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Processing Optimization and Physicochemical Properties of Collagen from Skate (*Okamejei kenojei*) Skin

by

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Pukyong National University

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홍어 (*Okamejei kenojei*) 껍질 유래 콜라겐의 추출 최적화 및 물리화학적 특성 해석

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요 약

본 연구는 홍어 (*Okamejei kenojei*) 껍질로부터 반응표면분석이라는 통계기법을 이용하여 콜라겐의 제조 조건을 최적화하고 홍어 껍질 콜라겐의 물리화학적 특성을 육상동물 유래의 콜라겐과 비교하는데 그 목적이 있다. 제조 조건의 최적화에는 중심합성계획법이 사용되었으며 독립변수로는 NaOH 농도 (X_1), 처리 시간 (X_2), pepsin 처리 농도 (X_3) 및 추출 시간 (X_4)으로 정하였으며, 종속변수로는 콜라겐 수율 (Y)이 선택되었다. 다중반응최적화에 의해 산출된 독립변수의 최적화 조건은 NaOH 농도 0.45 N, 처리시간 23.4 hr, 효소 가수분해 농도 0.88%, 처리시간 46.8 hr로 나타났으며, 예상되는 collagen의 수율 (Y, %)은 6.94%로 실제 추출한 결과 6.56±0.3%과 큰 차이를 보이지 않았다. 홍어 껍질 콜라겐의 물리화학적 특성을 알아보기 위해 아미노산분석, SDS-PAGE, FT-IR, NaCl과 pH에 따른 용해도와 변성온도를 육상동물인 소 껍질에서 추출한 콜라겐과 비교한 결과, imino acid (Hyp+Pro)의 함량은 1,000 잔기 중 152이었으며, SDS-PAGE 결과 두

개의 α-chain (α_1 and α_2)과 β-component와 γ-component로 구성되어 있었는데, 우피 콜라겐과 비슷한 결과를 나타냈지만, 이동도가 약간 달랐다. FT-IR 분석결과 skate skin collagen과 calf skin collagen의 amide A, I, II 및 III 영역의 최대 peak band는 각각 3284 cm^{-1}, 3313 cm^{-1} (amide A), 1640 cm^{-1}, 1650 cm^{-1} (amide I), 1547 cm^{-1}, 1549 cm^{-1} (amide II) 그리고 1237 cm^{-1}, 1238 cm^{-1} (amide III)로 비슷한 파장에서 peak band를 나타내었다. NaCl 농도에 따른 용해도를 알아본 결과 2% 까지는 완만한 용해도의 감소를 보이다가 3% 이후 급격한 용해도의 감소를 보여 6-7%의 농도에서는 거의 0에 가까운 용해도를 나타내었으며, pH 농도에 따른 용해도의 영향을 알아본 결과, 용해도는 pH 6까지는 서서히 감소하다가 pH 7, 8, 9의 영역에서 가장 낮은 용해도를 나타내었고, pH 10에서 약간 증가하는 경향을 보였다. 홍어껍질 콜라겐의 변성온도를 측정한 결과 23℃로 나타났으며, 우피 콜라겐의 변성온도보다 약 10℃정도 낮게 나타났다.

I. Introduction

Collagen is the richest protein that possesses 20~30% of total protein of vertebrates (Harkness, 1961), and composes more than 70% of the dry weight of skin, tendon, and cartilage (Grant & Jackson, 1976).

Collagen has a diameter of 1.5 nm × 30 nm and the average 300 KDa of polypeptide in connected structure. Structure of collagen is the primary structure of polypeptide called α-chain which is triple spiral structure (Kalder et al., 1996; Nimni & Harkness, 1988), and the primary structure of α-chain has repeated sequence of -Gly-X-Y-. Glycine is placed in the middle of triple spiral structure, and by proline (Pro, P) on X and 4-hydroxyproline (Hyp, O) on Y, it is occupied alternately. 4-Hydroxyproline is crucial to stabilize the triple spiral structure (Bella et al., 2006; Myllyharju & Kivirikko, 2004; van der Rest & Garrone, 1991).

Type of collagen is categorized according to the distribution, structure, function to fibril-forming collagen (type I, II, III, V, XI, XXIV, and XXVII), network collagen (type IV, VIII, and X), filamentous collagen (type VI), and FACIT collagen (fibril-associated collagens with interrupted triple helices) (type IX, XII, XIV, XVI, XIX, XX, XXI, XXII, and XXVI), and it has been reported that there are 29 types currently (Bailey, 1998; Gelse et al., 2003; Myllyharju & Kivirikko, 2004; Veit et al., 2006; Söderhäll et al., 2007).

Collagen is being used in various industries like food, medicine, cosmetics (Wood et al., 2008). By adding it into meat, it improves food texture

(Light, 1987), and it has been used for preference to the casing of sausage (Hood, 1987). Also, by putting it on burns, it absorbs exudates of tissue, adequately conserves humid environment and helps regeneration of epithelium from injury (Chvapil, 1982). Type II collagen is placed in cartilaginous tissue, and it can diminish pain by restraining activity of articular rheumatism patient's T-cell (Li et al, 2006), and can prevent arthritis (Hu et al, 2003). Also, study and range of its use are getting extended to things like wrinkle control (Kwon et al., 2007), recovery and rebirth of blood vessel (Tonnesen et al., 2000), artificial internal organs of vessel or ligament (Lee et al., 2001).

On the other hand, currently circulating collagen products are originated from land animals like cow or pig, and because customer's apprehension about the hygiene safety from the danger of body transfer and foot-and-mouth disease (FMD) such as transmissible spongiform encephalopathies (TSE) and bovine spongiform encephalopathy (BSE) is socially rising, the study about new raw material with guaranteed natural safety is needed (Trevitt & Singh, 2003; Epstein, 2005; Helcke, 2000; Friess & Lee, 1996).

Skates are kinds of chondrichthyes that are widely distributed throughout worldwide sea areas from shallow shore to deep sea of over 3,000 meter, and there are about 280 kinds that have been reported (Jeong, 1999). Chondrichthyes like skates contain elements, ammonia, and trimethyl amine in its body which gives them unique smell (Cho et al., 2004a). Skates have about 30% of skin and bone (Cho et al., 2006), so it is becoming the reason for waste of resource and environmental pollution. Therefore, optimal

extraction of collagen using skin of skate is very meaningful.

There are many studies regarding characteristics of physicochemical properties about collagen on fish skin like mink whale (*Balaenoptera acutorostrata*) (Nagai et al., 2008), ocellate puffer fish (*Takifugu rubripes*) (Nagai et al., 2002), nile perch (*Lates niloticus*) (Muyonga et al., 2004), deep-sea redfih (*Sebastes mentella*) (Wang et al., 2008a), bronstripe red snapper (*Lutjanus vitta*) (Jongjareonrak et al., 2005), black drum (*Pogonias cromis*) and sheepshead seabream (*Archosargus probatocephalsu*) (Ogawa et al., 2003). Optimal studies using response surface methodology about central composite design are reported to be abstraction of collagen from yellowfin tuna (*Tunnus albacares*) dorsal skin (Woo et al.. 2008), abstraction of gelatin from yellowfin tuna (*T. albacares*) skin (Cho et al., 2005) and shark (*Isurus oxyrinchus*) cartilage (Cho et al., 2004b), etc.

The objectives of this study are to investigate the optimum condition for collagen extraction from skate (*Okamejei kenojei*) skin using response surface methodology by central composite design and to examine its characteristics of physical chemistry.

II. Materials and methods

1. Materials

In this experiment, skate skin which is by-product of processed skate was supplied by Songho Food Inc. (Busan, Korea). Skin has been washed, chopped into 1~2cm, and then frozen to -18℃ for storage. Also, to compare the characteristics of collagen extracted from skate skin with those of collagen originated from land animal, calf skin collagen (234112-250MG, collagen from calf skin, Calbiochem Co., U.S.A.) has been used, and all reagents used in this study were analytical grade for research.

