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# Optimization of Alcalase for Krill Byproduct Hydrolysis and Antioxidative Activities by Response Surface Methodology

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**Abstract**

**Krill byproduct was hydrolyzed with Alcalase 2.4L to produce functional ingredients for high antioxidative activities against 1,1-dimethyl-2-picryl-hydrazyl (DPPH) radical and Fe. The objective of this study was to investigate the optimum condition for degree of hydrolysis and antioxidative activity of enzymatic hydrolysate produced with the commercial Alcalase using response surface methodology (RSM) with a central composite rotatable design (CCRD). The ranges of independent variables were pH 7.6～10.4 for initial pH and 50.9～79.1°C for hydrolysis temperature and their dependent variables were degree of hydrolysis, Brix, amount of phenolic compounds, DPPH-scavenging activity and Fe-chelating activity. RSM with CCRD was well designed to investigate the optimum condition for functional ingredients with high antioxidative activities using Alcalase 2.4L because of their high $R^2$ values of the range of 0.93～0.99 except the $R^2$ value of 0.50 for the amount of total phenolic compounds. The optimum hydrolysis conditions were pH 9.5 and 62°C for degree of hydrolysis (DH) and pH 9.1 and 64°C for DPPH-scavenging activity by response surface methodology. The yield of DH and DPPH-scavenging activity were 14.1±0.5% and 10.5±0.2%, respectively. It is advantageous to determine the optimum hydrolysis conditions of krill and its by-products for the creation of different kinds of food products, as well as to increase the usage of marine protein sources.**

**Key words:** krill byproduct, degree of hydrolysis, Alcalase 2.4L, antioxidative activity, optimization, response surface methodology

## INTRODUCTION

About 6 billion tons of krill (*Eupausia superba*), also called antarctic shrimp, are caught yearly around the world (1). Krill is mainly used as feed and fishing material, even though it contains many functional ingredients. Also, although krill has high protein content, which is beneficial for improving the health of the elderly, infant growth and patient recovery, its use as a food for humans is very low, because of a generally low opinion of krill, its browning tendency, its bad smell and the hard job of deshelling it. With lots of research into making more valuable food products and functional ingredients from krill, its use can be more widely applied so that it will help to provide more food resources in the future (2).

Two main sources for protein hydrolysates are plant and animal proteins. Recently, fish protein hydrolysates have been investigated as the replacement for plant or other animal proteins (3). The enzymes used for enzymatic hydrolysis of fish protein were Neutrase 0.5L, complex enzyme 2000, Alcalase 2.4L, protease A, protease S, protease P, protease APL and Optimase APL (4). Alcalase 2.4L is commonly used for industrial processes because it is cheap and the reaction rate is fast under moderate conditions (5). Lots of protein sources, including fish protein, have been studied for their potential functional ingredients, but there are few papers on the enzymatic hydrolysate of krill.

Response surface methodology (RSM) is a method for investigating the optimum condition for the enzymatic reaction of protease. To perform RSM, there are independent variables and dependent variables which should be designed by using several models. The main independent variables for enzymatic hydrolysis are hydrolysis temperature, pH, time, enzyme concentration, and ratio of substrate to the added water. The important dependent variables are degree of hydrolysis (DH) and antioxidative activity (6). In this study, two main factors of hydrolysis temperature and pH were used, as well as the five dependent variables of DH, Brix, the amount of phenolic compounds, DPPH radical-scavenging activity and Fe-chelating activity. Therefore, the objectives

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of this study were to investigate the optimum hydrolysis of Alcalase 2.4L to maximize DH and antioxidative activities from krill byproduct and to study the relationship between DH and other factors.

## MATERIALS AND METHODS

### Materials

Krill byproduct used for this study was purchased from Dongwon F&B Co., Ltd. (Changwon, Korea). Krill was deshelled by automatic pilot plant processing procedures. The byproduct was delivered by the frozen state and it was freeze-dried. The freeze-dried krill byproduct was kept at a packed state in a deep freezer of -50°C.

