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# Changes in an Ammonia-like Odor and Chondroitin Sulfate Contents of Enzymatic Hydrolysates from Longnose Skate (*Rasa rhina*) Cartilage as Affected by Pretreatment Methods

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**Abstract** To reduce ammonia-like odor in chondroitin sulfate, longnose skate (*Rasa rhina*) cartilage was processed by washing, autoclaving, and alkali pretreatments. Content of total volatile basic nitrogen (TVB-N), index of ammonia-like odor, of raw skate cartilage without pretreatment was 254 mg/100 g, whereas those of skate cartilage pretreated with washing and autoclaving increased to 630 and 636 mg/100 g, respectively. TVB-N of skate cartilage pretreated with sodium hydroxide sharply decreased to 15 mg/100 g at optimal condition of 0.12 M and 3.6 volume of NaOH, as determined by surface response methodology of central composite design for optimization. Alkali pretreatment resulted in 97.6% deodorizing. Washing and autoclaving pretreatments had almost no effect on the yield of chondroitin sulfate (approximately 30%), whereas decreased to 16.0% after alkali pretreatment, showing chondroitin sulfate of skate cartilage as chondroitin sulfate C.

**Key words:** chondroitin sulfate, longnose skate (*Rasa rhina*) cartilage, total volatile basic nitrogen (TVB-N), response surface methodology (RSM), alkali treatment

## Introduction

Skate, along with ray and shark, is a typical cartilaginous fish, and is commonly eaten in Korea after being fermented for approximate one week at a low temperature (below 10°C). The quality of a well fermented skate is determined by its special flavor of ammonia-like odor formed during fermentation due to its higher concentrations of nitrogen components such as trimethylamine oxide (TMAO) and urea than other fishes (1-3). TMAO and urea are degraded into volatile bases of trimethylamine (TMA), ammonia, and dimethylamine (DMA) by degradation actions of microorganisms and enzymes during fermentation (1-5). The ammonia-like odor produced from these low molecular volatile bases bestows a good flavor quality to fermented skate; however, the byproducts of cartilages containing chondroitin sulfate have disadvantages as functional materials due to the strong smell and low threshold for acceptability.

In addition to the already known applications of chondroitin sulfate in cosmetics, functional foods, medicines, and other products (6), numerous recent studies have investigated the use of chondroitin sulfate with glucosamine for the prevention and treatment of arthritis (7-9). Chondroitin sulfate is a sulfated mucopolysaccharide and is classified into chondroitin sulfate A, C, D, E, and K according to the binding site of the sulfate group and the unit of the component (10-12). It has a complicated structure, which is combined to the core protein, and is found in tissues such as cartilage as a structural component of proteoglycans (6, 13, 14). The processing of skate, ray, and shark produce high content of chondroitin sulfate-rich cartilage as a byproduct (15-17), and chondroitin sulfate in the cartilage contributes significantly to the economic value of the fish as well as supplies important bioactive industrial and medicinal ingredients.

Pretreatment processing for the efficient removal of the unique flavors of volatile bases produced from skate fermentation is needed to produce chondroitin sulfate with acceptable sensory characteristics from fermented skates. Therefore, this study evaluated methods for the removal of these off-flavors during the processing of chondroitin sulfate from skate cartilage. The deodorizing process was optimized using response surface methodology to remove the ammonia-like odor through alkali pretreatment of skate cartilage before the processing of the enzymatic hydrolysates including chondroitin sulfate. Effect of alkali pretreatment on the yield of chondroitin sulfate was also investigated.

## Materials and Methods

**Materials** Frozen longnose skate (*Raja rhina*) was obtained from Youngsanpo Food Co. (Youngsanpo, Korea). To remove the remaining tissue and others contaminants surrounding the cartilage, skate cartilage was treated with hot water for a few minutes, and the heat-denatured protein was removed by hand-washing with cold water. The washed longnose skate cartilages were placed in a tray and dried at room temperature for 30 min and homogenized with a homogenizer (AM-5, Nihonseiki Kaisha LTD., Tokyo, Japan). Crushed cartilages (200 g) were packaged in plastic bottles and stored in a freezer at -20± 1°C until used.