2. Methods

2.1. Determination of proximate components

The measurements of general components for skate skin was done by the following methods: moisture was done by dry heating method, fat by Soxhelt method, protein by kjeldahl method, and ash content by dry-type method, and all these experiments were repeated three times.

2.2. Extraction of collagen from skate skin

The process of collagen abstraction was shown in Fig. 1. After washing and chopping, to remove protein except collagen from freed skate and to

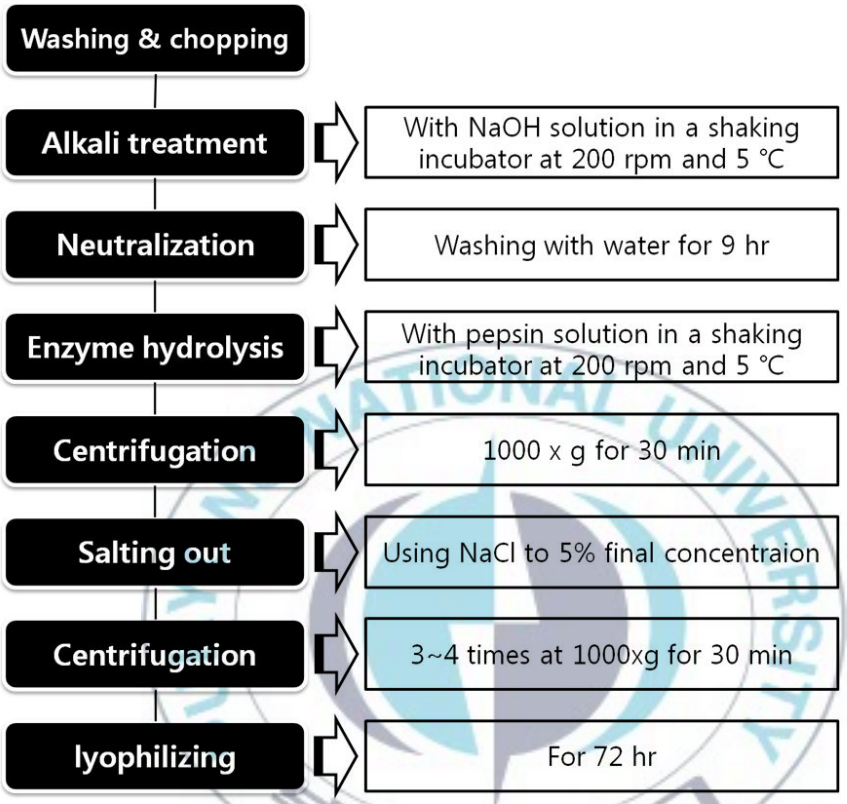

Fig. 4. Flow chart for collagen preparation from skate (*Okamejei kenojei*) skin.

swell the structure, with NaOH solution of 0.1, 0.2, 0.3, 0.4 and 0.5 N concentration in 10 times in a shaking incubator (HB-201SF, Hanbaek Scientific Co., Korea) at 200 rpm and 4℃, it was shaken for 12, 18, 24, 30 and 36 hours in 6 hour interval. After NaOH treatment, to neutralize its pH, it was washed and neutralized with water for 9 hours. Then, to extract collagen by enzyme hydrolysis, on top of 20 times material weight contrast of 0.5 N acetic acid solution, with pepsin solution of 0.7, 0.9, 1.1, 1.3 and 1.5% concentration in a shaking incubator at 200 rpm and 4℃, it was shaken and hydrolyzed for 24, 36, 48, 60 and 72 hours in 12 hour interval. For the abstracted solution, residue has been separated by using centrifugation at 10,000×g and 4℃ for 30 minutes. From 25% NaCl solution to 5% final concentration, the abstracted solution was salted out. After salting out, 6 N NaOH solution was treated and it was centrifuged at 10,000×g and 4℃ for 30 minutes to withdraw precipitate, and then distilled water was used for 30 minutes centrifugation at 10,000×g and 4℃. The experiments with distilled water were repeated 4 times to remove salt. The sample was vacuum lyophilized. All the process to abstract collagen was done under 10℃ to prevent heat degeneration.

2.3. Experimental design and data analysis

To optimize the best manufacturing condition of collagen, experiment section was established by central composite design (CCD) (Box & Wilson, 1951) (Table 1), and SAS (statistical analysis system 9.01) program was used for response surface methodology (RSM). Among collagen manufacturing conditions, total 4 independent variables have been selected,

Table 1. Experimental ranges and values of independent variables in central composite design for optimization of collagen processing from skate (*Okamejei kenojei*) skin

Independent variables	Symbols	Range and levels				
		-2	-1	0	1	2
NaOH concentration (N)	X_1	0.1	0.2	0.3	0.4	0.5
Treatment time (hr)	X_2	12	18	24	30	36
Additional ratio of pepsin (%)	X_3	0.7	0.9	1.1	1.3	1.5
Hydrolysis time (hr)	X_4	24	36	48	60	72

and each variable was designed to experiments in 5 ranges. It has been experimented in all 27 sections, and experimental plan was organized as 16 of factorial points, 8 of axial points (α = 2), and 3 times of central value.

Manufacturing process of collagen can be divided into two important processes: alkali (NaOH) treatment, and enzyme hydrolysis using pepsin. Therefore, independent variables have been decided as concentration of NaOH (N, X_1), its processing time of NaOH (hr, X_2), concentration of material weight contrast pepsin (%, X_3) and its processing time (hr, X_4). Ranges and levels of each variable were shown in 5 different sections according to the following formula (1).

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (1)$$

where x_i is coded value of independent variable, and X_i is practical value of independent variable. X_0 is central value of actual independent variable, and $\triangle X_i$ is showing the change range of X_i.

Central value and range of independent variable in this study was determined according to the result of preliminary experiment, and changes of subordinating variable in independent variable were measured.

Based on the result of response surface analysis using RSREG procedure of SAS system (Version 9.01, SAS Institute Inc., U.S.A.), adequate response surface model was found in 95% of considerate standard (2).

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \qquad (2)$$

Where Y, subordinate variable, is rate percentage of collagen, and β_0 is invariable, β_i, β_{ii}, β_{ij} are regression coefficients, and X_i, X_j is level of independent variables.

Using this, response surface plot was shown as a three dimensional graph in Maple software (Maple 11, Waterloo Maple Inc., Canada), and when influence of two independent variables is needed to be shown, the amounts of other two independent variables that were not shown in the graph was set in an optimal condition.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was practiced with a little modified version of Laemmli (1970) method. Six percent separating gel which was added with 30% of 2 mL acrylamide, 2.5 mL of 0.75 M Tris-HCI (pH 8.8) buffer, 4.9 mL of distilled water, 10% of 0.1 mL SDS solution, 10% of 0.5 mL ammonium persulfate and 10 uL of TEMED, was charged onto glass plate for slab gel, isopropyl alcohol was added a little bit on upper part, and then it was left on the room temperature to polymerize to high molecules. Then, after charging 5% stackiong gel executed using 30% of 0.83 mL acrylamide, 0.25 M of 1.26 mL Tris-HCI (pH 6.8) buffer, 2.8 mL of distilled water, 10% of 0.05 mL SDS solution, 10% of 0.05 mL ammonium persulfate, and 10 uL of TEMED, it was left on the room temperature to polymerize to high

molecules. Sample was made to the concentration of 10 mg/ml, then after mixing it with tracking dye which was made with 0.25 M of 1 mL Tris-HCI (pH 6.8) containing 10% SDS, 0.4 mL of 2-mercaptoethanol, 50% of 3.2 mL glycerol, 1% of 1 mL bromophenol blue, 2.4 mL of distilled water in 1:1 ratio, it was heated at 100℃ for 5 minutes. After heating it, the mixed sample solutions were loaded onto each gel and elctrophoresed in a slab gel of 10 mA/gel. Dyeing of gel after electrophoresis was 0.25% (w/v) of coomassie brilliant blue R-250, and pre-stained marker (BioPrince™) for broad-range protein was compared with calf skin collagen (type I), and then the amount of the molecule was measured with GL-100 (Kodak, Japan).

