CHAPTER IV
RESULTS

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

Extraction of collagen from skin and scale of giant gouramy fish was carried out by pepsin soluble method using 2 grams of starting material. Pepsin-solubilized collagen either from skin and scale was precipitated with 2.6 M NaCl at neutral pH. Extraction yield determined by Modified Lowry's method and percent extraction of collagen from skin and scale of giant gouramy were shown in Table 4.1. 1.64 gram and 0.49 gram were obtained from 2 grams of pulverized lyophilized skin and scale respectively, resulting in 82.15% and 24.78% extraction, respectively.

Table 4.1. Extraction yield, percent extraction of collagen extracted from skin and scale of giant gouramy, determined by Modified Lowry's method

Raw material	Starting material lyophilized weight (g)	collagen concentration (mg/ml)	Total volume (ml)	Extraction yield (g)	% Extraction
Fish skin	2	4.477	367	1.64	82.15
Fish scale	2	2.253	220	0.49	24.78

The concentration of collagen extracted from skin and scale of giant gouramy were determined by Modified Lowry's method using bovine skin collagen type I of known concentration as reference. Standard curve plotted between averaged of standard collagen concentration and the averaged of absorbance of duplicated sample was revealed in Fig 4.1. Standard curve was linear within the concentration of collagen between 0.1-0.5 mg/ml, with $R^2 = 0.9956$

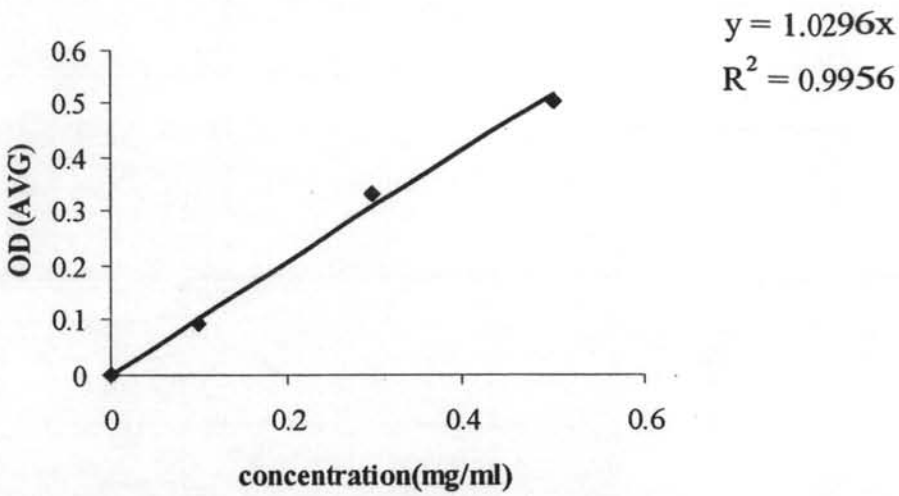

Figure 4.1. Standard curve of collagen. Bovine skin collagen type I was used as reference at concentrations of 0.1, 0.3 and 0.5 mg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The pepsin-solubilized collagen (PSC) isolated from giant gouramy skin and scale were analysed by 6% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Figure 4.2). PSCs from both sources were shown to comprise of three different protein bands with the molecular weight of approximately 205, 130 and 116 kDa. These three protein bands had the same electrophoretic mobility as those of porcine tendon collage type I, used as a reference protein (lane no.1). There was no significant difference in migrating patterns between skin and scale collagen.

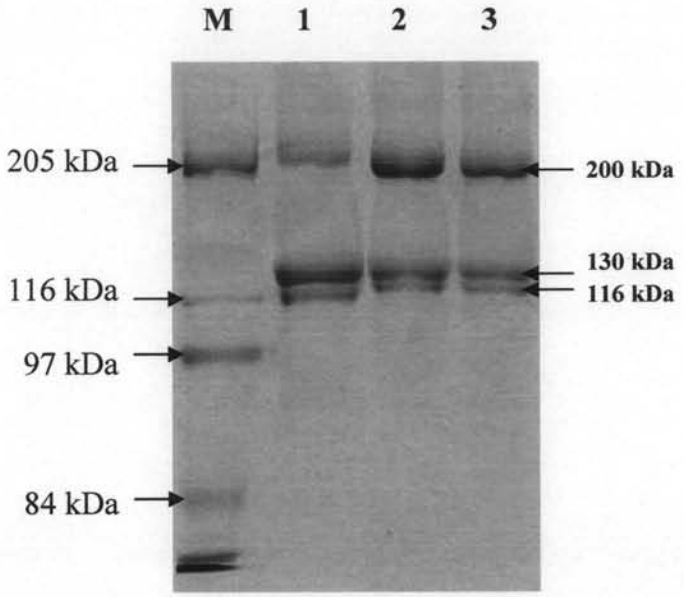

Figure 4.2. Electrophoretic patterns of giant gouramy collagen on 6% SDS-PAGE. M : molecular weight marker (Sigma®); lane no.1: porcine tendon collagen type I; lane no.2: giant gouramy skin collagen; lane no.3: giant gouramy scale collagen

Subunit composition of collagen extracted from skin and scale of giant gouramy

The denatured pepsin-solubilized collagen samples isolated from skin and scale of giant gouramy were applied to a cation excahange HiTrap CM-FF column chromatography after being eluted with linear gradient of 0-0.2 M NaCl. Both of the denatured collagens were eluted into two peaks with partial separation (Figure 4.5a and 4.6a). Several fractions, as indicated by numbers were analysed by SDS-PAGE at 6% gel concentration. These two elution peaks revealed SDS-PAGE patterns of typical type I collagen, containing of three protein bands of MW 250, 130 and 116 kDa approximately. The fraction in the second peak posed three protein bands with different intensity. Between two lower protein bands, the more intense one had the same migrating rate as standard protein of 110 to 120 kDa. On the other hand, the faint band had electrophoretic mobility similar to reference protein of 120 to 130 kDa as shown in Figure 4.5b and 4.6b.

