimura (1992) had studied the fish collagen and reported that the α3 chain was widely found in many teleosts such as eel, sardine, chum salmon, rainbow trout, carp, angler, Alaska Pollack, cod, halfbeak, common mackerel, Tilapia, red barracuda, northern dab and file fish. Besides these teleost, the α3 was distributed in various marine organisms such as paper nautilus outer skin (Nagai and Suzuki, 2001) edible jellyfish exumbrella, rhizostomous jellyfish mesogloea, ayu bone and C. arakawai arm (Nagai et al., 2002). However, α3 was not found in the present study.

On the other hand, many observations were in agreement with the present result including cuttle fish outer skin (Nagai et al., 2001) puffer fish skin (Nagai et al., 2002) nile pearch skin (Muyonga et al., 2004), great blue shark and dogfish shark skin (Hwang et al., 2005).

Peptide mapping

The collagen extracted from giant gouramy skin and scale were hydrolyzed and subjected to 10% gel SDS-PAGE. Peptide mappings of giant gouramy collagen were achieved from hydrolysis of *Staphylococcus aureus* endopeptidase (V8 protease) and lysyl endopeptidase (Figure 4.3, 4.4). Both enzymes are specific enzyme cutting at the specific amino acid.

The hydrolysate demonstrated the intense band at 37 and 35.8 kDa and the protein fractions at MW of 27.4 to 35 kDa. The band with the same MW and intensity as the electrophoretic pattern of enzyme is not protein fraction of collagen. The peptide mappings of fish and mammalian collagen were quite different. α and β chains of porcine tendon collagen were hydrolyzed into smaller fraction than the fish

collagen but the peptide mappings of both the collagen isolated from skin and scale giant gouramy were not different. This result revealed that fish collagen was less tolerated to *Staphylococcus aureus* proteinase than the porcine tendon collagen, as found in other fish reported (Kittiphattanabawon et al., 2005). Moreover, it is also related to the degree of glutamic residue in fish collagen because this enzyme activity is very specific in cleavage between peptide bond of glutamic acid and aspartic acids in the collagen molecule. Porcine tendon collagen like other mammalian collagens such as calf skin collagen, might have glutamic acid less than giant gouramy collagen (Cui et al., 2007).

Lysyl endopeptidase digestion would cleave specifically the peptide bond of lysine with other amino acid (C-terminus). The electrophoretic pattern of mammalian collagen and fish collagen were different. The high molecular weight crosslink and triple helix chain of porcine tendon collagen were more susceptible to hydrolyze than fish skin and scale collagen as demonstrated by the decreased of intense band and concurrence with several faint band appearances. Similar to V8 proteinase, both skin and scale collagen extracted from giant gouramy had the same peptide mapping. This observation suggested that fish collagen was more resistant to lysyl endopeptidase activity than porcine tendon collagen. However, this result was differ from Brown back toad fish skin collagen (Senaratne et al., 2005) Bigeye snapper collagen (Kittiphattanabawon et al., 2005), Brownstrripe red snapper ((Jongjareonrak et al., 2005) and grass carp skin (Zhang et al., 2007).

Both hydrolysate from skin and scale giant gouramy type I collagen gave similar peptide maps. Variation of susceptible bonds in collagen structure to enzyme activity is caused by discrepancy of their amino acid sequence and hydroxylation degree (Jongjareonrak et al., 2005). Therefore, the sequencing of glutamic acid and degree of hydroxylation in both tissues of fish collagens were not different. Moreover, Zhang and co-workers (2007) reported that the difference of collagen peptide maps is associated with extracted sources and species. Therefore, the differences of two protein maps peptide between giant gouramy skin and scale collagen and porcine tendon collagen revealed differences of amino acid sequence, primary structure, and degree hydroxylation of both species (Kittiphattanabawon et al., 2005).