2.5. Amino acid analysis

Amino acid was analyzed by HPLC method with AccQ-Tag which is modified from the method of Wang et al. (2008b). After putting 1 mg of sample into an ampule, 200 uL of 6 N HCI + 1% phenol was added, and after filling the ampule with nitrogen, seal it quickly. It is left to react in 105℃ oven for 20 hours. After reaction, it was melted with 50 uL of borate buffer from waters accQ-flour reagent kit and it was diluted. It was analyzed using amino acid analyzer (Waters 2690, Waters 747, USA).

2.6. FT-IR (Fourier transform infrared) spectroscopy

FT-IR (Fourier transform infrared) spectroscopy was measured by Woo et al. (2008) method. Using FT-IR spectrophotometer (Bruker, IFS88, Germany)

to measure 2 cm^{-1} of data acquisition rate from 600 to 4000 cm^{-1}, collagen from skate skin was compared with calf skin collagen (type 1) from land animal.

2.7. Effect of NaCl concentration on collagen solubility

Collagen solubility on NaCl concentration was measured by a little modified method from Montero et al. (1991). In 0.1 N acetic acid, skate skin collagen and calf skin collagen were made in 1 mg/mL concentration, respectively. The solution was melt by using the condition of 200 rpm and 4℃ in a shaking incubator (HB-201SF, Hanbaek Scientific Co., Korea). In 5 mL of 0.1 N acetic acid, NaCl was melted in for 0%, 2%, 4%, 6%, 8%, 10%, 12%, and 14% (w/v), 5 mL of melted collaged solution was added, and the final concentration of NaCl in each solution was made to 0%, 1%, 2%, 3%, 4%, 5%, 6%, and 7%. This solution was slowly stirred at 4℃ for 30 minutes and centrifuged at 10,000×g and 4℃ for 30 minutes with a centrifuge (SUPRA 30K, Hanil Co., Korea). The protein concentration of the upper solution was measured using SMART BCA protein assay kit (iNtRON Biotechnology Inc., Korea). For the solubility, when the protein concentration at 0% NaCl concentration is assumed to be 100 protein concentration, the protein concentration at each NaCl concentration was relative value to the protein concentration of 100 at 0% NaCl concentration.

2.8. Effect of pH on collagen solubility

Collagen solubility on pH was measured by a little modified method from

Montero et al. (1991). In 0.1 N acetic acid, skate skin collagen and calf skin collagen were made in 1 mg/mL concentration, respectively. The solution was melt by using the condition of 200 rpm and 4℃ in a shaking incubator (HB-201SF, Hanbaek Scientific Co., Korea). In 5 mL of melted collagen solution, using 0.1 N NaOH and 0.1 N HCI, the collagen solution was adjusted to the several pHs from 3 to 10 and it was made to the final volume up to 10 mL with distilled water. This solution was slowly stirred at 4℃ for 30 minutes, and was centrifuged at 10,000×g and 4℃ for 30 minutes with a centrifuge (SUPRA 30K, Hanil CO., Korea). The protein concentration of the upper solution was measured using SMART BCA Protein Assay kit. For the solubility, when the protein concentration at pH 3 was assumed to be 100, the protein concentration at each pH was relative value to the protein concentration of 100 at pH 3.

2.9. Degeneration temperature for relative viscosity

Degeneration temperature of collagen was measured by a little modified method from Woo et al. (2008). It was analyzed by difference between temperature change and relative viscosity. Viscosity was measured using concentric cylinder geometry and cylinder sensor (Z20 DIN Ti, Haake Co., Ltd., Germany) of rheometer. After melting skate skin collagen and calf skin collagen into 0.1 N acetic acid at 4℃ and 6 mg/mL concentration, respectively, it was observed from 3 Pa, gap 4.2 mm at 15℃ to 0.5℃/min at 40℃. An analysis of relative viscosity was shown as 100 at 15℃ and denaturation temperature was 1/2 point of the section where change in viscosity can abruptly diminish.

III. Results and discussion

1. Determination of proximate components

The analytic result of general components of skate skin used in this study was shown in Table 2. Among general components, moisture content occupied 66.5%, protein with collagen occupied 30.2%, and dry weight of crude protein occupied such high content as 90.1%. And since content of impurities like lipid and ash, are low, 2.1% and 0.5% each, skate skin is considered as suitable material for collagen abstract.

2. Optimal manufacturing condition for collagen

2.1. Statistic analysis of collagen manufacture

It was experimented in total 27 sections, and subordinate variable (Y, %) of each section was shown in Table 3. Data from the experiment was put in adequate quadratic polynomial model based on response surface methodology result using RSREG procedure of SAS software.

To check significance of linear term (X_1, X_2, X_3, X_4), secondary term (X_{11}, X_{22}, X_{33}, X_{44}) and all variable terms related to cross-term, t-statistic and each conjecture coefficient model were shown in Table 4. X_1 (P = 0.0109), X_3 (P = 0.0225) of linear coefficient showed significance on $P < 0.05$ level; however, X_2 (P = 0.9833) and X_4 (P = 0.0971) did not show significance on $P > 0.05$. Also, when all the secondary term showed significance on as high level as $P < 0.0001$, all the cross-term did not show any significance

Table 2. Proximate composition of raw skate (*Okamejei kenojei*) skin

Component	Content (%)[a]
Moisture	66.5±1.5
Crude protein	30.2±0.5 (90.1±1.5)
Crude lipid	2.1±0.9 (6.3±2.7)
Crude ash	0.5±0.2 (1.5±0.6)

[a] The value is the average value and standard deviation of 3 time replications and the value of parenthesis is the one of dry basis.

Table 3. Central composite design and responses of the dependent variables for collagen processing from skate (*Okamejei kenojei*) skin to the independent variables

Run No.	Coded levels of variables[a]				Response
	X_1	X_2	X_3	X_4	Y[a]
1	-1	-1	-1	-1	1.62
2	-1	-1	-1	+1	1.49
3	-1	-1	+1	-1	1.15
4	-1	-1	+1	+1	0.74
5	-1	+1	-1	-1	2.43
6	-1	+1	-1	+1	2.64
7	-1	+1	+1	-1	1.55
8	-1	+1	+1	+1	0.81
9	+1	-1	-1	-1	3.11
10	+1	-1	-1	+1	3.24
11	+1	-1	+1	-1	2.91
12	+1	-1	+1	+1	1.69
13	+1	+1	-1	-1	2.97
14	+1	+1	-1	+1	3.04
15	+1	+1	+1	-1	2.50
16	+1	+1	+1	+1	1.69
17	-2	0	0	0	2.09
18	+2	0	0	0	1.96
19	0	-2	0	0	1.76
20	0	+2	0	0	0.95
21	0	0	-2	0	1.69
22	0	0	+2	0	1.76
23	0	0	0	-2	3.38
24	0	0	0	+2	2.30
25	0	0	0	0	6.94
26	0	0	0	0	6.77
27	0	0	0	0	6.97

[a]*Y*, X_1, X_2, X_3, and X_4 are collagen yield (%), NaOH concentration (N), NaOH treatment hour, additional ratio of pepsin (%), and extraction hour.