### Procedure of enzymatic hydrolysate

The freeze-dried krill byproduct was ground and used for enzymatic hydrolysis. The used commercial protease was Alcalase 2.4L (Novozymes Korea Limited, Seoul, Korea), kept at 5°C. Enzymatic hydrolysate of krill byproducts was made by a little modified method from Gildberg and Stenberg (7). Six gram of krill sample was chopped and 40 mL distilled water was added. The autolytic enzyme of krill solution was inactivated by heating it at 85°C for 15 min. Its pH was adjusted with 2 M HCl or NaOH. 40 μL Alcalase 2.4L was added and the sample solution was hydrolyzed for 30 min in the adequate pHs and temperatures of RSM as shown in Table 1 and 2. Then, the hydrolyzed krill solution was inactivated by heating it to 85°C for 15 min. The solution was centrifuged at 4,000 rpm for 40 min. The supernatant was used for further research.

### Measurement of degree of hydrolysis of protein hydrolysate

Degree of hydrolysis was measured by a slightly modified method from Haslaniza et al. (8). One mL of enzymatic hydrolysate was put into a test tube and 2 mL of 0.3 M trichloroacetic acid was added. This was mixed with a Voltex mixer and the mixed solution was placed for 20 min at an ambient temperature. The solution was filtered with a filter paper (Whatmans No. 4) and into 25 μL of the filtered solution, 0.225 mL distilled water, 1.25 mL of 0.5 N NaOH, 0.25 mL of 1.0 N Folin & Ciocalteu's phenol reagent were added and mixed. The mixture was reacted for 15 min at 30°C. The insoluble materials of the reacted solution were removed by using a filter paper (Toyo no. 2). The absorbance of the filtered solution was measured at 750 nm and degree of hydrolysis was calculated by the below formula.

$$\text{Degree of hydrolysis } (\%) = \frac{D_t - D_0}{D_{max} - D_0} \times 100$$

Where $D_0$ is the absorbance of unhydrolyzed sample; $D_t$ is the absorbance of the sample hydrolyzed for t hours; and $D_{max}$ is the absorbance of the sample hydrolyzed with 4 mL 6 N HCl into 0.1 g sample for 24 hours at 110°C.

### Measurements of total phenols and DPPH-scavenging activity

The content of total phenolic compounds was measured according to the method of Whang et al. (9), with some modifications, in which the reaction of phenolic compounds with phophomolybdic acid to produce a blue color was used.

The antioxidative activity against DPPH was modified from the method of Šližyte et al. (10). Three mL of supernatant of the centrifuged sample was mixed with 0.15 mL DPPH reagent to make a sample solution. Control was 3 mL of the supernatant of the centrifuged sample and 0.15 mL MeOH. Blank test was the mixture of 3 mL MeOH : water (1.5:1) and 0.15 mL MeOH. For DPPH reagent, 10 mg DPPH was dissolved into 50 mL methanol. All treatments were covered with aluminum foil to protect from light and were reacted for 30 min at an ambient temperature. The absorbances of these solutions were measured at 519 nm, so $A_{Sample}$, $A_{Control}$, and $A_{Blank}$ are their absorbance values for sample, control and blank test. The antioxidative activity was calculated by the following formula.

$$\% \text{ Inhibition} = \frac{A_{Blank} - (A_{Sample} - A_{Control})}{A_{Blank}} \times 100$$

### Chelating activity against Fe (%)

Chelating activity of protein hydrolysate against Fe was measured using the method of Raghavan and Kristinsson (11). The 2.5 mL of krill hydrolysate solution was put into 12.5 mL methanol. It was centrifuged at 2,000 rpm for 10 min at 4°C and the supernant was used. Two mM $FeCl_2$ and 5 mM ferrozine were prepared. To a 3 mL supernant of sample solution, 0.1 mL $FeCl_2$ and 0.2 mL ferrozine were added, mixed and incubated at an ambient temperature for 30 min. The absorbance of the solution was measured at 561 nm. For blank test, a mixture of 3 mL MeOH, 0.1 mL $FeCl_2$ and 0.2 mL ferrozine was used. For control test, a mixture of 3 mL MeOH, 0.1 mL $FeCl_2$ and 0.2 mL ferrozine was used. Zero value was used with MeOH.