The thermal stability of collagen extracted from skin and scale of giant gouramy

The thermal stability of collagen extracted from giant gouramy skin and scale were determined by a differential scanning calorimeter (DSC). The thermal transition curves of both collagens were shown in Figure 4.7, 4.8. The melting temperature (T_m) of the skin was 36.4°C and that of the scale was 36°C. This is due to the fact that fish collagen are less stable than mammalian collagen such as calf skin collagen (40.8°C) (Zhang et al., 2007). Ogawa and co-workers (2004) suggested that imino acid composition played an essential role in stabilizing of collagen structure. In general, the major unit of its structure is $[Gly –X-Y]_n$ where X and Y site is imino acid Pro 38% and Hyp 25% respectively. Therefore, the two main types of collagen repeated peptide are Gly –Pro-Pro and Gly –Pro-Hyp. These forms have an impact on maintaining the triple helices structure by intramolecular crosslink. From previous studies, they indicated that these imino acid contents in marine collagen were lower when compared with mammalian counterparts leading to low thermal stability. Moreover, there was report indicated that the tripeptides having Hyp in the Y position had denaturation enthalpy twice as great as the other structure. This difference enthalpy is due to hydroxylation of proline in this site as well as pyrrolidine ring conformation. Owing to its position in the prrrolidine ring, the folding of triple helix and formation of all types of interaction such as hydrogen bonds and contact van der Waals interaction is more appropriate. Moreover, OH-group of Hyp is able to form additional hydrogen bond either to water molecules or to polar groups of adjacent polypeptide chains (Boryskina et al., 2007).

Besides the quantity of imino acids, T_m also relates to the physiological and environmental temperature of collagen source. This result was concomitant with the study of the Nile perch skin collagen (36°C) (Muyonga, 2004). Additionally the T_m of giant gouramy skin and scale collagen were higher than that obtained from other tropical fish such as Blak drum and Sheepshead bone and scale (33.6-34.9°C) (Ogawa et al., 2004), Bigeye snapper skin (31°C) and bone (31.5°C) (Kittiphattanabawon et al., 2005) and Brownstripe red snapper skin (31.02°C) (Jongjareonrak, et al., 2005) and much higher than the temperate and cold water fish collagen including Alaska pollack skin (16.8°C) and swim bladder (18.4°C) (Kimura and Ohno, 1987b), bullhead shark (25.0°C) japanease sea bass skin (26.5°C) Japanease sea bass fin (29.1°C) japanease sea bass bone (30°C), skipjack and ayu (29.7°C) chub mackerel (25.6 °C)and yellow sea bream and horse mackerel (29.5°C)

and (Nagai and Suzuki, 2000a). chum salmon (20.6°C), saury (24°C), common mackerel (26.9°C), eel (30.2°C), carp skin (31.7°C) and muscles (32.5°C) (Nagai et al., 2002)

Transition of triple-helix to random coil conformation results in denatured collagen. This process is strongly affected by the environmental conditions such as concentration, pH, and ionic strength of the medium as well as by the content of the conformationally restricted amino-acids, namely proline and hydroxyproline (Fessas et al., 2006). Swelling collagen fiber is result in T_m decreasing (Nielson-Marsh, et al., 2000). On the other hand, Purna Sai, and co workers (2001) reviewed about the factors influencing T_m variation. They reported that T_m could be changed by hydration status and pH of sample and enthalpy of dry collagen rose from -0.7kcal/mol to -3.6 kcal/mol resulted in declining of denaturation temperature from 210°C to 41.1°C. They assumed that the enthalpy of helix to coil transition in dry collagen involves in direct peptide-peptide hydrogen bond breakdown, whereas enthalpy of totally hydrated collagen was used in breakdown the hydrogen bond not only at peptide bond but also at hydration shell.

It is known that the inter- and intra- molecular crosslink of collagen is correlated with its viscosity. Increase of temperature results in collagen triple helices depolymerization. The extremely organized structure will become the random coil configuration of gelatin. The pH of solvent has an impact on this process. In acidic environment, viscosity of the collagen will be decreased. This is due to the possibility of peptide bond rupture in acidic pH is higher than in neutral pH.

Amino acid composition of collagen extracted from skin and scale of giant gouramy

The amino acid compositions of giant gouramy skin and scale PSC are demonstrated in table 4.2 and 4.3. Glycine (Gly), proline (Pro) and alanine (Ala) as well as glutamine (Glu) are rich in these collagens, which are characteristic of the extracellular matrix. The result was concomitant with the nature of collagen which generally consists of glycine approximately one third of composition. Gly is existed in every third amino acid because this space is very restricted. No any amino acid can be instead (Albert, 2002), except for the first 10 amino acids from C-telopeptide and first 14 amino acids from N-telopeptide (Senaratne et al., 2005). Substitution of other amino acids leads to the abnormality such as osteoporosis imperfecta (Yamauchi,

2002).