Table 4. Estimated coefficients of the fitted quadratic polynomial equation for the response of Y[a] (collagen yield, %) based on t-statistic

Parameter[a]	Parameter estimate	Standard error	*T*-value	*P*-value
Intercept	6.883333	0.331414	20.77	0.0001
X_1	0.352500	0.117172	3.01	0.0109
X_2	0.002500	0.117172	0.02	0.9833
X_3	-0.306667	0.117172	-2.62	0.0225
X_4	-0.210833	0.117172	-1.80	0.0971
X_1X_1	-1.195833	0.124280	-9.62	0.0001
X_1X_2	-0.198750	0.143506	-1.38	0.1913
X_1X_3	0.022500	0.143506	0.16	0.8780
X_1X_4	-0.047500	0.143506	-0.33	0.7464
X_2X_2	-1.363333	0.124280	-10.97	0.0001
X_2X_3	-0.097500	0.143506	-0.68	0.5098
X_2X_4	0.022500	0.143506	0.16	0.8780
X_3X_3	-1.270833	0.143506	-0.33	0.0001
X_3X_4	-0.216250	0.143506	-1.51	0.1577
X_4X_4	-0.992084	0.124280	-7.98	0.0001

[a]*Y*, X_1, X_2, X_3, and X_4 are collagen yield (%), NaOH concentration (N), NaOH treatment hour, additional ratio of pepsin (%), and extraction hour.

on P > 0.05.

Response surface model equation was shown in Table 5 except 8 units that did not approve significance in 95% level. Decision coefficient R^2 from independent variable of collagen was 0.9481 and P < 0.0001.

By analysis of variance (ANOVA), statistic significance of quadratic polynomial model was evaluated. Through ANOVA, significance of response model of Y (yield, %) according to subordinate variable was shown on Table 6. According to the result of ANOVA, linear term (X_1, X_2, X_3, X_4, P = 0.0154) and secondary term (X_{11}, X_{22}, X_{33}, X_{44}, P < 0.0001) except cross-term (P = 0.5872) approved significance in over 95% level.

2.2. Optimization of collagen manufacture

To find out the optimum manufacturing condition of skate skin collagen, central value was decided by central composite design (CCD; Box & Wilson, 1951). NaOH concentration (0.3 N, X_1), NaOH treatment time (24 hr, X_2), enzyme concentration (1.1%, X_3), and enzyme treatment time (48 hr, X_4) were shown as 4 independent variables, and the central values and ranges were determined through the preliminary experiment (Table 1).

The optimum coded values from response surface analysis, X_1 = 0.15, X_2 = -0.01, X_3 = -0.11, and X_4 = -0.10, into equation (1) were substituted to get the uncoded values. The uncoded values from the result of RSREG procedure were X_1 = 0.45 N, X_2 = 23.4 hr, X_3 = 0.88%, and X_4 = 46.8 hr. When it was done by the uncoded values, the expected value for collagen ratio (Y, yield, %) was 6.94% (Table 7), and actual manufactured result was 6.56%, showing about 0.3% lower than expected, which is not a big difference (Table 8).

Table 5. Response surface model of processing conditions on collagen yield from skate (*Okamejei kenojei*) skin

Response	Quadratic polynomial model[a]	R^2	*P*-value
Collagen Yield (%)	$Y = 6.883 + 0.353X_1 - 0.3067X_3 - 1.196X_1^2 - 1.363X_2^2 - 1.271X_3^2 - 0.992X_4^2$	0.9481	0.0001

[a]*Y*, X_1, X_2, X_3, and X_4 are collagen yield (%), NaOH concentration (N), NaOH treatment hour, additional ratio of pepsin (%), and extraction hour.

Table 6. Analysis of variance (ANOVA) for response of the dependent variable

Sources	DF[a]	SS[a]	MS[a]	*F*-value	*P*-value
Regression					
Linear	**4**	**6.3062**	**0.0827**	**4.78**	**0.0154**
Quadratic	**4**	**64.4009**	**0.8446**	**48.86**	**0.0001**
Cross-product	**6**	**1.5847**	**0.0208**	**0.80**	**0.5872**
Total model	**14**	**72.2917**	**0.9481**	**15.67**	**0.0001**
Residual					
Lack of fit	**10**	**3.9330**	**0.3933**	**37.34**	**0.0264**
Pure error	**2**	**0.0467**	**0.010533**	**-**	**-**
Total error	**12**	**3.9541**	**0.329505**	**-**	**-**
Total	**26**	**76.2458**		**-**	**-**
Factors[b]					
X_1	**5**	**34.1654**	**6.8331**	**20.74**	**<0.0001**
X_2	**5**	**40.4442**	**8.0888**	**24.55**	**<0.0001**
X_3	**5**	**37.6192**	**7.5238**	**22.83**	**<0.0001**
X_4	**5**	**22.8561**	**4.5712**	**13.87**	**0.0001**

[a]DF, SS, and MS mean degrees of freedom, sum of square, and mean of sum of square.

[b]X_1, X_2, X_3, and X_4 are NaOH concentration (N), NaOH treatment hour, additional ratio of pepsin (%), and extraction hour.

Table 7. Optimum condition of collagen processing from skate (*Okamejei kenojei*) skin

Dependent variable[a]	Independent variables[a]	Critical value		Predicted value	Stationary point
		Coded	Uncoded		
Y	X_1	0.15	0.45	6.94	Maximum
	X_2	-0.01	23.4		
	X_3	-0.11	0.88		
	X_4	-0.01	46.8		

[a]*Y*, X_1, X_2, X_3, and X_4 are collagen yield (%), NaOH concentration (N), NaOH treatment hour, additional ratio of pepsin (%), and extraction hour.

Table 8. Comparison of experimental and predicted results of the collagen yields for verification under the optimized condition

Dependent variables	Predicted value	Experimental value
Y (collagen yield, %)	**6.94**	**6.56±0.30**

The optimized condition is 0.45 N NaOH concentration, 23.4 treatment hours, 0.88 % additional ratio of pepsin, 46.8 extraction hours.

The effects of independent variables (X_1, X_2, X_3, X_4) on the dependent variable (Y, %) were shown in three dimensional graphs using regression equation from the result of response surface methodology with Maple software (Maple 11, Waterloo Maple Inc., Canada) (Fig. 2~4). When the effects of only two independent variables were shown, the values from other two independent variables shown on the graph were those at the optimal condition. The graph (A) shows the relationship between X_1 (NaOH concentration) and X_2 (NaOH treatment time). The graph (B) shows the relationship between X_1 (NaOH concentration) and X_3 (ratio of pepsin). The graph (C) shows the relationship between X_1 (NaOH concentration) and X_4 (pepsin treatment time). The graph (D) shows the relationship between X_2 (NaOH treatment time) and X_3 (ratio of pepsin). The graph (E) shows the relationship between X_3 (ratio of pepsin) and X_4 (pepsin treatment time). The graph (F) shows the relationship between X_2 (NaOH treatment time) and X_4 (pepsin treatment time). From the results on the response surface graphs, as all coded values get closer to 0, collagen yield (Y, %) gets higher. More X_1 (NaOH concentration) than X_4 (pepsin treatment time), more X_2 (NaOH treatment time) than X_1 (NaOH concentration), more X_3 (pepsin ratio) than X_2 (NaOH treatment time) had more influence on collagen yield. The factor of more X_3 (pepsin ratio) than X_2 (NaOH treatment time) had the most influence on collagen yield. Thus, when extracting collagen, adjusting X_3 (ratio of pepsin) adequately must be the most important factor among all other factors.

