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Optimization of Shark (*Squatina oculata*) Cartilage Hydrolysis for the Preparation of Chondroitin Sulfate

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Abstract Enzymatic hydrolysis of shark (*Squatina oculata*) cartilage (SC) was optimized by response surface methodology (RSM) for chondroitin sulfate (CS) preparation. Among 11 commercial proteases, Maxazyme NNP showed highest productivity (CS yield per enzyme cost) of CS. Optimal hydrolysis conditions determined by RSM were 1.63% and 2.87 hr for enzyme concentration and hydrolysis time ($r^2 = 0.9527$, $p<0.01$), respectively and highest yield of hydrolysate under the conditions was 42.3%. The yield (43.1±2.1%) and CS content (24.8±0.1%) of hydrolysate at optimal condition verified statistical optimization of SC enzymatic hydrolysis was valid.

Keywords: chondroitin sulfate, shark cartilage, hydrolysis optimization, RSM

Introduction

Chondroitin sulfates (CS), a mucopolysaccharide, is composed of D-glucuronic acid, N-acetyl-D- galactosamine, and sulfate group, and exists as a proteoglycan, a protein complex (1). CS is widely distributed in animal kingdom (2), not only in cartilages of vertebrata, but also in skin and blood of animals, and is responsible for the high elasticity and resilience of the tissues (3, 4). Recent researches on the physiological activities (5, 6) of CS showed its possibility as a marketable functional material, for applications in cosmetics, pharmaceuticals, as well as in functional foods. However, in spite of this functionality, mammalian CS is exposed to risks such as contamination with bovine spongiform encephalopathy and foot & mouth disease, therefore, researches on the replacement of mammalian CS with that from shark cartilage and other marine organisms are necessary (7). Gu *et al.* (8) assessed aquatic invertebrates for the sulfated mucopolysaccharides, and Park *et al.* (9) optimized the sea cucumber hydrolysis for the preparation of CS. Shark scapular cartilage hydrolysis by commercial proteases was also optimized, and CS was purified from the hydrolysate (10). Furthermore, Kralovec *et al.* (11) isolated immunostimulating proteoglycan from shark cartilage.

Therefore, to effectively utilize the ocean bio-resources rarely used, in this study the enzymatic hydrolysis of shark cartilage was optimized by response surface methodology (RSM) for the effective and economic preparation of CS.

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Materials and Methods

Materials Shark (*Squatina oculata*) cartilage (SC), composed of 11.1% moisture, 33.9% crude protein, 51.9% crude ash, 13.3% carbohydrate, and 0.9% crude lipid was purchased as dried powder from PT.INDO BIOTEKNIK SEJAHTERA Co. (Jawa Barat, Indonesia). Complex enzyme, Natoozyme (Daeho, 38°C, pH 5.5), and the following commercial proteases were used for hydrolysis, : Alcalase 2.4LFG (Novo, 60°C, pH 6.5-8.5), Flavourzyme 500 MG (Novo, 50°C, pH 5.0-7.0), Protamex 1.5MG (Novo, 40°C, pH 5.5-7.5), Neutrase 0.5L (Novo, 50°C, pH 5.5-7.5), Delvolase (Bision Biochem., 60°C, pH 9.5-10.5), Bromelain 2000GDU (Bision Biochem., 60°C, pH 4.5-5.0), Maxazyme NNP (Bision Biochem., 50°C, pH 6.5-7.5), Sumizyme LP (Bision Biochem., 50°C, pH 5.0-7.0), Collupulin (Bision Biochem., 60°C, pH 5.0-7.5), and Protease NP (Pacific Co., 50°C, pH 6.0-8.0). All reagents used were of analytical grade.

Preparation of enzymatic hydrolysate SC powder (15 g) was hydrolyzed with 10 volumes (v/w) of distilled water (DW) by 2% (w/w, dry weight) each protease at the optimum temperature and pH. The hydrolysate was then centrifuged (1400 g, 30 min), lyophilized, and pulverized.