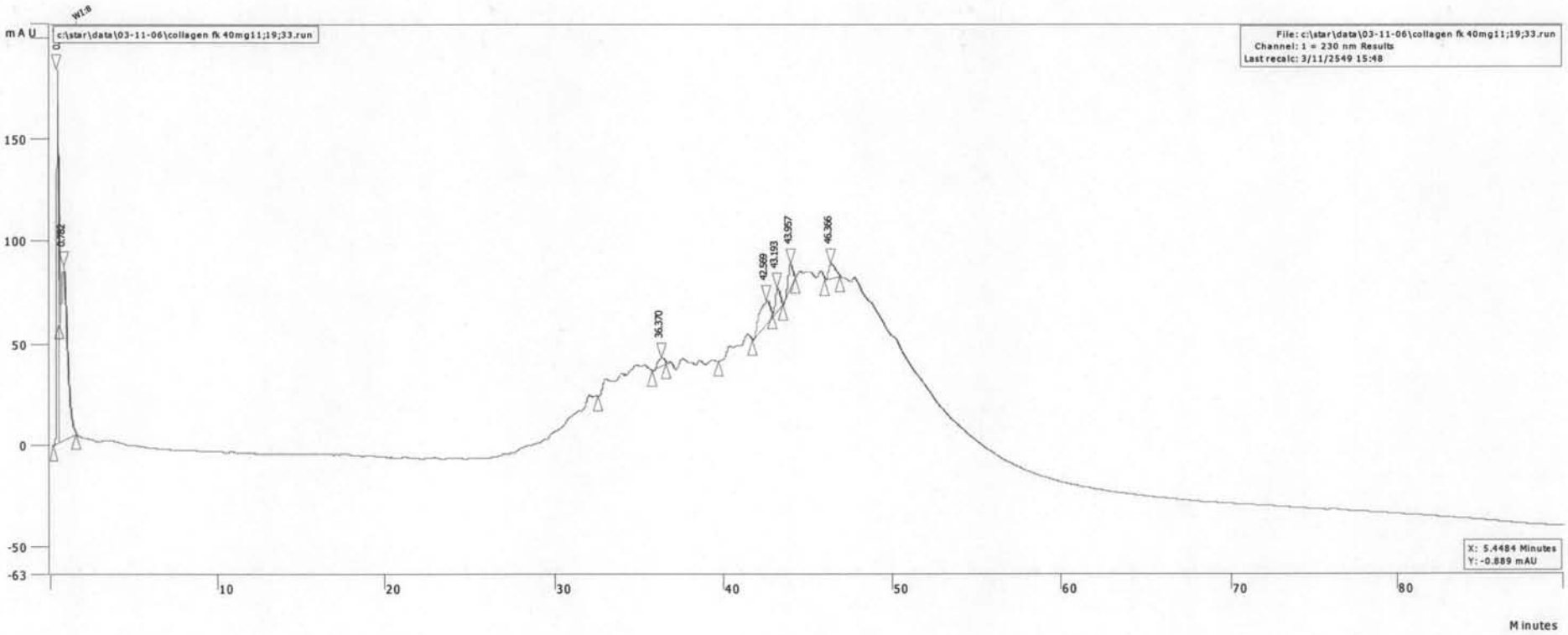

Figure 4.5a. Elution patterns of cation exchange column chromatography (Hitrap CM FF) of pepsin-solubilized collagen extracted from the skin of giant gouramy, The 40 mg of lyophilized collagen sample was dissolved in 1 ml of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea denatured at 45°C for 30 min, and then eluted from the column, which had been equilibrated with the same buffer, with a linear gradient of 0-0.2 M NaCl over a total volume 140 ml at a flow rate of 1.5 ml/min and maintained at 37°C. Unlined fractions were collected. The fractions indicated by numbers were analyzed by SDS-PAGE.

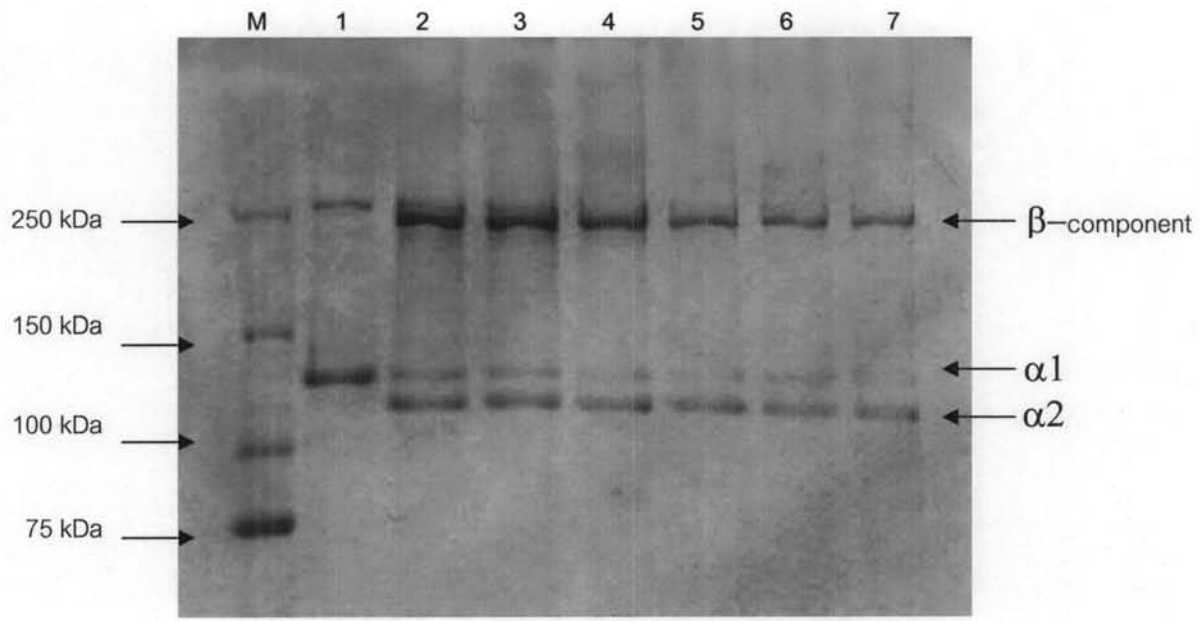

Figure 4.5b. SDS-PAGE electrophorogram of elution fractions giant gouramy skin collagen, M = molecular weight marker (BIORAD); lane 1. Fraction no.35 .; lane 2. Fraction no.45; lane 3. Fraction no.46 ; lane 4. Fraction no.47; lane 5. Fraction no.48 lane 6 Fraction no.49; lane 7. Fraction no.50