Like other collagens, skin and scale of giant gouramy collagen comprised the unique amino acids which were able to be found only in collagen including proline (Pro), hydroxyproline (Hyp), and hydroxylysine (Hyl). The level of imino acid (Pro and Hyp) in giant gouramy collagens (skin was 15.53% and scale was 17.78%; Table 4.5) were relativey low compared with other tropical fish such as Tilapia (25.4%) Nile pearch (20.0%) (Muyonga et al., 2004), Bigeye snapper skin (19.30%) and bone (16.3%) (Kittiphattanabawon et al., 2005), Brownstripe red snapper skin (22.10%) (Jongjareonrak et al., 2005), Blackdrum bone (19.16%) and scale (19.89%) Sheepshead bone (19.45%) and scale (19.94%) (Ogawa et al., 2004), grass carp skin collagen (18.6%) (Zhang et al., 2007). They are also lower than mammalian collagen including the pig skin collagen (22%) and calf skin collagen (21.5 %) Ocellate puffer fish (17%) (Nagai et al., 2002). This observation confirmed with many reports indicating fish collagen has the imino acid content lower than the mammalian counterpart resulting in less thermal stability (Zhang et al., 2007 and Lin and Liu, 2006). Non-covalent bonding from pyrrolidine ring of proline and its hydroxylation are important in maintaining polypeptide structure by limitation on changes in the secondary structure of the triple helix. The collagen molecule is more stabilized by hydroxyl group of hydroxyproline via synthesizing the hydrogen bond.

Moreover, the habitat and physiological temperature are also related to the denaturation temperature. Lin and Liu (2005) observed that low thermal stability of Frog and shark skin collagen were associated with their habitat and evolution reducing the quantities of Pro and Hyp content of collagen.

However, the degree of hydroxylation is also associated with helical maintenance (Senaratne et al., 2005). The hydroxyproline and hydroxylysine is the product of proline and lysine oxidation and they can be catalysed by proline hydroxylase and lysine hydroxylase respectively. In this study, skin and scale collagen contained degree of proline hydroxylation (Hyp/Pro+Hyp) at 36.8 %and 42.93% (Table 4.5) respectively, which were relatively high compared with other marine source collagen such as edible jellyfish exumbrella (32.8%).

The total degree proline and lysine hydroxylation [(Hyp+Hyl)/(amount of imino acid+Hyl+Lys)] for giant gouramy skin collagen (38.42%) and scale collagen (39.67%) were higher than that reported for Nile pearch (37.6%) pike (34%),

cod collagen (32%), sole (25.3%), megrim(25%) and hake (24.6%) (Muyonga et al.,2004)

The high thermal stability of giant gouramy collagen might be due to high level of total percent hydroxylation in collagen molecule.

Table 5.2 Imino acid content and percent hydroxylation and denaturation temperature comparison between tropical fish collagen and mammalian collagen

Source of collagen	Total imino acid (%)	% *Hydroxylation* Proline	Lysine	Total hydroxylation	Td (°C)
Giant gouramy					
skin PSC	15.53	36.80	26.54	38.42	36.00
scale PSC	17.78	42.93	29.10	39.67	36.40
Nile pearch[a]					
young fish	19.30	40.90	26.10	37.60	36.00
adult fish	20.00	40.20	27.50	37.60	36.00
Bigeye snapper[b]					
skin	19.30	39.90	24.39	37.18	31.00
bone	16.30	41.72	44.44	42.31	31.48
Brownstripe red snapper[c]					
skin ASC	21.20	38.21	21.43	35.43	31.52
skin PSC	22.10	38.91	30.61	37.41	31.02
blackdrum[d]					
bone PSC	19.16	44.15	24.50	45.88	34.90
scale PSC	19.89	44.19	19.68	40.30	35.30
Sheepshead[d]					
bone PSC	19.45	45.50	24.73	42.20	34.50
scale PSC	19.94	43.33	21.43	39.84	33.60
Ocellate puffer fish[e]					
skin PSC	17.00	39.40	ND	ND	28.00
Brown backed toadfish[f]					
Skin	17.00	45.30	24.20	41.80	28.00
Calf skin collagen[g]	21.50	43.72	21.21	40.73	36.30
Pig skin collagen[g]	22.00	55.91	20.59	40.94	ND

Muyonga et al., 2004[a], Kittiphattanabawon et al., 2005[b], Jongjareonrak et al., 2005[c], Ogawa et al., 2004[d], Nagai et al., 2002[e], Senaratne et al., 2005[f], Zhang et al., 2007[g]