**Autoclaving condition in pretreatment of skate cartilage** One hundred grams of the longnose skate cartilages were put into a flask, added with three-times distilled water, and autoclaved (DW-AC-930, Dong won Scientific Co., Busan, Korea) at 115°C for 10 to 120 min. The

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deodorized degree of autoclaved cartilage was measured in terms of the remaining total volatile basic nitrogen after the production of enzymatic hydrolysate.

**Water treatment in pretreatment of skate cartilage** Mixture of 100 g longnose skate cartilage and 3 volumes distilled water in a flask was shaken at 200 rpm for 1-7 hr at ambient temperature. The moisture of the pretreated cartilage was removed, and the degree of deodorization of the pretreated cartilage was measured based on the total volatile basic nitrogen remaining after the production of enzymatic hydrolysate.

**Alkaline treatment in pretreatment of skate cartilage** One hundred grams of longnose skate cartilage was put into flasks, and various concentrations of sodium hydroxide were added to each flask. The mixture was placed in a shaking incubator and shaken at 200 rpm for several hours at ambient temperature. The alkali-pretreated cartilage was washed with tap water until reaching pH 8.0. The pretreated cartilage was freeze-dried, and the degree of deodorization as the treatment variable was plotted against treatment time (hour) and concentration of sodium hydroxide as measured by total volatile basic nitrogen remaining after the enzymatic hydrolysate production.

**Preparation of enzymatic hydrolysate from longnose skate cartilage** One hundred grams of longnose skate cartilage was pretreated by chopping in a homogenizer (AM-5, Nihonseiki Kaisha Ltd., Tokyo, Japan), and 3 volumes (v/w) distilled water and 2% alcalase (v/w) were added to the sample. The mixture was hydrolyzed at 55 ± 1°C, pH 8.0, for 4 hr in a shaking incubator at 200 rpm (HB-201SF, Hanbaek Scientific Co., Bucheon, Korea). Enzyme activity was deactivated by heating the hydrolysate at 99 ± 1°C for 15 min. The hydrolysate was centrifuged at 1,400 × g for 30 min, and the supernatant was filtered through diatomaceous earth. The filtrate was concentrated to 10 brix in a vacuum rotary evaporator (N-N, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), dried at 60°C for 24 hr in a hot air drier, and crushed. The crushed material was used to extract chondroitin sulfate.

**Determination of total volatile basic nitrogen (TVB-N) and proximate components** TVB-N was measured as Conway units using the microdiffusion method of Ishizaka (18). Briefly, 0.4 gram (dry basis) sample was weighed and added with 50 mL distilled water. The solution was mixed for 5 min at 14,000 rpm, added with 10 mL of 20% trichloroacetic acid, and mixed. The mixed solution was held for 10 min to precipitate the water-soluble protein and filtered through a filter paper (Toyo No. 5A). The filtrate was put into a 100 mL mess flask, brought to volume with distilled water, and used for the test of TVB-N. Into the inner part of a Conway unit, 1 mL boric acid solution, composed of 10 g $H_3BO_3$, 200 mL ethanol, 700 mL distilled water, 10 mL mixed indicator with 0.066% methyl red, and 0.033% bromocresol green in 95% ethanol, showing light pink color indicative of a weak acid of the boric acid solution, was added. Into the outer part of the Conway unit, 1 mL saturated $K_2CO_3$ solution and 1 mL sample solution were added, and held for 80 min at 37°C. The mixed solution was titrated with 0.01 N $H_2SO_4$ for quantitative analysis. For the analyses of other proximate components, the contents of moisture, crude ash, crude lipid, and crude protein were determined by AOAC methods (19).

**Cellulose acetate membrane electrophoresis** Electrophoretic analysis of chondroitin sulfate was performed by the method of Hata and Nagai (20) on an electrophoresis system (EC-240, Advantec, Tokyo, Japan) using a cellulose acetate membrane (Seleca-V, 6 cm × 22 cm, Advantec, Tokyo, Japan) in 1.0 M acetic acid-pyridine (pH 3.5) at a constant current of 0.5 mA/cm width for 40 min. The membrane was stained with 0.5% (w/v) toluidine blue in 3% (v/v) acetic acid for 5 min, and destained with 1% acetic acid.