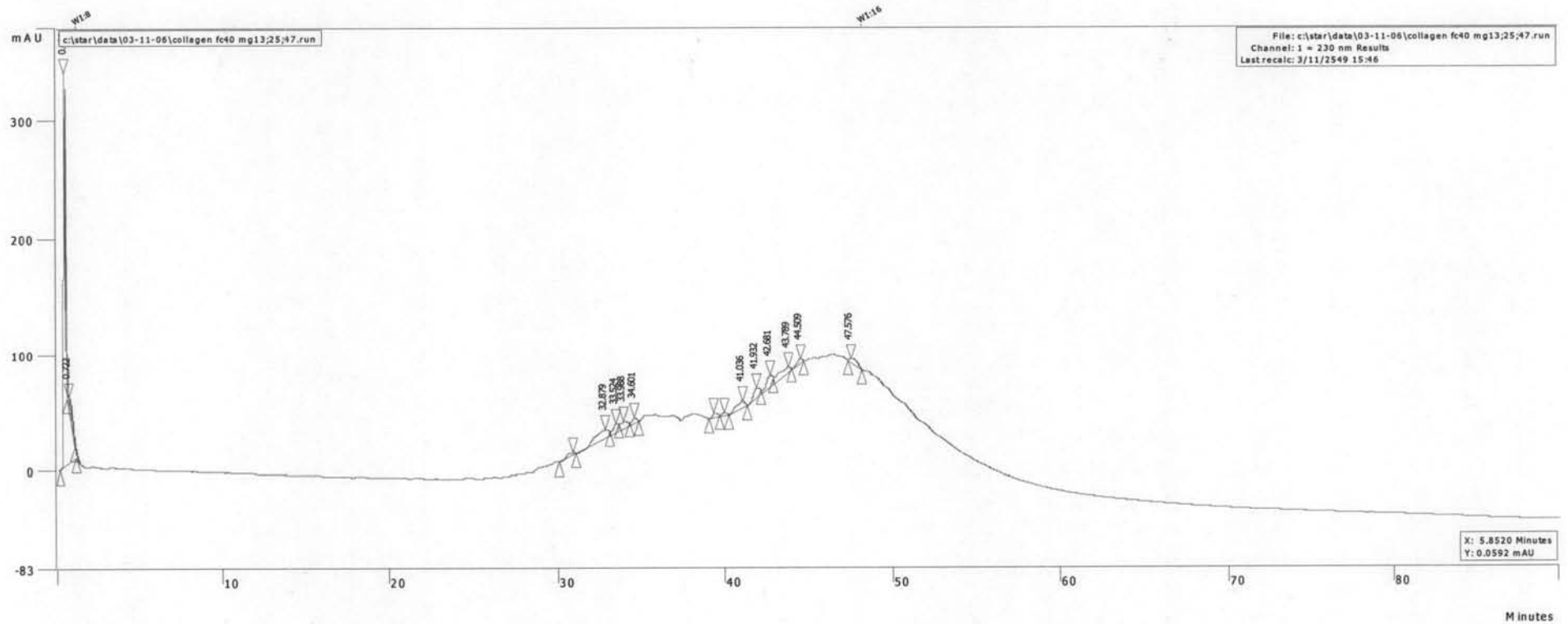

Figure 4.6a. Elution patterns of cation exchange column chromatography (Hitrap CM FF) of pepsin-solubilized collagen extracted from the scale of giant gouramy, The 40 mg of lyophilized collagen sample was dissolved in 1 ml of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea denatured at 45°C for 30 min, and then eluted from the column, which had been equilibrated with the same buffer, with a linear gradient of 0-0.2 M NaCl over a total volume 140 ml at a flow rate of 1.5 ml/min and maintained at 37°C. Unlined fractions were collected. The fractions indicated by numbers were analyzed by SDS-PAGE

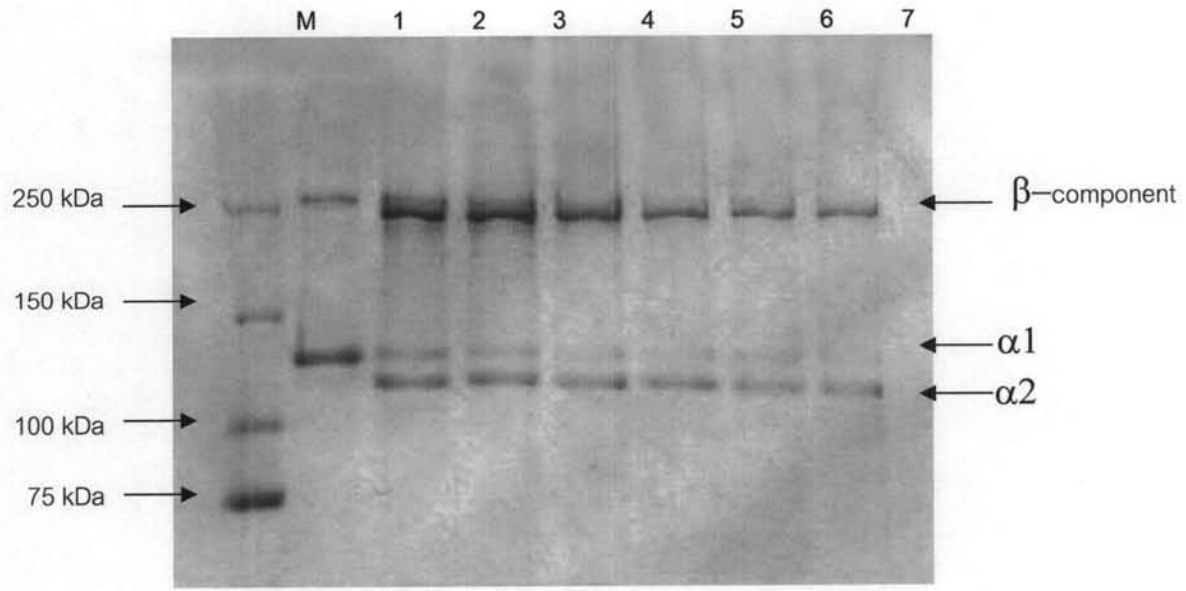

Figure 4.6b. SDS-PAGE electrophorogram of elution fractions giant gouramy skin collagen, M = molecular weight marker (BIORAD); lane 1. Fraction no.35 .; lane 2. Fraction no.45; lane 3. Fraction no.46 ; lane 4. Fraction no.47; lane 5. Fraction no.48 lane 6 Fraction no.49; lane 7. Fraction no.5

Peptide mapping of collagen extracted from skin and scale of giant gouramy

The peptide mapping on 10% gel SDS-PAGE of giant gouramy skin and scale collagen digested by *Staphylococcus aureus* endopeptidase and lysyl endopeptidase, in comparison with type I porcine tendon collagen were shown in Figure 4.3 and 4.4 respectively. After limited hydrolysis, band intensity of high molecular weight subunit identified as β and α chains were digested into small fractions. The band patterns of both digested fish collagens were similar but different from that of porcine tendon collagen.