$$\% \text{ Metal chelation} = \frac{A_{Blank} - (A_{Sample} - A_{Control})}{A_{Blank}} \times 100$$

**Table 1.** Uncoded values of coded levels for the optimization of Alcalase in hydrolysis of krill byproduct

| Hydrolysis variable | Coded levels | | | | |
|---|---|---|---|---|---|
| | -1.414 | -1 | 0 | +1 | +1.414 |
| Initial reaction pH | 7.586 | 8 | 9 | 10 | 10.414 |
| Reaction temperature | 50.86 | 55 | 65 | 75 | 79.14 |

**Optimization on hydrolysis of Alcalase and statistic analysis**

The enzyme concentration as the ratio of enzyme against substrate concentration (E/S, %) was determined by pre-experimental data. Reaction pH and temperature are the important factors for the yield of enzyme hydrolysis. The response surface method used was the central composite design of using independent variables and coded values (12). Five coded levels were -1.414, -1, 0, 1, and +1.414, as shown in Table 1. Based on the data of the optimum condition for Alcalase, the experimental ranges and central points were determined as shown in Table 2. Experimental results were statistically analyzed by using SAS software (version 9.1, SAS Institute Inc., USA). The second order regression equations were obtained and their three dimensional figures were drawn by using Maple 8 software (Waterloo Maple Inc, Waterloo, Ontario, Canada).

## RESULTS AND DISCUSSION

The antioxidative activities in DPPH radical-scavenging activity and iron-chelating ability of krill hydrolysate were investigated using a surface response methodology. Initial pH and reaction temperature of hydrolysis were the independent variables. The degree of hydrolysis (DH), Brix, phenolics content, DPPH radical-scavenging activity, and iron-chelating ability were dependent variables. The experimental values for five dependent variables on the two independent variables are shown in Table 2. The data was statistically analyzed to study the optimum hydrolysis condition of Alcalase 2.4L.

**Response surface methodology for enzymatic hydrolysis of krill byproduct with Alcalase 2.4L**

Table 2 was statistically analyzed with the SAS program and the results are shown in Table 3. For two dependent variables, there are 13 experimental runs, in-

**Table 2.** Coded level combinations and their results for a two variable central composite rotatable design for Alcalase hydrolysis of krill byproduct

| Run | Independent variables | | Dependent variables | | | | |
|---|---|---|---|---|---|---|---|
| | Reaction pH ($X_1$) | Reaction temp. (°C) ($X_2$) | Degree of hydrolysis (%) ($Y_1$) | Brix ($Y_2$) | Total phenolics (%) ($Y_3$) | DPPH-scavenging activity (%) ($Y_4$) | Fe-chelating activity (%) ($Y_5$) |
| 1 | 0 | 0 | 13.46±0.36[1)] | 8.47±0.15 | 2.83±0.15 | 10.61±0.04 | 9.62±0.34 |
| 2 | 0 | 0 | 13.37±0.38 | 8.20±0.20 | 2.80±0.10 | 10.51±0.34 | 9.56±0.19 |
| 3 | 0 | 0 | 13.55±0.59 | 8.27±0.12 | 3.00±0.10 | 10.33±0.52 | 9.40±0.15 |
| 4 | 0 | 0 | 13.43±0.21 | 8.43±0.06 | 3.03±0.06 | 10.61±0.25 | 9.67±0.37 |
| 5 | 0 | 0 | 13.37±0.42 | 8.60±0.10 | 2.93±0.15 | 10.30±0.44 | 9.83±0.15 |
| 6 | -1 | -1 | 7.17±0.59 | 4.17±0.06 | 2.23±0.15 | 4.12±0.42 | 2.66±0.41 |
| 7 | -1 | 1 | 8.18±0.49 | 3.60±0.10 | 2.53±0.21 | 2.94±0.41 | 4.44±0.27 |
| 8 | 1 | -1 | 10.94±0.31 | 7.47±0.06 | 2.73±0.31 | 6.22±0.38 | 7.58±0.25 |
| 9 | 1 | 1 | 8.22±0.20 | 5.20±0.10 | 2.33±0.12 | 3.80±0.28 | 4.44±0.21 |
| 10 | 0 | -1.414 | 10.15±0.30 | 6.37±0.15 | 2.67±0.06 | 4.69±0.43 | 7.06±0.18 |
| 11 | 0 | +1.414 | 6.07±0.71 | 2.47±0.06 | 3.80±0.17 | 3.59±0.28 | 3.25±0.08 |
| 12 | -1.414 | 0 | 4.62±0.30 | 2.14±0.12 | 2.77±0.06 | 4.15±0.42 | 3.32±0.11 |
| 13 | +1.414 | 0 | 12.94±0.48 | 7.93±0.12 | 3.27±0.15 | 8.19±0.31 | 9.06±0.12 |