3. Physicochemical characteristics of the skate skin collagen

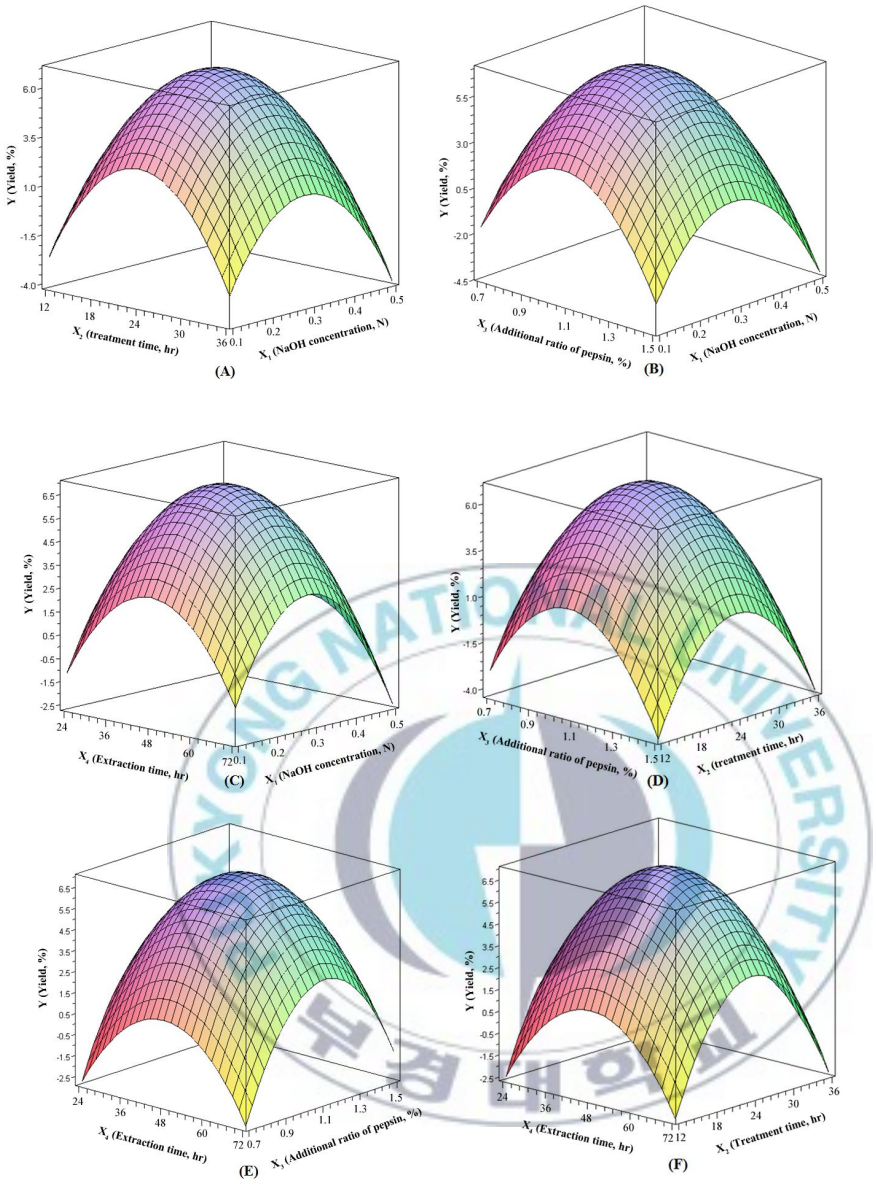

Fig. 2. Response surface plots for optimization of collagen extraction from skate (*Okamejei kenojei*) skin.

Y means collagen yield (%); X_1 is NaOH concentration (N) and X_2 is treatment hour for alkali (NaOH) treatment; X_3 is additional ratio of pepsin (%) and X_4 is extraction hour for enzymatic treatment.

3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Fig. 3 is the result of SDS-PAGE with skate skin collagen (B), compared with protein marker (A) and calf skin collagen (C). Skate skin collagen had α_1-chain of 126 KDa molecular weight and α_2-chain of 110 KDa, β -component (cross-linked dimer of α-chain) of 227 KDa, γ-component (cross-linked trimer of α-chain) of 367 KDa. As the amounts of β- and γ -components were bigger, the amount of cross-linking that has heat-stable was bigger (Love et al., 1976).

Band mobilities of skate skin and calf skin collagens were slightly different, and α_1-chain had a little stronger characteristics than α_2-chain. During electrophoresis, α_1-chain and α_2-chain have the same mobilities, so the bands appears on the same place. The α_1-chain band is stronger than the α_2-chain (Kimura et al., 1988; Nagai et al., 2004).

Collagens extracted from minke whale (*B. acutorostrata*) (Nagai et al., 2008), nile perch (*L. niloticus*) skin (Muyonga et al., 2004) and cuttlefish (*Sepia lycidas*) skin (Nagai et al., 2001) had α_1- and α_2-chains, and β-component. Collagens extracted from yellowfin tuna (*T. albacares*) dorsal skin (Woo et al., 2008), brownstripe red snapper (*L. vitta*) skin (Jongjareonrak et al., 2005), black drum (*P. cromis*) skin, sheepshead seabream (*A. probatocephalsu*) skin (Ogawa et al., 2003) and brown backed toadfish skin had α_1- and α_2-chains, and β- and γ-components like the skate skin collagen. Also, collagens from Japanese sea bass (*Lateolabrax japonicus*) caudal fin collagen (Nagai, 2004), yellow sea bream and horsemackerel bone had only α_1-chain and β-component. This reason is that there are much components that are cross-linked in and out of the molecule

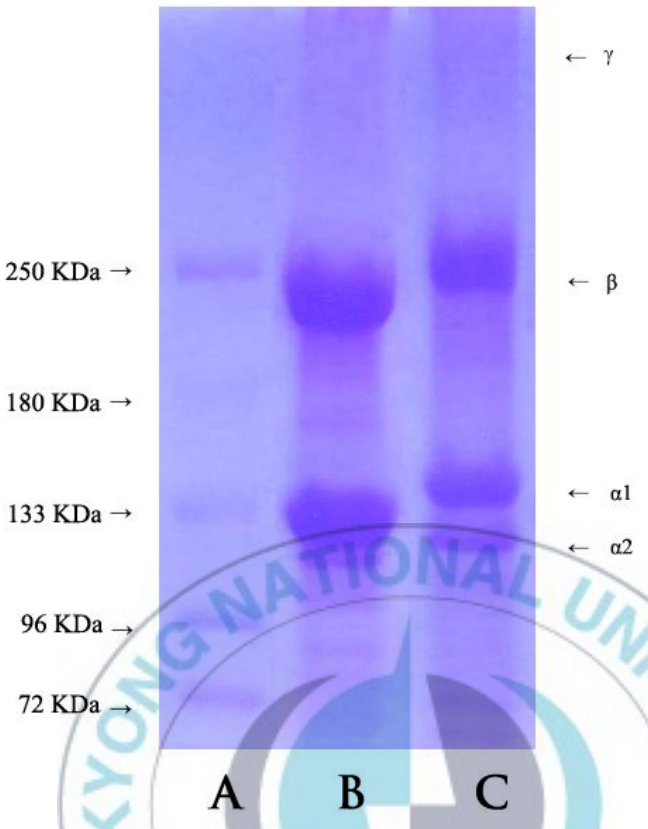

Fig. 3. SDS-polyacrylamide gel electrophoresis of the collagens from skate (*Okamejei kenojei*) and calf skins.

A, B, and C are protein marker, skate (*Okamejei kenojei*) skin collagen, and calf skin collagen, respectively.

(Nagai & Suzuki, 2000).