Effect of collagen on Human fibroblast cell culture

In vitro cytotoxicity test of collagen extracted from giant gouramy skin and scale were studied by MTT assay. The human dermal fibroblast (HDFs) used in this study is the primary cell line originating from normal human skin. Its life span is limited at 25th passages. Therefore, the 19th and 24th passage were used to demonstrate the effect of giant gouramy skin and scale collagens on growth and proliferation of normal cell that may respectively represent for adult and aging cell. In preliminary study, investigating the effect of serum on cell growth suggested that this cell could grow and proliferate in the presence and absence of FBS. In this study, the results reveal that the higher serum supplementation in medium, the more efficiency of HDFs growth in longer period of time. Since serum is one of the essential factors for cell growth, the serum free media was used as negative control in order to discriminate the response occurred from serum effect or collagen effect.

The numbers of adhesion and proliferation of HDFs using the absorbance unit from sample measured by MTT assay are revealed to cell response to collagen substrate. Generally, the cell numbers counted at 6 hours represent the number of cell attachment since starting of cell cycle must be about 24 hours. Thus, increase of cell number in 24 hours reveals to cell proliferation. In this experiment, dissimilar promoting effect of collagen in different passages of HDFs was observed. An adhesion effect was indicated in 19th passage while 24th passage was not observed. For the proliferation assay which is the results at 24 h or 48 h, the collagens had the effect on only the fist 24 hours of cell growth suggesting the collagen substrate driven the HDFs to enter the early growth phase of cell cycle.

After plating cell for 6 hours, the number of cells in 5% FBS were significantly less than that in all of the serum free media (a non-collagen coated and collagen-coated plates) significantly. However, HDFs rather grow and proliferate in 5% FBS at 24 and 48 hours than those were grew the presence of collagen substrate. Decrease of cell attachment in the presence of 5% FBS in first 6 hours may be due to unidentified various factors including protein inhibitor contaminant in serum. Therefore, the limit of serum usage is non-reproducibility because each batch of serum has the different effect either promote or inhibit cell growth (Gunrayanee and Nuananong, 2005). In this experiment, the cell number of 19th passage at 6 hours in porcine-tendon collagen, fish-skin collagen coated-plates) were higher than those

grew in non-collagen coated plate. The evidence suggests that porcine tendon collagen and fish skin collagen have promoting effect on the attachment of HDFs.

After 24 hours cell were plated, the number of HDFs in both types of the fish collagens were higher than those in the porcine tendon collagen. Moreover, there was no significant difference between cell number in fish collagen and 5% FBS control. However, these effects were not observed in all of the collagen at 48 hours afterwards (Figure 4.11). These result revealed that collagen substrate could promote cell attachment, even though only the fish collagens are able to promote the cell proliferation. The adhesion effect of collagen may be due to the engagement of integrin with minimal recognition sequences called RGD (Babylak, 2002). The engagement contributes to generate many cascades of intracellular signal pathway. Basically, the RGD which is short peptides sequence (Arg-Gly-Asp) found in $\alpha 1$ and $\alpha 2$ chains of type I collagen is recognized by many integrins of human dermal fibroblast such as $\alpha 1\beta 1$, $\alpha 2\beta 1$and $\alpha 11\beta 1$. Linking of RGD with specific integrin increase intracellular calcium level through tyrosine kinases signaling pathway. The activation of tyrosine kinase leads to phospholipase C activation and phosphoinositides releasing, contributing to opening of IP_3–sensitive calcium channels in intracellular calcium stores. Another possible pathway of interaction between cell receptor and extracellular matrix is mediated by small G-protein of Rho family. The GTPases of the Rho family are important in assembly of protein in focal adhesions the molecule (Mineur et al., 2005). They also play a role in organizing of the cytoskeleton structure (Reed et al., 2001 and Mineur et al., 2005). These activation pathways of integrin have an impact on adhesion, migration and gene expression of cell (Mineur et al., 2005).

However, the effect after engagement may be varied depending on type of cell, type of activated integrin and diversity of intracellular signal pathway. Since the study investigated only one type of cell, the different signal transduction effects may not have an impact on the present study. There were many studies demonstrated that activation of different integrin contributes dissimilar effect. For example, binding between collagen and $\alpha 1\beta 1$ integrin results in increase of cell proliferation by activation of extracellular signal regulated-kinase (ERK) in MAPK pathway, linked up to adapter protein (Shc). On the other hand, the cell proliferation is inhibited when $\alpha 2\beta 1$ integrin is linking to collagen in melanoma cells. The