**Determination of chondroitin sulfate** Content of chondroitin sulfate was determined by the method of Korea Food & Drug Administration (21). A sample solution was prepared as follows. About 0.3 g sample was dissolved in 100 mL distilled water, and 4mL aliquot of the solution was diluted to 20 mL with distilled water and then filtered. Subsequently, 5 mL sodium borate sulfuric acid solution was added to test tubes and cooled with ice water. The sample solution and 1 mL glucuronolactone standard solution were carefully added to each test tube and mixed while kept cool. The test tubes were then heated in a 100°C water bath for 10 min, and cooled with ice water immediately. Carbazole solution (0.2 mL) was added to each test tube, mixed, reheated for 15 min, and cooled. The yield of chondroitin sulfate was determined by measuring the absorbance at 530 nm.

**Experimental design for enzymatic hydrolysate using response surface methodology** The optimum NaOH pretreatment condition for removing ammonia-like odors in the processing of chondroitin sulfate from longnose skate cartilage was investigated using a central composite design (CCD) (22), which had $2^2$ factorial points, 4 axial points ($\alpha$=1.414), and 4 center points (Table 4). Among the three independent variables of NaOH concentration ($X_1$, M), volume ($X_2$, w/v), and treatment time (hr), the amount of TVB-N was almost removed for a short time of 1 hour treatment, so treatment time was fixed as one hour Therefore, the two important independent variables for NaOH pretreatment were NaOH concentration ($X_1$, M) and volume ($X_2$, w/v), and the dependent variable was TVB-N content ($Y$, mg/100 g) as an index of ammonia-like odor. The center point values and ranges were decided based on the results of preliminary experiments (Table 1). All experiments were performed in triplicates with a random order of experimental runs to reduce experimental errors.

The response surface regression (RSREG) procedure of SAS (Version 8.01, SAS Institute Inc., Cary, NC, USA) was used to fit a quadratic model for predicting the optimal point. The equation was expressed according to the following formula (1):

$$\hat{y}_i = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (1)$$

**Table 1. Experimental range and levels of the independent process variables**

| Independent variables | Symbol | | Range and levels | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | −1.414 | −1 | 0 | +1 | +1.414 |
| NaOH concentration (M) | $X_1$[a] | $x_1$ | 0.058 | 0.07 | 0.10 | 0.13 | 0.142 |
| NaOH volume (times, v/w) | $X_2$ | $x_2$ | 1.59 | 2 | 3 | 4 | 4.41 |

[a]where: $X_1=(x_1-0.1)/0.03$; $X_2=(x_2-3)/1$

where, $\hat{y}_i$ represents the response variable, $b$ is the coefficient, and $X_1$ and $X_2$ represent independent variables. Where possible, the model was simplified by eliminating statistically insignificant terms. All possible regression models were considered using Akaike Information Criteria (AIC) (23) and $C_p$ of $R^2$ procedure to test the models for goodness of fit.

The statistical significance of the regression coefficients was determined by Student's *t*-test. The second-order model equation was determined by Fischer's test, and the proportion of variance explained by the model was given by the multiple coefficient of determination, $R^2$. Three-dimensional graphs were made using the Maple software (Maple 7, Waterloo Maple Inc., Waterloo, Canada). In addition, significant differences were analyzed by ANOVA using F value with a SAS program (Version 8.01, SAS Institute Inc.), and multiple comparisons were carried out by Duncan's Range Test at a 95% confidence level.

## Results and Discussion

**Proximate compositions and total volatile basic nitrogen (TVB-N) of longnose skate cartilage** Proximate compositions of raw material and enzymatic hydrolysate of longnose skate cartilage are shown in Table 2. Unprocessed longnose skate cartilage had 80.5% moisture, 10.1% crude protein, 0.8% crude lipid, 7.6% crude ash, and 1.0% total carbohydrates. Enzymatic hydrolysate of longnose skate cartilage contained 4.1% moisture, 56.6% crude protein, 1.1% crude lipid, 8.5% crude ash and 29.7% total carbohydrates.