Determination of yield of enzymatic hydrolysate Yield of the enzymatic hydrolysate was determined based on the percentage (dry basis) of the hydrolysate in SC.

Determination of CS content CS content was determined by the Korean food code method (12). Three hundred milligrams of the hydrolysate was dissolved with D.W. and massed up to 100 mL. Aliquot (4 mL) of the solution was massed up again to 20 mL and filtered (5C, Advantec) for use as a sample solution. Aliquot (5 mL) of 1% (w/v)

sodium borate sulfuric acid solution was placed in a test tube and sufficiently cooled with icy water. Aliquot (1 mL) of the sample solution was carefully added to the sodium borate sulfuric acid solution, mixed with cooling, heated in a water bath (90°C, 10 min), and immediately cooled with icy water. Aliquot (0.2 mL) of 0.125% (w/v, absolute ethanol) carbazole solution was added to the test tube, mixed, heated (90°C, 15 min) again, and cooled to room temperature. Using the same method, standard and blank groups were prepared with glucuronolactone standard solution [1 mL of 0.04% (w/v) glucuronolactone was massed up to 20 mL with DW] and DW, respectively. The absorbance was measured at 530 nm, and the content of CS was calculated as follows:

CS content (%) = glucuronic acid (%) × 2.593

$$= \frac{\frac{A}{B} \times C(0.04g) \times 1.1023}{D(0.3g) \times 4} \times 100 \times 2.593$$

A : The absorbance of sample solution,
B : The absorbance of standard solution,
C : δ-glucuronolactone, D : Sample weight
2.593 : molecular weight of CS / molecular weight of glucuronic acid
1.1023 : molecular weight of glucuronic acid / molecular weight of δ-glucuronolactone

Statistical treatment Yields of enzymatic hydrolysates and CS contents were determined in triplicates. ANOVA test was performed using SAS software (Version 8.01, SAS Institute Inc., Cary, NC, USA) and significances were considered at $p < 0.01$.

Response surface methodology (RSM) For effective hydrolysis of SC, the optimal condition was established by RSM using central composite design (CCD) (13). Preliminary studies (10) revealed the two important independent variables were enzyme concentration and hydrolysis time. Cha *et al.* (14) confirmed that the degrees of hydrolysis were similar within the range of 0.1 to 0.5% E/S ratio in the hydrolysis of pen shell by-product, and Kim *et al.* (15) reported that the degree of hydrolysis was steady up to 0.3% E/S ratio in the hydrolysis of anchovy sauce & soy sauce by-products. Therefore, the amount of additional DW was fitted as 10 time (v/w) the volume of SC.

Experimental design CCD was adopted in the optimization of SC hydrolysis. Enzyme concentration and hydrolysis time were applied as independent variables, and yield of hydrolysate as a dependent variable. The range and center point values of the independent variables were based on the results of preliminary experiments (Table 1). The independent variables were coded to five levels of -1.414, -1, 0, 1, and 1.414. CCD in the experimental design consisted of 2^2 factorial points, four axial points (α = 1.414), and triplicates of the central point (Table 2). Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

Analysis of data The response surface regression (RSREG) procedure of the Statistical Analysis System software (Version 8.01, SAS Institute Inc.) was used to fit the following quadratic polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{p} \beta i Xi + \sum_{i=1}^{p} \beta ii Xi^2 + \sum_{i<j=2}^{p} \beta ij Xi Xj$$

where Y is the dependent variable (yield of hydrolysate), β_0 is constant, β_i, β_{ii}, and β_{ij} are regression coefficients, and X_i and X_j are levels of the independent variables. The response surface plots were developed using MINITAB statistical software (Version 13, Minitab Inc.). All data were statistically analyzed by SAS software (Version 8.01, SAS Institute Inc.).