[1)]The values mean average±standard deviation for three replication.

**Table 3.** Effect of independent variables by multiple linear regression for producing hydrolysate from krill byproduct

| Factors | Coefficients | | | | |
|---|---|---|---|---|---|
| | Degree of hydrolysis (%) ($Y_1$) | Brix ($Y_2$) | Total phenolics (%) ($Y_3$) | DPPH-scavenging activity (%) ($Y_4$) | Fe-chelating activity (%) ($Y_5$) |
| Intercept | -350.430762$^{***}$ | -232.680965$^{***}$ | -31.528656 | -375.370097$^{***}$ | -331.862852$^{***}$ |
| pH | 49.568395$^{***}$ | 32.906765$^{***}$ | 6.851853 | 53.676603$^{***}$ | 44.447822$^{***}$ |
| Temp. | 4.158602$^{***}$ | 2.739009$^{***}$ | 0.101478 | 4.414803$^{***}$ | 4.211596$^{***}$ |
| $pH^2$ | -2.333462$^{***}$ | -1.583720$^{***}$ | -0.321084 | -2.834704$^{***}$ | -1.935049$^{***}$ |
| pH×Temp. | -0.085992 | -0.042500 | -0.017500 | -0.030833 | -0.122917 |
| $Temp.^2$ | -0.026784$^{***}$ | -0.018930$^{***}$ | 0.000575 | -0.032322$^{***}$ | -0.024535$^{***}$ |
| $R^2$ | 0.93 | 0.96 | 0.50 | 0.99 | 0.95 |
| Probability>F | 0.0007 | <0.0001 | 0.41 | <0.0001 | 0.0002 |

$^{***}$Significantly different at $p<0.001$.

cluding 5 central points for a response surface methodology. The Alcalase 2.4L is a proteolytic enzyme produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. The enzyme was provided from Novozyme Korea (Busan, Korea). According to the product sheet, the optimum conditions are temperatures between 55°C and 70°C, depending on the type of substrate and pH values between 6.5 and 8.5 (13). Based on these data and a preliminary test, the central point for hydrolysis temperature and pH was 65°C and pH 9.0, respectively. The hydrolysis temperature (65°C) for the central point was in the range of the optimum conditions provided on the product sheet, but the reaction pH was a little higher than the range of the sheet. The value of pH 9.0 for the central point was determined by the preliminary test. The range of $R^2$ values for the experiment was 0.93～0.99, indicating the proper method of response surface methodology for Alcalase 2.4L optimization for functional hydrolysates from krill byproducts was used.

Other studies used response surface methodology to investigate several independent and dependent variables affecting the optimum enzymatic hydrolysis. Some important independent variables studied are pH, reaction temperature and time, enzyme concentration, the ratio of substrate and the added water. First of all, the most important variables are thought to be pH and reaction temperature. Lee et al. (14) investigated the optimum condition of Cryotin F with shrimp by-product. The independent variables were pH, reaction temperature and time, enzyme concentration, and the dependent variables were DH, DPPH-activity and Fe-chelating activity. The $R^2$ values for DH, DPPH-activity and Fe-chelating activity were 0.86, 0.97 and 0.95, respectively, showing a good relationship with RSM. Xia et al. (15) used RSM for the optimization of enzymatic hydrolysis of *Bellamya purificata* snail foot protein.