For *Staphylococcus aureus* digestion, lane no.1 was the porcine tendon collagen type I undigested molecule which is comprised of dominant protein band of 250, 130 and 116 kDa. Lane no.3 was porcine tendon collagen digested by *Staphylococcus aureus* endopeptidase resulting in dominant bands of MW 100 to 50 (Figure 4.3) while those of giant gouramy skin and scale collagen showed the prominent band only at approximately 67 kDa (lane no.4, 5). The faint bands from 50 to 37 kDa were observed in skin and scale collagen and both of the patterns were quite similar (Figure 4.3). Lane no.2 is the Staphylococcus aureus endopeptidase comprised of the dominant protein band of 37 to 25 kDa and the MW of approximately 34 kDa was protein band of enzyme found in all of the collagen tested.

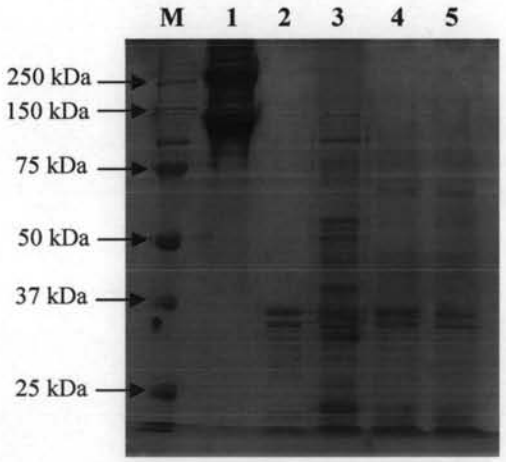

Figure 4.3. Peptide mapping on 10% gel of giant gouramy skin and scale collagen digested with *Staphylococcus aureus* endopeptidase. M : molecular weight marker (BIORAD®); lane no.1: undigested porcine tendon collagen; lane no.2: *Staphylococcus aureus* endopeptidase; lane no.3: digested porcine tendon collagen; lane no.4: digested giant gouramy skin collagen; lane 5: digested giant gouramy scale collagen

In addition, lysyl endopeptidase digestion of porcine tendon collagen resulted in 6 intense bands of 110, 43.5, 33, 29.8, 25 and 23 kDa approximately. (Figure 4.4; lane no.2). On the other hand, Giant gouramy collagen had more intense bands including approximately 85, 82.5, 80, 75, 72.5, 70, 57.5, 50, 35.8, 34.6 and 37 kDa as shown in Figure 4.4 (lane no. 3, 4).

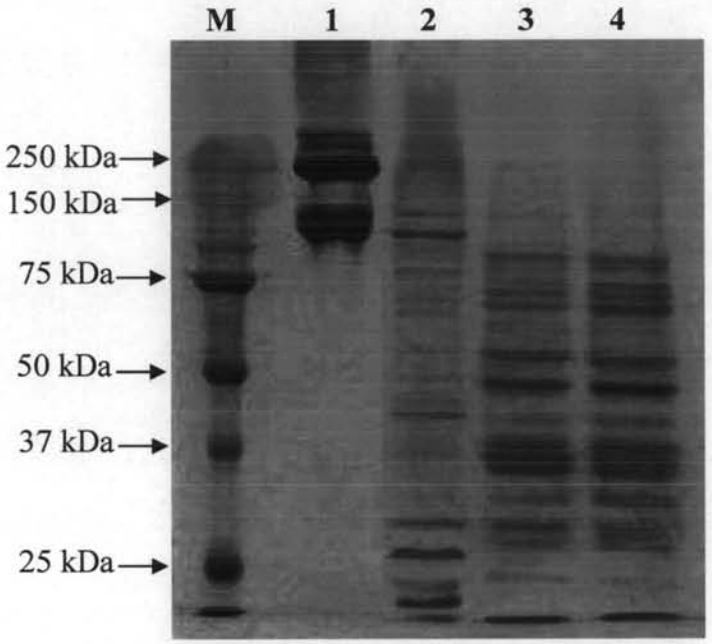

Figure 4.4. Peptide mapping on 10% gel of giant gouramy skin and scale collagen digested with Lysyl endopeptidase. M: molecular weight marker (BIORAD®); lane no.1: undigested porcine tendon collagen; lane no.2: digested porcine tendon collagen; lane no.3: digested giant gouramy skin collagen; lane 4: digested giant gouramy scale collagen

Thermal stability of collagen extracted from skin and scale of giant gouramy.

The collagen from both sources were rehydrated in sterile distilled water The PSC from giant gouramy skin had collagen-gelatin transition curve with a peak at 36.4°C whereas that of giant gouramy scale posed peak at 36°C. (Figure 4.7 and 4.8).

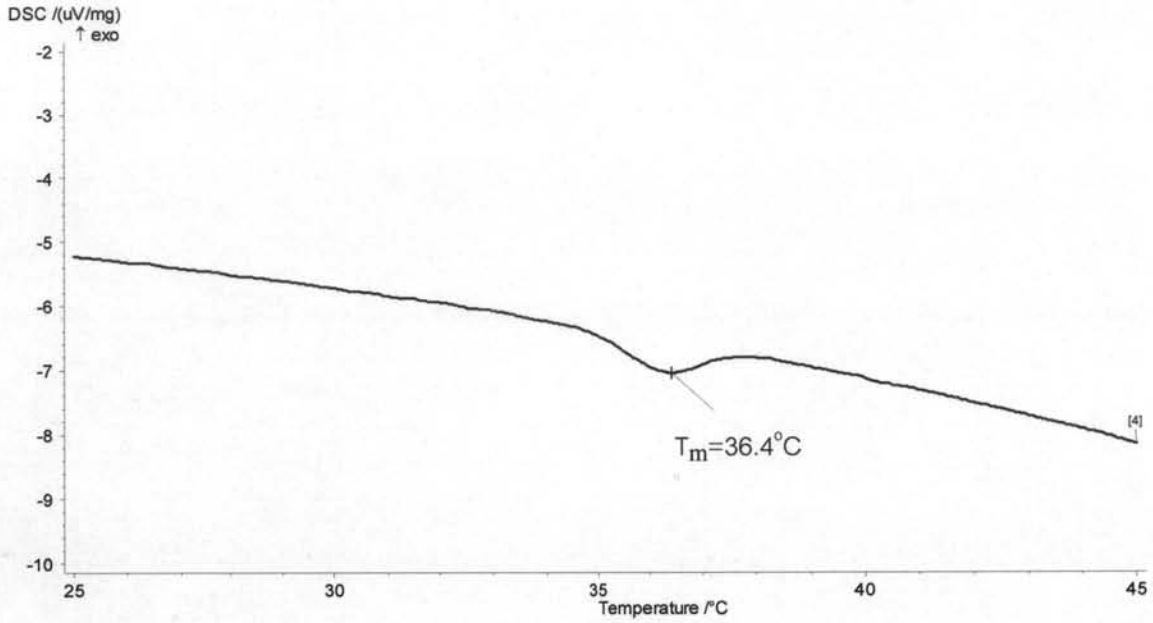

Figure 4.7. Thermal transition curve of giant gouramy skin collagen determined by DSC