TVB-N of longnose skate raw cartilage was 204 mg/100 g, while that of the enzymatic hydrolysate was 620 mg/100 g (Table 2), showing a sharp increase in the ammonia-like odor. The main off-flavor compounds against ammonia-like odor from longnose skate cartilage were ammonia and trimethylamine, which were representative TVB-Ns. Ammonia and trimethylamine are produced from the degradation of urea and TMAO (1, 2). TVB-N generally increases as the freshness of fish and shellfish decreases (24, 25). Like the above fishes, TVB-N of elasmobranchs such as skate, ray, and shark sharply increased with decreasing freshness. Because TVB-N is an index of ammonia-like odor in longnose skate cartilage, for the production of chondroitin sulfate with low ammonia-like odor, pretreatment of longnose skate cartilage is required to reduce TVB-N amount.

**Table 2. Proximate composition of longnose skate (*Rasa rhina*) cartilage and its enzymatic hydrolysate**

| Component | Skate cartilage | |
|---|---|---|
| | Raw | Enzymatic hydrolysate |
| Moisture | 80.5±0.8 | 4.1±0.9 |
| Crude protein (N×5.56) | 10.1±0.4 | 56.6±0.6 |
| Crude lipid | 0.8±0.1 | 1.1±0.2 |
| Crude ash | 7.6±0.4 | 8.5±0.5 |
| Total carbohydrate[a] | 1.0±1.7 | 29.7±2.2 |
| TVB-N (mg/100g) | 204.0±11.0 | 620.0±17.0 |

[a]100 – ( moisture + crude protein + crude lipid + crude ash )

**Effects of pretreatment methods on deodorization of longnose skate cartilage** Chondroitin sulfate of the cartilage exists in a bound state to proteins and, therefore, needs to be released into a free state using a protease during processing to produce pure chondroitin sulfate (13, 14). The enzymatic hydrolysis of the cartilage increased the ammonia-like odor of the hydrolysate, causing a strong off-odor (Table 2). The compounds related to the ammonia-like odor in skate cartilage have low thresholds; thus, migration of the off-odor to the final product of chondroitin sulfate is a major problem to be solved. Therefore, a pretreatment for deodorizing the cartilage is required before the release of chondroitin sulfate from the cartilage by protease. Effects of several pretreatments for removing ammonia-like odors from longnose skate cartilage on TVB-N level are shown in Fig. 1. Range of TVB-N levels of the ingredients hydrolyzed enzymatically after autoclaving the cartilage for several hours varied from 599 to 664 mg/100 g, showing that heat treatment alone could not remove the off-odor of the cartilage. As the second pretreatment trial for removing ammonia-like

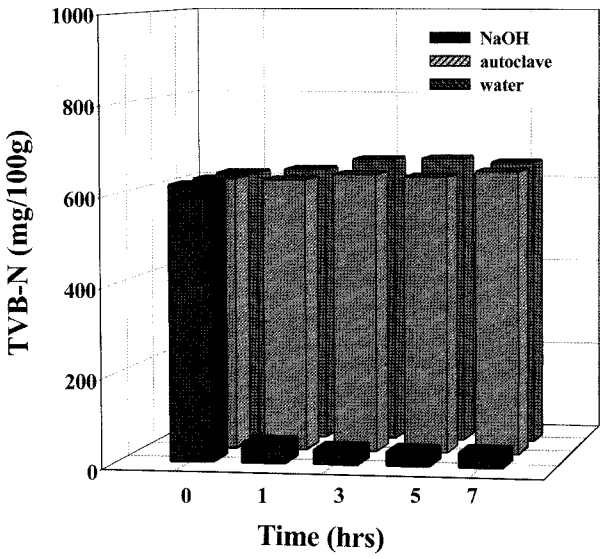


**Fig. 1. Change in total volatile basic nitrogen (TVB-N) of enzymatic hydrolysates from longnose skate (*Rasa rhina*) cartilage as affected by alkali, distilled water and autoclaving pretreatments for different times.**