**Degree of hydrolysis and brix of hydrolysate of krill byproduct with Alcalase 2.4L**

Protease breaks protein to peptides and, finally, to amino acids. During enzymatic hydrolysis, pH is lowered and Brix is increased as DH is increased. Table 3 shows the SAS results on degree of hydrolysis, Brix and pH of hydrolysate of krill byproduct from the data of Table 2. Their values of $R^2$ are 0.93 and 0.96, indicating a good RSM design. The parameters are significant in the $\alpha<0.001$ except the term for pH×temperature in Table 3. The variable of cross product of pH×temperature was not significantly different in the 95% significance level. Fig. 1 has three dimensional figures which show the effect of reaction pH and reaction temperature at the fixed value of 1% enzyme concentration and 30 min hydrolysis time from the formulas ($Y_{DH} = -400.736 + 49.5684 \times pH - 2.333462 \times pH^2 + 4.15860 \times Temp - 0.026784 \times Temp^2$; $Y_{BRIX} = -257.543 + 32.9068 \times pH - 1.58372 \times pH^2 + 2.739009 \times Temp - 0.018930 \times Temp^2$) which was obtained from Table 3. As shown in Fig. 1, the degree of hydrolysis showed the highest value. The optimum ranges for reaction pH and temperature of Alcalase in krill byproduct were pH 9.1～9.9 and 60～64°C, respectively, so the best point for the optimum condition was pH 9.5 and 62°C. At the optimum condition, the predicated yield was 14.1±0.5%. For the Brix results, the ranges for the optimum condition were pH 9.2～10.0 and 59～64°C reaction temperature, so their optimum values were pH 9.6 and 62°C. The Brix of the hydrolysate in the optimum condition was 9.0 ±0.3.

Depending on the substrate, the optimum temperature was changed. The optimum temperature for whey protein was 70°C and that for soy protein was 55°C. However, in the case of shrimp byproduct of such crustaceans family as krill, Holanda and Netto (16) had a similar research as this study. They hydrolyzed shrimp byproducts with Alcalase in the reaction condition of 3% ratio of

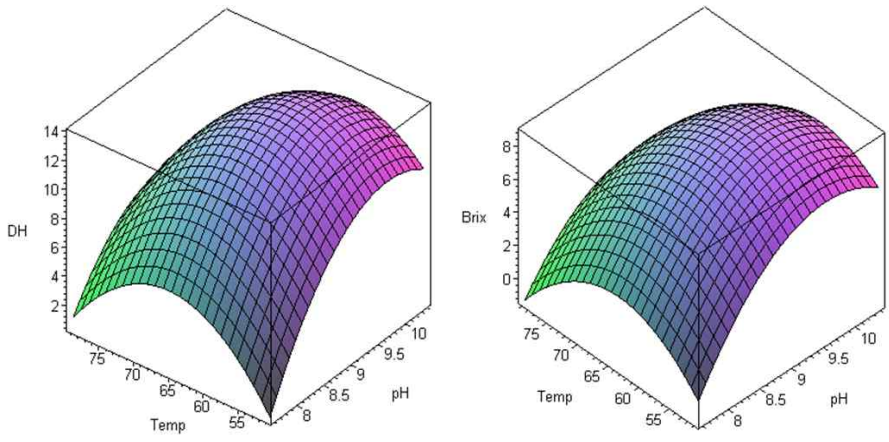


**Fig. 1.** Relationship between reaction pH and temperature on degree of hydrolysis and Brix by hydrolysis of krill byproduct in the fixed hydrolysis condition of 1% enzyme concentration of Alcalase 2.4L and 30 min hydrolysis time.