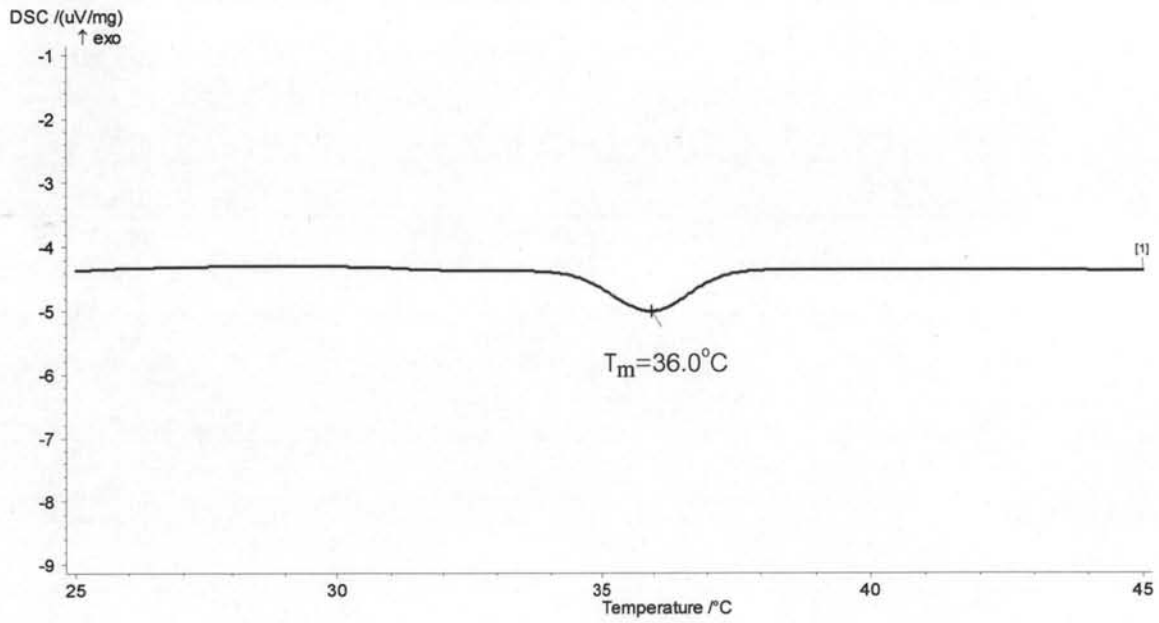

Figure 4.8 Thermal transition curve of giant gouramy scale collagen determined by DSC

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

Amino acid analysis of skin and scale collagen revealed a present of glycine at 29.74% and 34.51% respectively. Giant gouramy scale collagen contained higher level of proline alanine and glutamic acid residues (10.27%, 9.07% and 8.72% respectively) compared to skin collagen (9.12%, 8.08% and 7.86% respectively). Besides, skin and scale collagens of giant gouramy are also comprised of hydroxyproline, lysine and hydroxylysine (Table 4.2 and 4.3).

The amino acid composition of giant gouramy skin and scale collagen compared with that of bovine skin collagen were shown in Table 4.4

Table 4.5 demonstrated total imino acid and percent hydroxylation of collagen extracted from skin and scale giant gouramy

Table 4.2. Total amino acid composition of fish skin collagen

Amino acids	mg /100 mg
Hydroxyproline	6.41
Aspartic acid	4.14
Serine	2.40
Glutamic acid	7.86
Glycine	29.74
Histidine	1.42
Arginine	6.71
Threonine	2.14
Alanine	8.08
Proline	9.12
Tyrosine	0.81
Valine	1.64
Hydroxylysine	0.99
Isoleucine	0.92
Leucine	2.09
Lysine	2.74
Phenylalanine	1.96

Table 4.3. Total amino acid composition of fish scale collagen

Amino acids	mg /100 mg
Hydroxyproline	7.51
Aspartic acid	4.51
Serine	2.79
Glutamic acid	8.72
Glycine	34.51
Histidine	1.64
Arginine	7.42
Threonine	2.46
Alanine	9.07
Proline	10.27
Tyrosine	0.89
Valine	1.86
Hydroxylysine	1.26
Isoleucine	1.08
Leucine	2.36
lysine	3.07
Phenylalanine	2.21

Table 4.4 Amino acid composition of giant gouramy skin and scale compared with bovine skin collagen

Amino acids	mg / 1000 mg		
	Fish skin collagen	Fish scale collagen	Bovine skin collagen*
Hydroxyproline	64.1	75.1	129.1
Aspartic acid	41.4	45.1	40.3
Serine	24.0	27.9	2.4
Glutamic acid	78.6	87.2	18.4
Glycine	297.4	345.1	411.8
Histidine	14.2	16.4	10.1
Arginine	67.1	74.2	26.1
Threonine	21.4	24.6	10.9
Alanine	80.8	90.7	146.6
Proline	91.2	102.7	49.8
Tyrosine	8.1	8.9	2.7
Valine	16.4	18.6	17.1
Hydroxylysine	9.9	12.6	ND
Isoleucine	9.2	10.8	26.8
Leucine	20.9	23.6	37.1
Lysine	27.4	30.7	55.2
Phenylalanine	19.6	22.1	11.7
½ Cysteine	ND	ND	2.4

*(Lin and Liu ,2005)

Table 4.5 Total imino acid and % hydroxylation of giant gouramuy collagen.

	Fish skin	Fish scale
Total imino acid (%)	15.53	17.78
% Hydroxylation		
Proline	41.20	42.23
Lysine	26.54	29.10
Total hydroxylation	38.42	39.67

Effect of collagen on human dermal fibroblast cell culture

In order to determine the percent of serum growth factor and growth of human dermal fibroblast, the HDFs passages of 20^{th} which could represent the cell ages between 19^{th} and 24^{th} were tested in various concentrations of fetal bovine serum (FBS) (0%, 2%, 5% and 10% FBS). The number of viable cells which were represented by the $OD_{570/620}$ unit of sample at 6, 24, 48 and 72 hours was measured (Table 4.6). In addition, the absorbance indices for substrate coated and non-coated sample medium without cells hovered around 0.03, with no significant variation ($p>0.05$) (data not shown).