3.2. Amino acid composition

The analytic result of amino acid from skate and calf skin collagens are shown in Table 9. The result of the skate skin collagen was similar to that of calf skin collagen. Their contents of glycine, glutamic acid, arginine, proline, hydroxyproline were high, but their contents of histidine, methionine, tyrosine were relatively low. Also, the content of essential amino acids (valine, leucine, isoleucine, methionine, threonine, lysine, phenylalanine) from skate skin collagen was 223 per 1,000 amino acid residual, so the skate skin collagen has higher nutritional value than 164 of calf skin collagen.

Glycin content in the skate skin collagen was 217 per 1,000 amino acid residual; its hydroxyproline content was 61 per 1,000 amino acid residual: and the content of imino acids (proline+hydroxyproline) was 152; therefore, compared with 226 of calf skin collagen, it was 74 lower. Content of imino acids plays the crucial influence on quality of the gelatin. As the content of imino acid is high, viscoelasticity and gel intensity increase (Gómez-Guillén et al., 2002), and pyrrolidine ring of proline and hydroxyproline helps triple helix structure of collagen get stronger (Riesle et al., 1998).

Content of imino acids in flat fish skin collagen was 163 per 1,000 residual (Giraud-Guille et al., 2000); their content of brown backed toadfish (*L. gloveri*) skin collagen was 170 (Senaratne et al., 2006); their content of yellowfin tuna (*T. albacares*) dorsal skin collagen was 205 (Woo et al., 2008); their contents of acid-soluble and pepsin-solubilized collagens of

Table 9. Amino acid composition of the collagens from skate (*Okamejei kenojei*) and calf skins

(residues/1,000 residues)

Amino acid	Skate skin collagen	Calf skin collagen
Hydroxyproline	61	93
Aspatic acid	57	57
Threonine	38	19
Serine	56	32
Glutamic acid	106	101
Proline	91	133
Glycine	217	229
Alanine	75	93
Valine	33	23
Isoleucine	21	23
Leucine	43	33
Tyrosine	13	10
Phenylalanine	26	21
Lysine	38	37
Histidine	15	6
Arginine	87	84
Methionine	24	8
Imino acid[a]	**152**	**226**
Essential amino acid[b]	**223**	**164**

[a] Imino acids mean proline and hydroxyproline.

[b] Essential amino acids mean valine, leucine, isoleucine, methionine, threonine, lysine, and phenylalanine.

black drum (*P. cromis*) skin was 199.8 and 197.1, respectively; their contents of acid-soluble and pepsin-solubilized collagens of sheepshead seabream (*A. probatocephalsu*) skin was 205.1 and 198.1, respectively, (Ogawa et al., 2003); and contents of imino acids in acid soluble collagen of young and adult nile perch (*L. niloticus*) skin were 193 and 200, respectively (Muyonga et al., 2004).

3.3. FT-IR (Fourier transform infrared) spectroscopy

Fig. 4 is the result of FT-IR spectra about skate and calf skin collagens. Fourier transform infrared (FT-IR) is used to study a secondary structure of collagen and gelatin. Amide A, I, II, III areas of spectrum are directly related to polypeptide form, and also related to cross-linked and degeneration temperature of collagen (Paschalis et al., 2001; Friess & Lee, 1996). Peak of amide A (3200-3600 cm^{-1}) area is related to N-H stretching vibration which is linked to hydrogen bond (Sai & Babu, 2001); peak band of amide I (1600-1700 cm^{-1}) area is related to stretching vibrations of carbonyl group in peptide and is also useful when researching about a secondary structure of protein. Also, peak band of amide II (1500-1600 cm^{-1}) area is related to N-H bending and C-N stretching; peak band of amide III (1200-1300 cm^{-1}) appears by C-N and N-H stretching, and is related to the triple spiral structure of collagen (Table 10) (Kong & Yu, 2007; Krimm & Bandekar, 1986; Jakobsen et al., 1983; Abe & Krimm, 1972).

As a result of FT-IR, the highest peak bands of amide A, I, II, II area of skate and calf skin collagens were 3284 cm^{-1}, 3313 cm^{-1} (amide A),

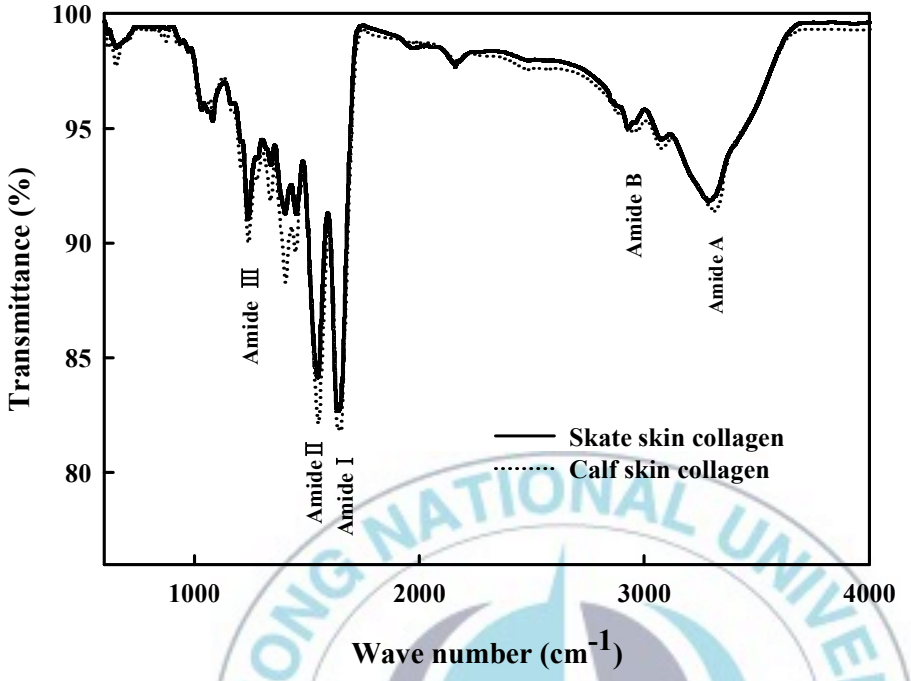

Fig. 4. FT-IR spectra of the collagens from skate (*Okamejei kenojei*) and calf skins.

The regions were amide A (3200-3600 cm^{-1}), amide B (2500-3200 cm^{-1}), amide I (1600-1800 cm^{-1}), amide II (1500-1600 cm^{-1}), and amide III (1200-1300 cm^{-1}).

Table 10. Peak positions and assignments of FT-IR spectra for the collagens from skate (*Okamejei kenojei*) and calf skins

Region	Peak wave number (cm^{-1})		Assignment	Reference
	Skate skin	Calf skin		
Amide A	3284	3313	NH stretch, coupled with hydrogen bonding	Sai & Babu (2001)
-	2925	2930	CH_2 asymmetrical stretch	Abe & Krimm (1972)
Amide I	1640	1650	C=O stretch/hydrogen bonding coupled with COO-	Jackson et al. (1995)
Amide II	1547	1549	NH bend coupled with CN stretch	Jackson et al. (1995)
-	1451	1450	CH_2 bend	Jackson et al. (1995)
-	1402	1403	CH_2 wagging of proline	Jackson et al. (1995)
Amide III	1237	1238	NH bend	Jackson et al. (1995)
-	1080	1082	C-O stretch	Jackson et al. (1995)

1640 cm^{-1}, 1650 cm^{-1} (amide I), 1547 cm^{-1}, 1549 cm^{-1} (amide II) and 1237 cm^{-1}, 1238 cm^{-1} (amide III), respectively, and they showed peak bands at the similar wavelengths. The highest peak bands of amide A, I, II, III of yellowfin tuna (*T. albacares*) dorsal skin collagen were 3424, 1651, 1544, and 1240 cm^{-1}, respectively (Woo et al., 2008), and the highest peak bands of amide A, I, II, III of deep-sea redfish (*S. mentella*) skin, scale, bone collagens were 3425, 1658, 1552, and 1240 cm^{-1} for skin; 3296, 1653, 1541, and 1242 cm^{-1} for scale; 3300, 1654, 1541, and 1240 cm^{-1} for bone (Wang et al., 2008a). The highest peak bands of amide A, I, II, III of young nile perch (*L. niloticus*) skin collagen were 3434, 1650, 1542, and 1235 cm^{-1}, respectively, and for adult nile perch skin collagen, they were 3458, 1654, 1555, and 1238 cm^{-1}, respectively (Muyonga et al., 2004).