Results and Discussion

Selection of enzyme for hydrolysis Because CS exists as a complex with protein in organisms, SC was hydrolyzed with 11 commercial proteases, and the most effective protease was determined. Yields and CS contents of the hydrolysates are shown in Fig. 1. The yields of hydrolysates were in the order of Sumizyme and Flavourzyme, Alcalase and Protamex, Delvolase and

Table 2. Responses of dependent variables for the yield of hydrolysate from shark(*S. oculata*) cartilage to independent variables

Exp. No.	Variable levels		Responses, %
	X_1	X_2	
1	-1	-1	37.92
2	1	-1	40.36
3	-1	1	35.92
4	1	1	38.19
5	-1.414	0	37.27
6	1.414	0	39.53
7	0	-1.414	38.00
8	0	1.414	37.74
9	0	0	41.81
10	0	0	42.43
11	0	0	42.23

Table 1. Experimental range and value of the independent variables in the design for the hydrolysis of shark(*S. oculata*) cartilage

Process	Independen variables	Symbol	Range and levels				
			-1.414	-1	0	1	1.414
Maxazyme hydrolysis	Maxazyme concentration (%)	X_1	0.5	1.0	1.5	2.0	2.5
	Hydrolysis time (hr)	X_2	1.0	2.0	3.0	4.0	5.0

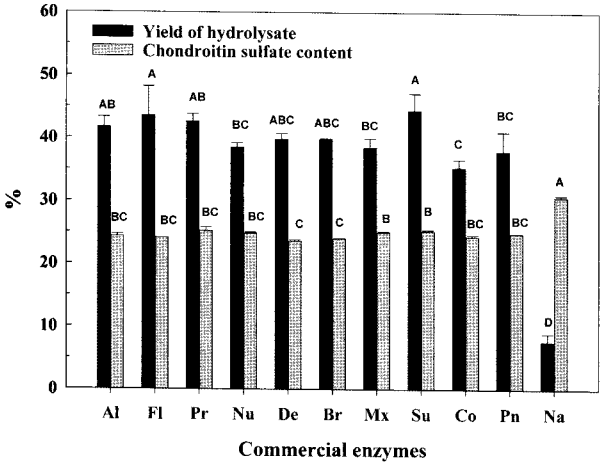

Fig. 1. Yields and chondroitin sulfate contents of shark cartilage hydrolysates by comercial proteases. Shark cartilage was hydrolyzed with 10 volumes (v/w) of D.W. by 2% (v/w or w/w; dry basis) each enzyme at the optimal temperature for 5 hr. Each bar shows the mean±S.D. (n=2-3). Means with the same letter are not significantly different (p<0.01). Al: Alcalase, Fl: Flavourzyme, Pr: Protamex, Nu: Neutrase, De: Delvolase, Br: Bromelain, Mx: Maxazyme, Su: Sumizyme, Co: Collupulin, Pn: Protease NP, Na: Natoozyme

Bromelain, Neutrase, Maxazyme, and Protease NP, Collupuline, Natoozyme in the ranges of 43.5-44.4, 41.7-42.6, 39.8, 37.9-38.5, 35.3, and 7.6%, respectively (p< 0.01). CS contents were in the order of Natoozyme, Maxazyme and Sumizyme, Alcalase, Flavourzyme, Protamex, Nutrase, Collupuline, and Protamex, Delvolase and Bromelain in the ranges of 30.6, 25.0-25.2, 24.3-24.8, and 23.6-24.0%, respectively (p<0.01). The lower yield of hydrolysate and higher CS content in Natoozyme were probably due to the specific type (complex) of the enzyme, the complex enzyme contained not only protease but also cellulase and xylase.

However, Sumizyme showed the highest efficiency in the yield of CS (yield of hydrolysate × CS content, YCS), the productivity (yield of CS per enzyme cost, YCS/C) was low (Table 3). Reasonable cost should be one of the most important factors to determine the most efficient enzyme. Thus, the productivity of CS depending on the enzyme used was analyzed (Table 3). While yields of CS in all enzymes used, except Natoozyme, were similar (10±1.4 %), cost of Maxazyme NNP was the lowest and, therefore, the productivity of CS in Maxazyme NNP was the highest (5.11), followed by Neutrase (3.49). This result indicated, among the tested enzymes, Maxazyme NNP was the most economically efficient for the hydrolysis of SC.