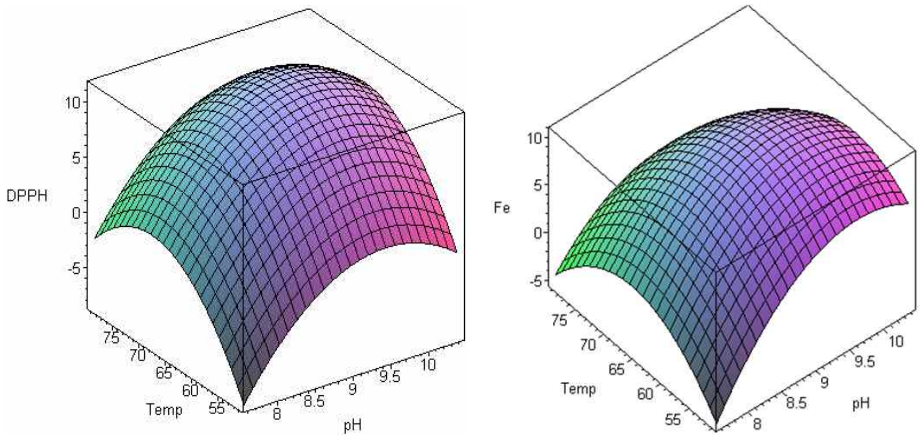


**Fig. 2.** Relationship between reaction pH and temperature on DPPH-scavenging and Fe-chelating activities by hydrolysis of krill by-product in the fixed hydrolysis condition of 1% enzyme concentration of Alcalase 2.4L and 30 min hydrolysis time.

enzyme/substrate (E/S), 10 min reaction time and 60°C reaction temperature and the final yield was 15%, showing the similar result of 13% in krill byproducts.

**Antioxidative activity of hydrolysate of krill by-product against DPPH radical**

The data from Table 2 was statistically analyzed and the SAS results are presented in Table 3, which shows the DPPH radical-scavenging ability of enzymatic hydrolysates. The meaningful data can be applied to make the relationship between reaction pH and reaction temperature for antioxidative activity on DPPH. The formula of "$Y_{DPPH} = -393.407 + 53.6766 \times pH - 2.834704 \times pH^2 + 4.414803 \times Temp - 0.032322 \times Temp^2$" is obtained and the relationship is shown in Fig. 2. Lee et al. (14) found that DPPH-scavenging activity of enzymatic hydrolysate from shrimp byproduct was more affected by reaction pH than by reaction temperature, showing higher activity at low pH of hydrolysis. Guérard et al. (17) reported that reaction pH and temperature (T) for enzymatic hydrolysis of yellowfin tuna byproducts were the two most important factors during hydrolysis for the analysis of variance in RSM ($p<0.001$). They reported that the optimum hydrolysis condition (temperature, pH and Alcalase 2.4L concentration) was pH 9.7, 66.2°C, enzyme concentration=68.1 Anson units (AU)/kg crude protein to obtain a hydrolysate with strong antioxidant activity measured using DPPH discoloration assay.

**Chelating activity of hydrolysate of krill byproduct against Fe**

The Fe-chelating activity of the hydrolysate from krill byproduct is shown in Table 2 and the data was statistically applied to obtain Table 3, which shows the significance level of the two independent variables for the optimum chelating activity. Then, the formula of "$Y_{Fe} = -403.769 + 44.447822 \times pH - 1.935049 \times pH^2 + 4.211596 \times Temp - 0.024535 \times Temp^2$" is obtained and the Maple result is given in Fig. 2, which shows the relationship between reaction pH and reaction temperature for Fe-chelating activity. The low molecular weight peptides and amino acids formed during hydrolysis of krill byproducts with Alcalase 2.4L are expected to be responsible for the antioxidant activity. In addition, krill byproducts contain astaxanthin, so the amount and activity of phenolic compounds including astaxanthin were expected to have some role in the total antioxidative activities. However, the amounts of phenolic compounds did not show a relationship with hydrolysis, even though astaxanthin has very strong antioxidative activity. Lee et al. (14) reported that Fe-chelating activity of enzymatic hydrolysate was more affected by reaction pH than by reaction temperature. The chelating activity is increased with increasing alkalinity.

## CONCLUSION

Response surface methodology was applied to investigate the optimum hydrolysis condition of Alcalase 2.4L for krill byproduct. The optimum condition of DH is pH 9.5 and 62°C, showing the almost same tendency as Brix. Therefore, Brix can be used to study the maximum hydrolysis instead of measuring DH. Difference of the initial pH and the final pH may be thought to have a relationship with the change of DH. With this study in future, the optimum condition can be easily measured. With this optimum condition, krill byproducts can be hydrolyzed to make functional ingredients.

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