3.4. Effect of NaCl concentration on collagen solubility

Fig. 5 is the result of effects of solubility on NaCl concentration of skate and calf skin collagens. Solubilities of skate and calf skin collagens showed the similar tendency up to 2% and were slowly decreased. After 3%, they decreased rapidly and at 6~7%, they almost hit 0.

Protein solubility is related to hydrophilic and hydrophobic interactions, and solvent in food is mostly water (Pelegrine & Gasparetto, 2005). Generally, to provide emulsifying, gel, and foam capacities that affect food quality, protein with high solubility is being used. Therefore, protein gives bad influence on food quality, when its solubility is low (Nakai & Li-chan, 1985; Vojdani, 1996).

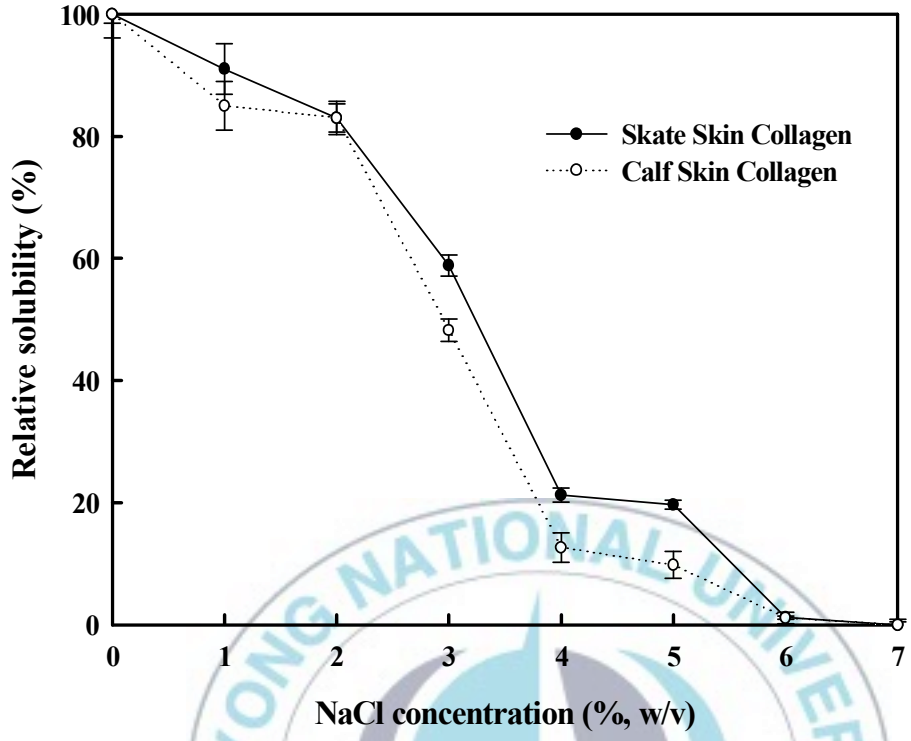

Fig. 5. Effect of NaCl concentration on relative solubilities of the collagens from skate (*Okamejei kenojei*) and calf skins.

The reason on decrease of collagen solubility by NaCl concentration is thought to be due to salting out phenomenon. As NaCl concentration gets higher, collagen solubility gets lower. This reason is because protein precipitates from competition of ion about water due to increase of hydrophobic interaction (Vojdani, 1996).

Collagen solubilities on NaCl concentration in other fish collagens such as bigeye snapper (*Priacanthus tayenus*) skin and bone collagens showed the lowest solubility at 4~6% NaCl concentration (Kittiphattanabawon et al., 2005); yellowfin tuna (*T. albacares*) dorsal skin collagen showed the slow decrease at 1~3% NaCl and the lowest solubility at 4~6% NaCl (Woo et al., 2008); collagens from dusky spinefoot (*Siganus fuscescens*), sea chub (*Kyphosus bigibbus*), eagle ray (*Myliobatis tobijei*), red stingray (*Dasyatis akajei*), yantai stingray (*Dasyatis laevugata*) skin showed the highest solubility at 0~4% NaCl, but the lowest at over 6% (Bae et al., 2008); and solubilities of acid-soluble collagen (ASC) and pepsin-solubilised collagen (PSC) from brownstripe red snapper (*L. vitta*) skin were rapidly decreased at 3% NaCl for ASC and 4% NaCl for PSC (Jongjareonrak et al., 2005).

3.5. Effect of pH on collagen solubility

Fig. 6 is the result of the effects of pH on solubilities of skate and calf skin collagens. Both skate and calf skin collagens showed the slow decrease up to pH 6, and the lowest solubility at pH 7~9. At pH 10, their solubilities increased a little bit. Collagen showed high solubility in acidic pH. Their different protein solubilities on pH are thought to be due to

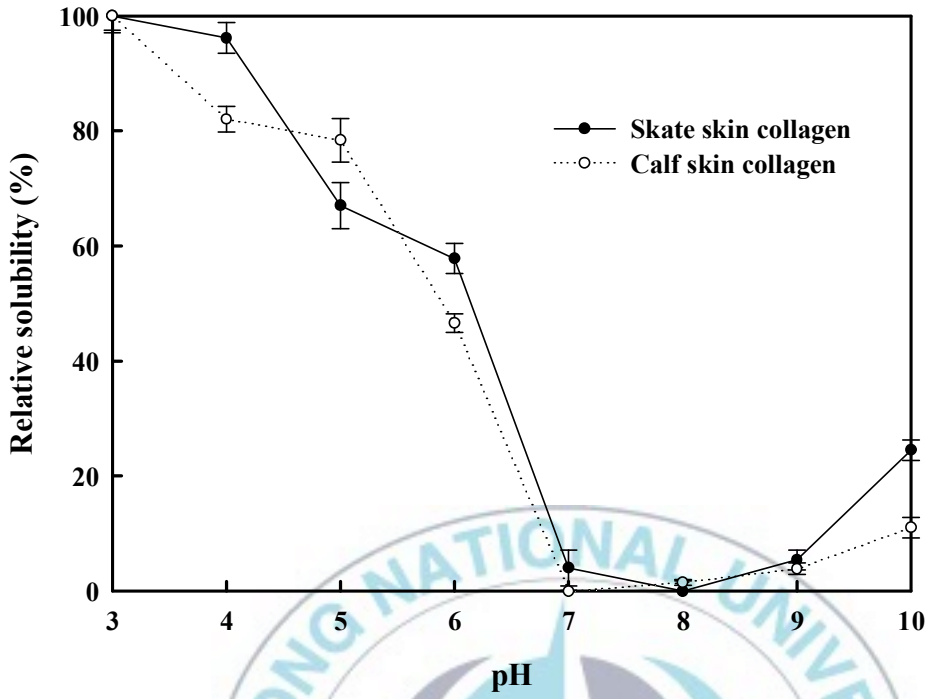

Fig. 6. Effect of pH on relative solubilities of the collagens from skate (*Okamejei kenojei*) and calf skins.

differences in their isoelectric points. If pH is higher or lower than isoelectric point, repulsive power between electric charge of protein molecules increase, and protein solubility increases as well. On the other hand, at isoelectric point, hydrophobic-hydrophobic interlink increases. As protein charge become neutral, protein precipitates and coagulation (Wong, 1989).