Table 4. Estimated coefficients of the fitted quadratic polynomial equation for response based on t-statistic

	Y (Yield of hydrolysate)	
	Coefficient	P-value
Intercept	42.1567	0.0001
X_1	0.9884	0.0091
X_2	-0.5673	0.0641
X_1X_1	-1.8880	0.0012
X_1X_2	-0.0425	0.9051
X_2X_2	-2.1531	0.0006

X_1(concentration of maxazyme, %), X_2(hydrolysis time, hr.)

Fitted model and analysis of variance The RSREG procedure through SAS was employed to fit the quadratic polynomial equation to the experimental data. The coefficients of linear (X_1, X_2), quadratic (X_{11}, X_{22}), interaction (X_{12}) regression, and intercept were calculated for significance (Table 4). The quadratic coefficients and X_1 term were highly significant (p<0.01), whereas, interaction and X_2 coefficients were not significant (p> 0.05). To develop the fitted response surface model equation, all insignificant terms (p>0.05) were eliminated, resulting in the fitted model of : $Y = 42.1567+0.9884X_1-1.8880X_1^2-2.1531X_2^2$, (Table 5). Determination coefficient (R^2) of the equation was 0.9527, and the fitted model was significant at p = 0.01, which indicates that the model is

Table 3. Evaluation of commercial proteases for the productivity (YCS/c) of chondroitin sulfate from shark (*S. oculata*) cartilage

Enzymes	Opt. pH	Opt. temp.	Cost(c)[1)]	Yield of CS (YCS)[2)]	Productivity (YCS/c)
Neutral					
Alcalase 2.4LFG	7.5±1.0	60°C	5.25	10.1	1.92
Flavourzyme 500MG	6.0±1.0	50°C	4.63	10.5	2.27
Protamex 1.5MG	6.0±1.5	40°C	6.25	10.7	1.71
Neutrase 0.8L	6.0±1.5	50°C	2.75	9.6	3.49
Bromelain 2000GDU	4.8±0.2	60°C	15.63	9.6	0.61
Maxazyme NNP	7.0±0.5	50°C	1.88	9.6	5.11
Sumizyme LP	6.0±1.0	50°C	11.88	11.2	0.94
Collupulin	6.5±1.0	60°C	4.25	8.6	2.02
Protease NP	7.0±1.0	50°C	2.88	9.4	3.26
Alkaline					
Delvolase	10.0±0.5	60°C	3.38	9.4	2.78
Acidic					
Natoozyme	5.0±0.5	38°C	1.00	2.3	2.30

[1)]Least expensive enzyme was given a value of 1.00.
[2)]It was calculated from yield of hydrolysate × CS content as shown in fig. 1.

Table 5. Response surface model for the hydrolysis of shark(*S. oculata*) cartilage

Response	Quadratic polynomial model	R^2	*p*-value
Y (Yield of hydrolysate)	$Y = 42.1567+0.9884X_1-1.8880X_1^2-2.1531X_2^2$	0.9527	0.0025