**Table 3. Experimental design and results according to the central composite design arrangement and responses**

| Runs | Independent variables | | Responses |
|---|---|---|---|
| | $X_1$ | $X_2$ | TVB-N (mg/100 g) |
| 1 | −1 | −1 | 123.2 |
| 2 | −1 | +1 | 57.5 |
| 3 | +1 | −1 | 73.3 |
| 4 | +1 | +1 | 21.4 |
| 5 | −1.414 | 0 | 125.8 |
| 6 | +1.414 | 0 | 29.0 |
| 7 | 0 | −1.414 | 127.8 |
| 8 | 0 | +1.414 | 26.5 |
| 9 | 0 | 0 | 32.2 |
| 10 | 0 | 0 | 33.2 |
| 11 | 0 | 0 | 29.9 |
| 12 | 0 | 0 | 30.5 |

odor, the cartilage was washed with water for 1 to 7 hr. TVB-N levels of the enzymatic hydrolysates increased from 618 to 642 mg/100 g and were not significantly different, indicating that the washing pretreatment was not effective for removing the ammonia-like off-odor. Pretreatment by autoclaving was also ineffective for removing urea and trimethylamine oxide as precursors of ammonia-like odor; the precursors could have been degraded into TVB-N by consecutive enzyme hydrolysis, resulting in a high TVB-N level. In addition, pretreatment by autoclaving or washing was not effective for removing ammonia and trimethylamine formed from the precursors. On the other hand, the TVB-N level of the enzymatic hydrolysate from skate cartilage pretreated with alkali was very low (Fig. 1), indicating that alkali pretreatment could be a very effective method for removing the off-odors from the cartilage, possibly because urea and trimethylamine oxide (TMAO) as precursors of ammonia-like odor, and ammonia and trimethylamine as TVB-Ns were very alkali-soluble and could be easily removed by alkali pretreatment. Therefore, alkali pretreatment was determined to be the most effective method for reducing the TVB-N level of longnose skate cartilage.

**Optimization of alkali pretreatment for deodorization of longnose skate cartilage by response surface methodology** To reduce TVB-N level, sodium hydroxide pretreatment was optimized using a central composite design, which had two independent variables, sodium hydroxide concentration (M, $X_1$) and volume (times, $X_2$), and a dependent variable of TVB-N (mg/100 g) as precursors of the ammonia-like odor. The fitted quadratic polynomial equation was obtained by the RSREG procedure of SAS.

**Table 4. Results of the regression analysis using the central composite design and analysis of variable (ANOVA)**

| Term | TVB-N (mg/100 g) | | | |
|---|---|---|---|---|
| | Coefficient | Standard errors | $t$-value | P |
| intercept | 31.5 | ±4.8 | 6.54 | 0.0006 |
| $X_1$ | −27.9 | ±3.4 | −8.19 | 0.0002 |
| $X_2$ | −32.6 | ±3.4 | −9.58 | <.0001 |
| $X_1X_1$ | 20.9 | ±3.8 | 5.48 | 0.0015 |
| $X_1X_2$ | 3.5 | ±4.8 | 0.72 | 0.5004 |
| $X_2X_2$ | 20.7 | ±3.8 | 5.45 | 0.0016 |

All linear ($X_1$, $X_2$), quadratic ($X_{11}$, $X_{22}$), and interaction coefficients were calculated for significance with $t$-statistic, and the estimated coefficients of each model are presented in Table 4. Except for the coefficient $X_1X_2$ ($P > 0.05$), all coefficients in the model were highly significant ($P<0.01$). To develop the fitted response surface model equations, insignificant terms were eliminated, and the fitted model was expressed according to Eq. (2):