At 6 hours, the number of viable cells in 2% FBS was significantly higher than the cell in other serum concentrations ($p<0.05$). At 24, 48 and 72 hours, the number of HDFs in the presence of FBS were higher than those in serum-free media, significantly ($p<0.05$) (Figure 4.9 a & b)

Table 4.6. Average percent growth of 20^{th} passage HDFs cell culture at 6, 24, 48 and 72 hours from serum free medium

Serum concentration	6 hours	24 hours	48 hours	72 hours
0% FBS	100.00 ± 5.41	100.00 ±5.38	100.00 ±3.78	99.79 ±5.62
2%FBS	113.33 ±1.98	121.41 ±2.98	121.30 ±5.08	135.74 ±5.16
5%FBS	89.84 ±1.93	121.89 ±1.51	130.07 ±8.71	178.91 ±7.22
10%FBS	83.17 ± 4.67	115.08 ± 3.77	134.33 ±5.17	183.13 ±1.59

*Data are presented by percent change of standard medium (5% FBS) ± SE.

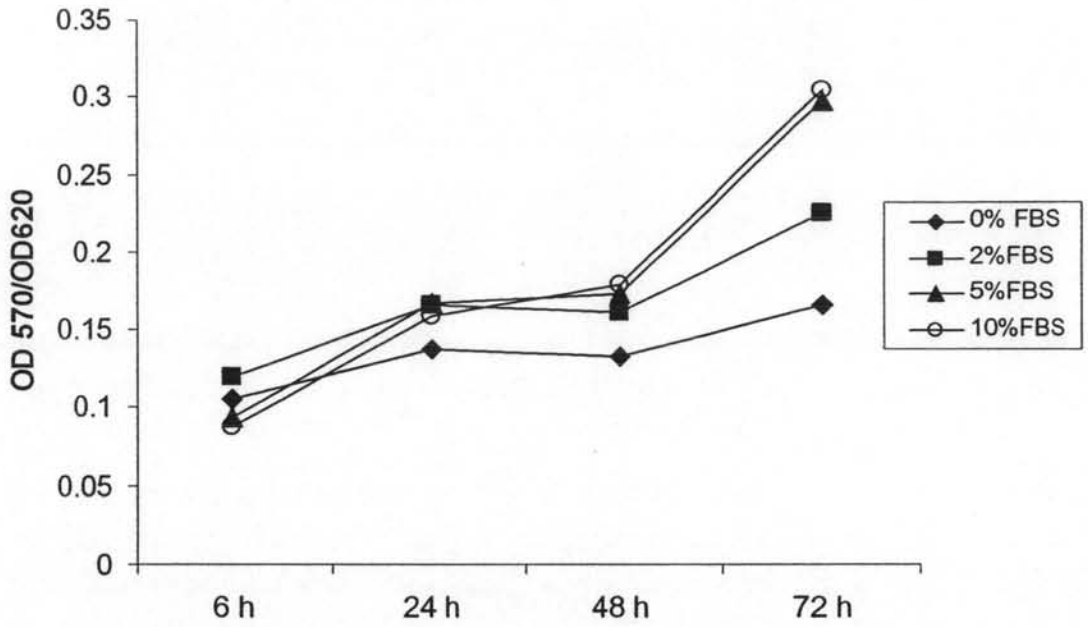

Figure 4.9a. Effect of serum concentration on the growth of 20th human dermal fibroblast

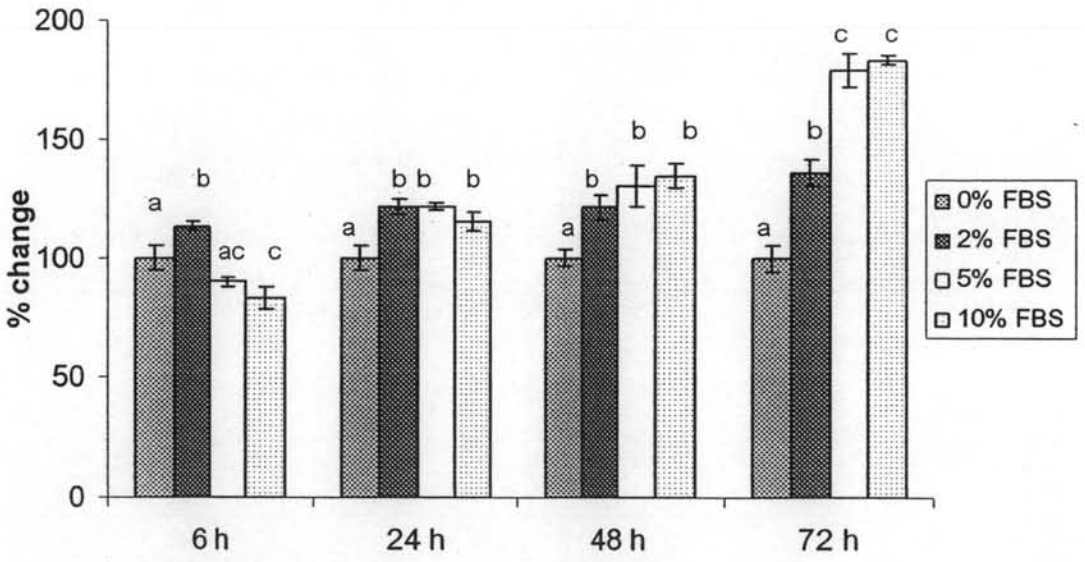

Figure 4.9b. Histogram represents the average percent changes of $OD_{570/620}$ ratio of effect of serum concentration on the growth of 20th human dermal fibroblast *

*The same alphabet refers to no significant differences between two data ($p>0.05$).
The different alphabet refers to significant differences between two data ($p<0.05$).

The adhesive and proliferative effect of collagen substrate on 19th passage of HDFs is maintaining in standard culture medium (DMEM media with of 5% FBS). The results revealed that percent cell adhesion (at 6 hr) were 124.1, 150.26, 135.38 and 135.38 for non-coated collagen, porcine tendon collagen, fish skin collagen and fish scale collagen versus the 5% FBS was 100% (Table 4.7). There was significant differences between number of cell in 5% FBS and serum free media (0% FBS), (Table 4.7). When compare with serum free media, the adhesion cell in porcine tendon collagen and fish skin collagen coated plate was higher than non-collagen coated plate significantly ($p<0.05$). However, there was no significant difference in number of cell adhering in fish skin and fish scale collagen coated plate ($p>0.05$) (Figure 4.10a & b).