mechanism is associated with α2 dependent up-regulation of p27 KIP which is an inhibitor of CDK/cyclin (cyclin dependent kinase /cyclin) in the cell cycle during G1/S transition. Although same integrin is activated, its outcome may be different. For instance, the proliferation effect can also arise when α2β1 activation by cyclinE/CDK2 inducion (Lundgren-Akerlund and Gullberg, 2002). Therefore, the proliferation effect at 24 hours of porcine tendon collagen did not be seen although it gave the best adhesion effect at 6 hours. Similarly the expected proliferation effect of giant gouramy collagen would be considered in that recognition of different integrin type or different intracellular signal pathway from giant gouramy collagen. Basically, the different signal pathway activation produce a variation of target gene expression. Increasing of gene expression is concerned with regulation of many type of protein synthesis. There was a research reported about the effect of collagen gel on cell culture to produce metalloproteases, which are matrix degradation and collagen synthesis. They found that MMP synthesis is up-regulated whereas collagen synthesis is down regulated. The increasing of MMPs production is suggested to mediate by α11β1 and α1β1 activation but down regulation of collagen synthesis is occurred via α1β1 (Lundgren-Akerlund and Gullberg, 2002).

Growth factor is other products synthesized from fibroblast and able to function as paracrine effect. It is obvious that extracellular matrix can function as a reservoir of many growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Babylak, 2002). Kanematsu and co-workers (2004) have studied the effect of collagen on bFGF. They found that collagen could protect bFGF from trypsin digestion. The phenomenon was due to incorporate of bFGF molecule in collagen fiber resulting in conformational change of this growth factor. For these reasons α11β1 and α1β1 integrin on HDFs might have affinity to trap with porcine tendon collagen rather than fish collagen contributed to higher MMPs production leading to inhibit cell proliferation afterwards. In addition, it is possible that giant gouramy collagen might protect growth factors better than porcine tendon collagen. The more growth factors were served, the more signal was occurred, which contributes to the enhanced proliferation effect. These possible reasons could help us to explain why giant gouramy collagens gave the better proliferative effect than that of porcine tendon collagen.

However, the protective effect of collagen might not enough for cell to growth in extensive period, which represented by number of viable cells at 48 hours. The cell viability plated on 5% bovine serum albumin medium was significantly higher than in serum-free collagen significantly either collagen-coated or non-collagen coated plate. The result may be due to difference concentration of growth factor which are 5% fetal calf serum added or growth factor synthesis in serum-free collagen coated plate.

For 24^{th} passage, which may represent the aging cell, all of the collagens did not promote cell adhesion but they increased growth and proliferation which the results was detected starting at 24 hours of growth curve. However, result at 48 hours was not different from 19^{th} passage. The different effect at first 6 hours between two passages might come from technical error. Although MTT assay, the in vitro universal method that use for measurement of cell viability and proliferation, still has the some steps that have to be concern such as changing reagent steps. Generally, MTT assay have to be remove cell culture media before adding the MTT reagent and MTT will be subsequently reduced by dehydrogenase enzyme of active cells and form formazan crystals, which can be solubilized by DMSO reagent. As mentioned above, it has the possibility that attachment between 24^{th} passage HDFs and collagen at first 6 hours was not enough to resist a suction power during changing reagent. For this reason, even though the collagens promote adhesion of HDFs, the adhesive effect of the collagens on HDFs could not be detected. However, the effect of collagen to 24^{th} passage was uncertain. It has to be investigated with other techniques such as [^{3}H]-thymidine uptake in further study.

In the present study, it is indicated that giant gouramy collagens did not have the cytotoxic effect to HDFs. However, mechanisms of adhesion and proliferation of HDFs were still unclear. It has to be considered whether or not it would be used as the effectiveness in cell adhesion and proliferation in patient's wound repairing.

In conclusion, pepsin solubilized type I collagen was extracted from skin and scale of tropical fish, giant gouramy, at the yield of 82.15% and 24.78%, respectively. Subunit analysis revealed a presence of only two $\alpha 1$ and one $\alpha 2$ polypeptides chains in collagen molecule of which lysine and glutamic acid sites were different from porcine tendon collagen using *Staphylococcus aureus* V8

endopeptidase and lysyl endopeptidase digestion. Melting temperature (Tm) of the fish skin collagen (36.4°C) and scale collagen (36°C) was lower than that of mammalian counterpart due to less amount of imino acid content in the molecule obtained by amino acid analysis. In addition, no cytotoxicity effect of giant gouramy of observed in HDF cell culture.