$$Y=31.5-27.9X_1-32.6X_2+20.9X_1^2+20.7X_2^2 \quad (2)$$

Based on canonical analysis of RSREG, all eigenvalues of the response model were positive and showed a stationary point of a minimum value. The coefficient of determination ($R^2$) for $Y$ was 0.9721 with significance at $P$=0.0001. The very high $R^2$ value was believed to be an experimental design which was well designed after preliminary tests. As observed in the canonical analysis results of RSREG, the expected critical values for coded values were 0.61 and 0.74 M, whereas uncoded values were 0.12 and 3.74 M NaOH concentration ($X_1$) and 3.74 NaOH volume ($X_2$), respectively. As concentration and volume of NaOH increased, the TVB-N value decreased. However, removal of the off-odor from TVB-N resulted in decreased chondroitin sulfate yield. The predicted value based on optimal conditions was 11.0 (mg/100 g), whereas the experimental value was 15.0 (mg/100 g), exhibiting a modest difference between values obtained through the predicted and experimentally confirmed optimal conditions (Table 5). Chondroitin sulfate obtained from the pretreated skate cartilage with alkali showed a 97.6% higher deodorizing effect than the TVB-N value (620 mg/100 g) of the chondroitin sulfate obtained without any pretreatment. Although higher concentrations and greater volumes of NaOH resulted in greater reductions in TVB-N values, treatments above the optimal concentration and volume of NaOH did not result in further removal of the off-odor. In addition, too high NaOH concentrations and volumes lowered the yield and quality of the product (Fig. 3).

**Table 5. Optimal alkali pretreatment conditions for deodorization of longnose skate (*Rasa rhina*) cartilage**

| Dependent variables | Independent variables | Critical value | | Predicted value | Experimental value |
|---|---|---|---|---|---|
| | | Coded | Uncoded | | |
| Y | $X_1$ [a] | 0.61 | 0.12 | 11 | 15 |
| | $X_2$ | 0.74 | 3.74 | | |

[a]$X_1$ (concentration of NaOH, M), $X_2$ (volume, w/v)

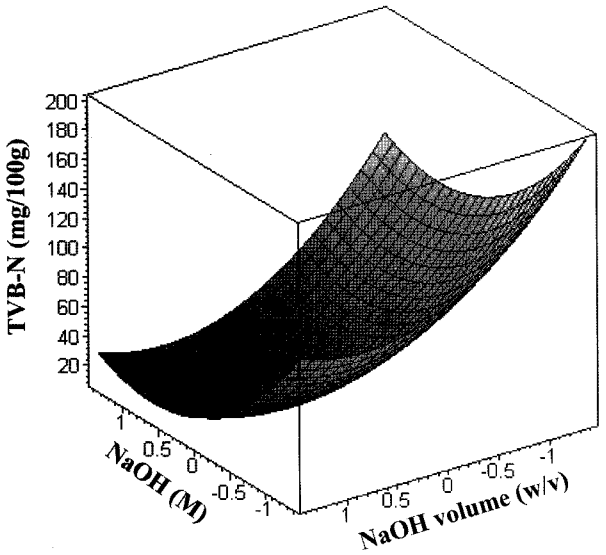


**Fig. 2. Response surface plots on effects of NaOH concentration and volume with 1hr treatment for deodorization optimization of longnose skate (*Rasa rhina*) cartilage.**

Three-dimensional graphs of response surface plots were drawn using Maple software (Maple 7, Waterloo Maple Inc., Canada) (Fig. 2). The level of TVB-N (mg/100 g) as the Y value decreased with increasing concentration and volume of NaOH. The yield of chondroitin sulfate was similarly affected by two independent variables of $X_1$ (NaOH concentration, M), and $X_2$ (NaOH volume, w/v). The independent variable of $X_2$ (NaOH volume, w/v) had slightly higher effect on the yield of chondroitin sulfate than $X_1$ (NaOH concentration, M). Therefore, $X_2$ (NaOH volume) is a more important variable for removing the ammonia-like odor during the processing of chondroitin sulfate from longnose skate cartilage.