After plating cell for 24 hours, the number of viable cell in 5% FBS was significantly higher than those in non-coated and porcine tendon collagen-coated plate ($p<0.05$). However, there were no significant difference between number of HDFs grew in fish collagens substrate and non-coated plate supplemented with 5% FBS ($p>0.05$) (Figure 4.10a & b).

In addition, at the time point later than 48 hours, the cell numbers of sample from 5% FBS supplementation were significantly higher than those in the other plates ($p<0.05$).

Table 4.7 Average percent growth of 19th passage HDFs cell culture at 6, 24 and 48 hours from standard culture medium*

	6 hours	24 hours	48 hours
Standard medium (5%FBS)	100.00 ± 5.02	100.00 ± 2.39	100.00 ± 1.68
Non-coated plate (NC)	124.10 ± 2.09	76.41 ± 0.87	59.25 ± 4.13
Porcine tendon collagen (PTC)	150.26 ± 2.54	74.80 ± 1.89	60.19 ± 3.81
Fish skin collagen (FK)	135.38 ± 2.61	86.90 ± 2.99	72.76 ± 5.21
Fish scale collagen (FC)	135.38 ± 3.32	86.09 ± 2.58	72.88 ± 3.21

*Data are presented by percent change of standard medium (5% FBS) ± SE.

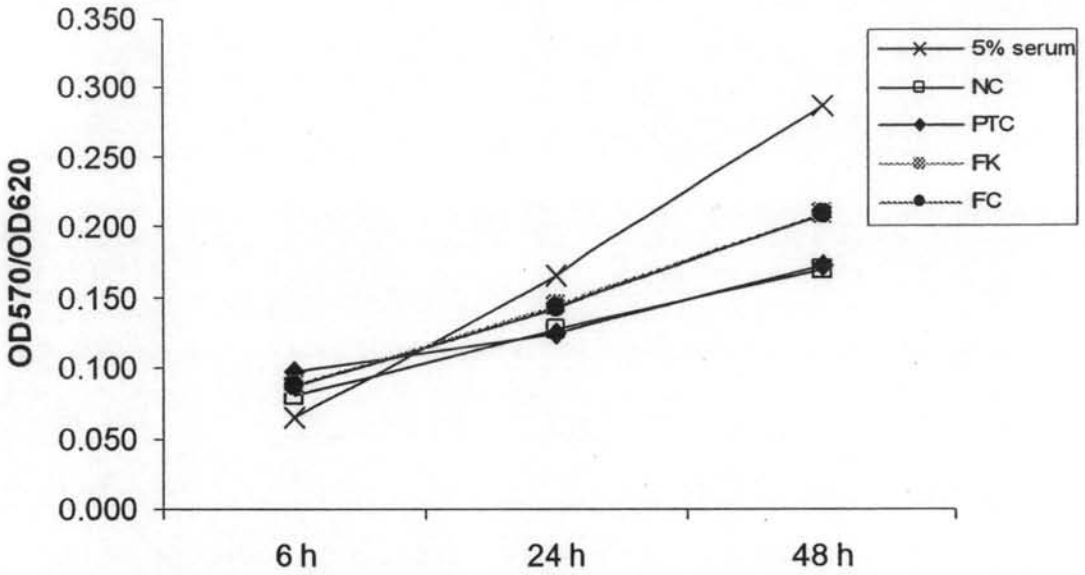

Figure 4.10a. MTT proliferation assay represented in $OD_{570/620}$ ratio of the sample from the 19th passage of HDFs at 6, 24 and 48 hours after plating into 48-well culture dishes (NC = non-coated plate; PTC = porcine tendon collagen-coated plate; FK = fish skin collagen coated plate; FC = fish scale collagen coated plate)

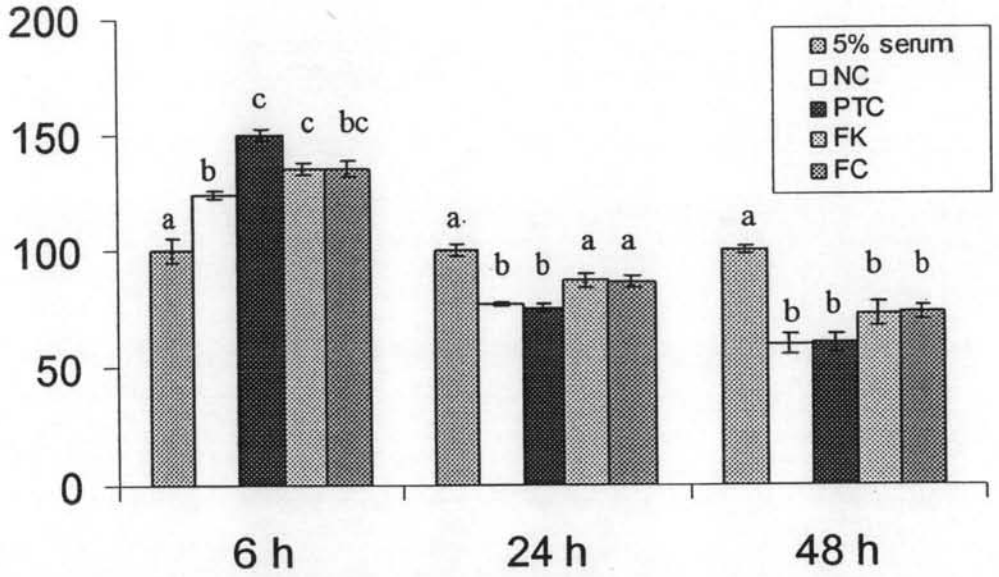

Figure 4.10b. Histogram represents the average percent changes of $OD_{570/620}$ ratio of the 19th passage of HDFs sample grew in collagen-coated plate containing serum-free culture medium from the 19th passage of HDFs sample grew in standard culture medium at 6, 24 and 48 hours after plating into 48-well culture dishes (NC = non-coated plate; PTC = porcine tendon collagen-coated plate; FK = fish skin collagen coated plate; FC = fish scale collagen coated plate)*

*The same alphabet refers to no significant differences between two data ($p>0.05$).
The different alphabet refers to significant differences between two data ($p<0.05$).