Solubility of calf skin collagen was the lowest when pH was 5~8 range. When it goes over this range, solubilities in acid and alkali pHs increased. As the solution goes to more salt concentration, collagen solubility is more increased (Ranganayaki et al., 1982). Skin and bone collagens of Bigeye snapper (*P. tayenus*) had the lowest solubility at pH 7~9 range (Kittiphattanabawon, 2005); dorsal skin collagen of yellowfin tuna (*T. albacares*) showed a rapid decreased solubility at pH 6~9 (Woo et al., 2008); skin collagen of dusky spinefoot (*S. fuscescens*) had a decreased solubility at pH 6~10; collagens from sea chub (*K. bigibbus*), eagle ray (*M. tobijei*); and red stingray (*D. akajei*) skins had the lowest solubility at pH 7~10; skin collagen of yantai stingray (*D. laevugata*) had the lowest solubility at pH 8~10 (Bae et al., 2008), and had the similar tendency as this study. Also, solubility of fish collagen did not depend on the age and nutritional condition of sample, compared with that of mammal collagen (Love et al., 1976).

3.6. Degeneration temperature for relative viscosity

Fig. 7 is the result of the measured degeneration temperature(T_d) of skate and calf skin collagens. Degeneration temperature of skate and calf skin

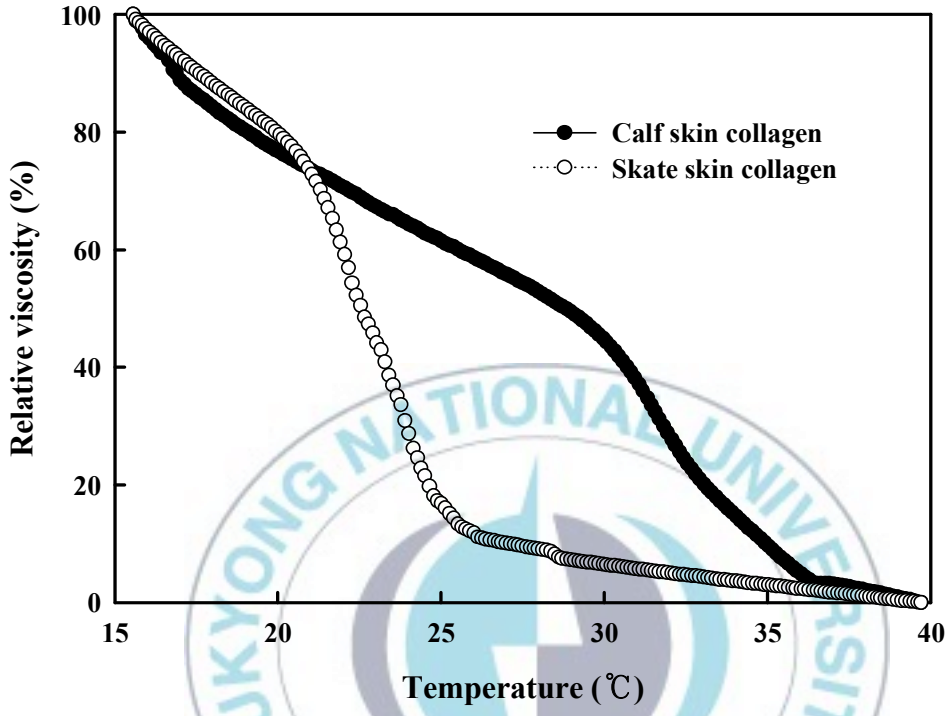

Fig. 7. Relative viscosities at different temperatures of the collagens from skate (*Okamejei kenojei*) and calf skins.

collagens are 23℃ and 33℃, respectively, showing that T_d of skate skin collagen was about 10℃ lower than that of calf skin collagen.

This result is thought to be due to the content of imino acids (proline and hydroxyproline) which is known by the amino acid composition of skate skin collagen. Their contents of imino acids of skate and calf skin collagens were 152 and 226 per 1,000 residual, respectively, which were proportional to their degeneration temperatures. It is known that T_d of collagen is proportional to hydroxyroline content (Doty & Nishihara, 1958), and also both proline and hydroxyproline contents were proportional to T_d of collagen (Piez & Gross, 1960). As such amino acid combinations as Gly-Pro-Ala and Gly-Pro-Hyp was little, T_d got lower (Kimura, 1971), and T_d was reported to be proportional to total pyrrolidine content of collagen (Josse & Harrington, 1964).

Degeneration temperatures of fish skin collagens were 28.7℃, 29.2℃, 34.1℃, 33.2℃, 32.2℃, and 28.0℃ for dusky spinefoot (*S. fuscescens*), sea chub (*K. bigibbus*), eagle ray (*M. tobijei*), red stingray (*D. akajei*), yantai stingray (*D. laevugata*), and ocellate puffer fish (Nagai et al., 2002). Degeneration temperatures of dorsal skin collagen of yellowfin tuna (*T. albacares*), collagen of rhizostomous jellyfish (*Rhopilema asamushi*) were 32℃ and 28.8℃ (Nagai et al., 2000), respectively, and T_d of minke whale (*B. acutorostrata*) collagen was 31.5℃ (Nagai et al., 2008). Degeneration temperatures of acid-soluble collagen (ASC) and pepsin-solubilized collagen Degeneration temperatures of ASC and PSC of sheepshead seabream (*A. probatocephalsu*) were 34.0℃ and 34.4℃, respectively (Ogawa et al., 2003). Degeneration temperatures of acid-soluble collagens of young and adult nile

perch (*L. niloticus*) were 36.0℃ and 36.5℃, respectively (Muyonga et al., 2004). Also, degeneration temperature of the collagen of *Ophiocephalus striatus* that breathes through lung was 32.9℃, and this is thought to be due to the different contents of imino acids (proline+hydroxyproline). Another reason is thought to be due to their different environmental temperatures (Rama & Chandrakasan, 1983). The reason is reported that the melting temperature of the collagen of the fish that was caught at 2~4℃ around 2,000 meter under the water was lower than that of the fish caught at 16~18℃ around 200 meter under the water (Rigby & Prosser, 1975).

IV. Conclusion

The processing of collagen from skin of skate (*Okamejei kenojei*) was optimized by the response surface methodology and the central composite design. The optimal condition included the following independent variables: 0.45 N NaOH concentration, 23.4 NaOH treatment hours, 0.88% additional ratio of pepsin, and 46.8 extraction hours. The collagen content estimated under the optimal condition was 6.94%, and the actual experimental collagen content was 6.56%. Physicochemical properties of collagen extracted from skate skins were characterized by amino acid analysis, SDS-PAGE, FT-IR, solubility and denaturation temperature. Collagen from skate skin had an imino acid (Hyp+Pro) of 152 residues/1,000 amino acids. The SDS-PAGE pattern of the skate collagen indicated two different α-chains (α_1 and α_2), β -component and γ-component. This pattern is similar to that of calf skin collagen, but the skate collagen has a different mobility. FT-IR of collagen from skate and calf skins showed such regions of amide A, I, II and III as 3284 and 3313 cm^{-1} (amide A); 1650 and 1640 cm^{-1} (amide I); 1549 and 1547 cm^{-1} (amide II); 1238 and 1237 cm^{-1} (amide III), for collagens of skate and calf skins, respectively, exhibiting similar wave numbers to each other. Solubility of the skate skin collagen on NaCl concentration is hardly changed up to 2%, and then it was decreased from 3% to 5%. Solubility of the skate skin collagen on pH variation was high in the range of pH 3~5 and then sharply decreased up to pH 7. Denaturation temperature of the skate skin collagen was 23℃ which was lower by about 10℃ than that of calf skin collagen.

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