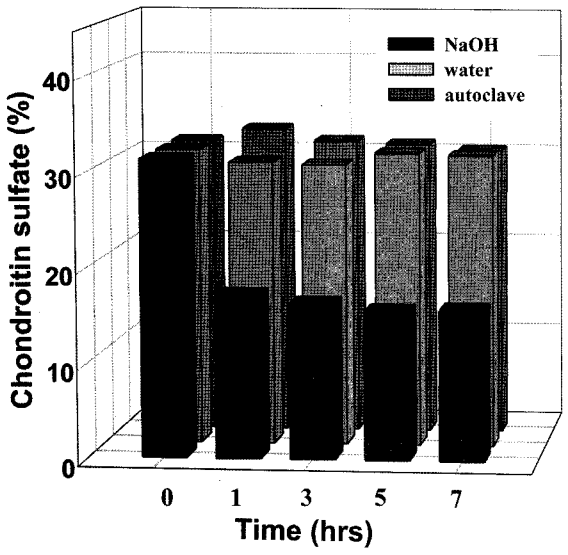


**Fig. 3. Change in chondroitin sulfate contents of enzymatic hydrolysate from longnose skate (*Rasa rhina*) cartilage as affected by alkali, distilled water and autoclaving pretreatments for different times.**

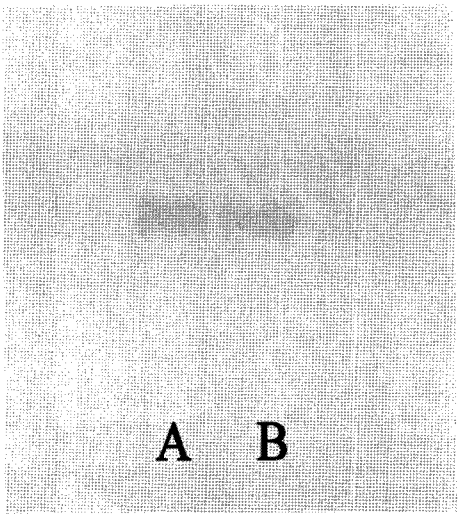


**Fig. 4. Cellulose acetate membrane electrophoretic pattern of chondroitin sulfate from longnose skate (*Rasa rhina*) cartilage.** (A) Authentic chondroitin sulfate C, (B) Alkali pretreatment hydrolysate.

**Effects of pretreatment methods on chondroitin sulfate contents of enzymatic hydrolysates from longnose skate cartilage** Recovery of chondroitin sulfate after several pretreatments and enzymatic hydrolysis of longnose skate cartilage are shown in Fig. 3. The yield of chondroitin sulfate in raw cartilage was 0.6%, a relatively low value, whereas up to 30% in the enzymatic hydrolysate from the cartilage without any pretreatment, probably because chondroitin sulfate was changed into a free state from the bound state to protein by the enzymatic treatment of the cartilage. In the case of shark cartilage and *Cioma intestinalis*, enzyme-treated samples had higher yields of chondroitin sulfate than those with no treatment (26, 27).

Longnose skate cartilage with alkali pretreatment contained 16.0 ± 2.0% chondroitin sulfate. In samples with only autoclaving pretreatment, the concentration of chondroitin sulfate was 30.3%-32.8% depending on treatment time, showing no big differences. In addition, in the case of water washing as a pretreatment, the enzyme hydrolysate contained 29.9%-31.2% chondroitin sulfate, also showing no big differences. Although the pretreatments of autoclaving and washing has lower effect than alkali pretreatment in reducing TVB-N level, they produced much better yields of chondroitin sulfate (Fig. 2 and 3). After the optimal alkali pretreatment for odor reduction, the yield of chondroitin sulfate was greatly reduced compared with autoclaving and washing pretreatments (16.0% vs. 30-32%). These results, therefore, showed alkali pretreatment effectively reduced TVB-N, improving the flavor quality of the ingredient, while significantly reduced the yield of chondroitin sulfate, compared with autoclaving and washing pretreatments. The cause for the reduction in the yield of chondroitin sulfate by the alkali pretreatment is believed to be due to the solubilization with alkali pretreatment and removal of chondroitin sulfate protein-bound in cartilage. Mobility of chondroitin sulfate isolated from longnose skate cartilage by cellulose acetate membrane electrophoresis was the same as that of the authentic chondroitin sulfate C (Fig. 4).

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