For 24th passage of HDFs, the number of viable cells in 5% FBS at 6 hours were lower than those in serum-free media significantly ($p<0.05$). There was no significant difference of the viable cell samples from non-coated and coated collagen plate (Figure 4.11 a & b). In addition, the number of cells at 24 hour from all of the collagen coated plates were higher than those of non-collagen coated plate or plate supplemented with 5% FBS significantly. Similar to the result of 19th passage, the numbers of HDFs grew in the serum medium were significantly higher than those grew in all of the serum free media with or without collagen coating at 48 hours ($p< 0.05$).

Table 4.8 Average percent growth of 24[h] passage HDFs cell culture at 6, 24 and 48 hours from standard culture medium*

	6 hours	24 hours	48 hours
Standard medium (5%BSA)	100.00 ± 2.79	100.00 ± 4.81	100.00 ± 4.10
Non-coated plate (NC)	145.16 ± 3.36	99.60 ± 4.15	70.16 ± 3.48
Porcine tendon collagen (PTC)	154.84 ± 4.27	113.64 ± 0.99	83.77 ± 4.14
Fish skin collagen (FK)	143.01 ± 5.99	115.81 ± 1.54	67.54 ± 4.51
Fish scale collagen (FC)	151.61 ± 1.61	113.44 ± 0.20	68.38 ± 0.99

*Data are presented by percent change of standard medium (5% FBS) ± SE.

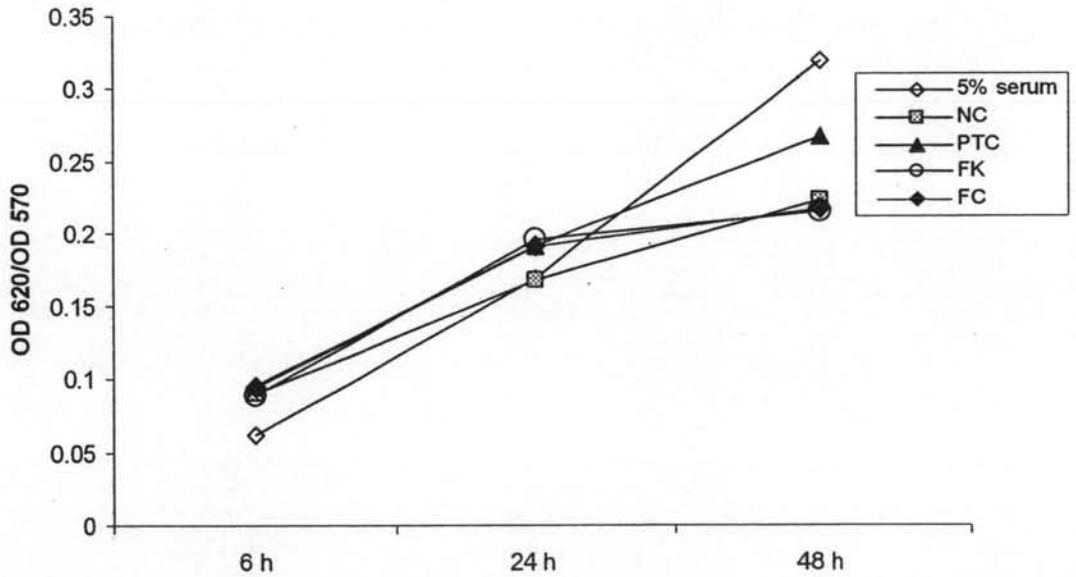

Figure 4.11a MTT proliferation assay represented in $OD_{570/620}$ ratio of the sample from the 24[th] passage of HDFs at 6, 24 and 48 hours after plating into 48-well culture dishes (NC = non-coated plate; PTC = porcine tendon collagen-coated plate; FK = fish skin collagen coated plate; FC = fish scale collagen coated plate)

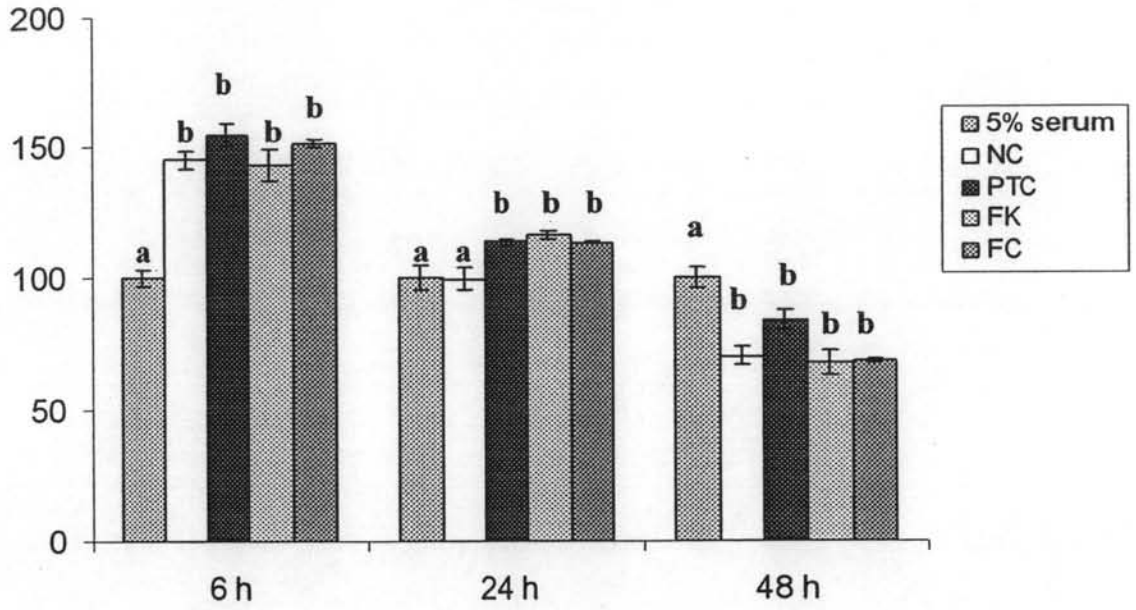

Figure 4.11b. Histogram represents the average percent changes of $OD_{570/620}$ ratio of the 24th passage of HDFs sample grew in collagen-coated plate containing serum-free culture medium from the 19th passage of HDFs sample grew in standard culture medium at 6, 24 and 48 hours after plating into 48-well culture dishes (NC = non-coated plate; PTC = porcine tendon collagen-coated plate; FK = fish skin collagen coated plate; FC = fish scale collagen coated plate)*

*The same alphabet refers to no significant differences between two data ($p>0.05$).
The different alphabet refers to significant differences between two data ($p<0.05$).