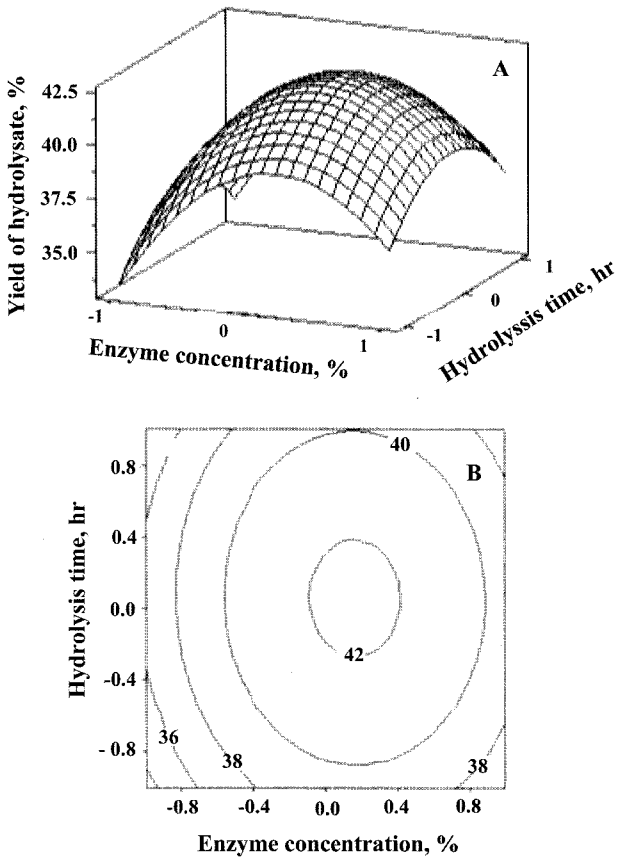

Fig. 2. Response surface (A) and contour (B) plot for hydrolysis of shark (*Squatina oculata*) cartilage by Maxazyme NNP as affected by enzyme concentration and hydrolysis time. Shark cartilage was hydrolyzed with 10 volumes (v/w) of D.W. at 60°C for 5 hr.

suitable to represent the real correlation among the reaction parameters. This result confirmed the adequacy of the experimental design based on the preliminary test. Table 6 shows the analysis of variance (ANOVA) for the fitted model that explains the response of the dependent variable, Y (yield of hydrolysate). Cross-product term was not significant (p = 0.9051), whereas, linear and quadratic terms were significant at 95 and 99% probability levels, respectively.

Table 7. Optimal conditions of enzymatic hydrolysis of shark (*s. oculata*) cartilage

Dependent variables	Independent variables	Critical value		Predicted value	Stationary point
		Coded	Uncoded		
Y (Yield of hydrolysate)	X_1	0.263	1.63	42.3%	Maximum
	X_2	-0.134	2.87		
Experimental value				43.0%	

X_1(concentration of maxazyme, %), X_2(hydrolysis time, hr.)

Optimal hydrolysis condition and verification SC was hydrolyzed (Table 2), and the resultant response by RSM is shown in Fig. 2. CS contents were almost equal (Fig. 1). Therefore, the optimization of SC hydrolysis could be determined from the factors for the highest yield of hydrolysate. Stationary point was a maximum from negative eigenvalues, which were calculated based on the results of statistical treatment through SAS. Optimal condition for the highest yield was determined by canonical analysis, X_1 (enzyme concentration) and X_2 (hydrolysis time) were 0.26 and -0.134, as the coded values, and could be converted into 1.63% and 2.87 hr, respectively, as experimental values. The highest yield of hydrolysate at this optimal condition was 42.3% as the predicted value (Table 7). To verify the statistically predicted optimal condition, shark cartilage was repeatedly hydrolyzed six times under the optimal condition. Yield of the hydrolysate was 43.1±2.1% and CS content was 24.8± 0.2%, which confirmed the validity of the statistically optimal conditions. The optimum conditions of enzymatic hydrolysis for purification of CS from shark (*Isurus oxyrinchus*) scapular cartilage was 2% (w/w) Alcalase for 10 hr at 55°C, and 0.5% (w/w) flavourzyme for 4 hr at 50°C (10), thus confirming that the optimization of hydrolysis condition of the shark (*Squatina oculata*) cartilage was successfully developed by RSM for the preparation of CS.

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

Response	Source	DF	SS	MS	F-value	*p*-value
Y (Yield of hydrolysate)	Model	5	46.294	9.269	20.16	0.0025
	Linear	2	10.388	5.194	11.31	0.0139
	Quadratic	2	35.899	17.950	39.09	0.0009
	Cross-product	1	0.007	0.007	0.02	0.9051
	Residual	5	2.296	0.459	-	-
	Lack of fit	3	2.096	0.699	6.98	0.1279
	Pure error	2	0.200	0.100	-	-
	Total	10	48.590	4.859	-	-

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