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# Optimization of Enzymatic Hydrolysis Conditions for Production of Angiotensin-I Converting Enzyme Inhibitory Peptide from Casein

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**Abstract** This study was carried out to investigate an optimum condition for the high angiotensin-I converting enzyme (ACE) inhibitory activity and the yield on enzyme concentration, casein concentration, and hydrolysis time. The optimum condition was performed by response surface methodology for acquirement of casein hydrolysate of milk which shows high ACE inhibitory activity. Among 8 tested enzymes, Protamex showed the highest activation degree with 77.03 unit/g from casein. Their hydrolysis degrees of flovourzyme 500MG, protamex, mixture from 1% casein were 85.5, 88.5, and 93.5%, respectively. The ranges of enzyme concentration (0.25-1.25%), casein concentration (2.5-12.5%), and hydrolysis time (20-100 min) as 3 independent variables through preliminary experiments of the yield of casein hydrolysate and ACE inhibitory activity, and it shows optimum response surface at a saddle point. It shows enzyme concentration (0.64%), casein concentration (8.38%), and hydrolysis time (55.81 min) in the yield aspect and showed the highest activity at enzyme concentration (0.86%), casein concentration (5.97%), and hydrolysis time (63.86 min) in ACE inhibitory aspect. The $R^2$ value of a fitted optimum formula on the hydrolysis yield was 0.9751 as the significant level of 1%. The $R^2$ value of a fitted optimum formula on ACE inhibitory activity is 0.8398, and the significance is recognized in the range of 5%.

**Keywords**: casein, angiotensin-I converting enzyme (ACE) inhibitory activity, response surface methodology (RSM), hydrolysis, optimization

## Introduction

Hypertension is known to induce such direct cardiac circulating diseases as cardiac incompetence, decrease in kidney function, cerebral apoplexy. The reason of hypertension has been explained by a biochemical mechanism of renin-angiotensin system. Histidine-leucine of C-end of angiotensin-I in blood is degraded by the action of ACE, and then, angiotensin-I is changed to angiotensin-II of a hormone related to strong increase of blood pressure. Aldosterone in adrenal cortex is actively released, which increases blood pressure by inhibiting release of water and $Na^+$ (1). To reduce the increase of the blood pressure, inhibition of ACE activity is known to be important. ACE-inhibiting peptide is first found in snake toxic study. The activation mechanism between ACE and carboxypeptidase A was similar and proline residue of C-end is found to be important for ACE-inhibition. Based on the above studies, the chemical compounds including captopril and enarapril for ACE inhibition were chemically synthesized, so medicine for curing hypertension has been commercialized by using these chemicals (2). However, these ACE-inhibiting chemicals affect other cells and produce such bad effects as headache, inappetence, gestation change, decrease in leukocyte. The studies on screening and development for more safe and efficient ACE inhibitors are demanded from natural resources (3).

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Researches on several biofunctional ingredients have been actively found from food materials, so new biofunctional foods have been developed. When such adult diseases as hypertension and atherosclerosis are mostly thought to closely relate with dietary habit, for disease prevention, it is very meaningful to screen the effective components for drop of blood pressure from normally dietary food components (4). ACE-inhibiting effect in food components has been studied from the peptides obtained from the proteolytic hydrolysates of several food proteins which were α-zein (5), sardine (6), mackerel (7), syconus latex (8), and soybean (9-11).

ACE-inhibiting peptides of casein were studied from a fraction of trypsin-degrading peptides (10). Yamamoto *et al.* (13) isolated ACE-inhibiting peptides from sour milk which was fermented with the starter of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. The ACE-inhibiting peptides obtained from trypsin hydrolysates of casein showed the effect of drop of blood pressure in intravenous injection (13), but there was no effect on oral experiment of trypsin hydrolysates to SHR in the results of Yamamoto *et al.* (13). Though the peptides separated from sour milk have an effect of drop of blood pressure, particular microorganism related to processing of fermented milk should be used and the fermentation should be long-time fermentation. The method has a problem that is difficult to apply to short-term fermentation. Also, its storage is difficult because yeast as well as lactic acid bacteria are used. The amount of the peptide which has the effect of ACE-inhibiting activity was very small, so it has a problem in isolating and purifying the peptide and

applying to food ingredients for food and nutraceutical industries. In order to solve the above problems and obtain peptides of ACE-inhibiting activity, casein hydrolysates were tried. The objective of this study was to investigate the optimum hydrolysis condition for high hydrolysis yield and maximum ACE-inhibiting activity by treating casein with proteases.

## Materials and Methods

**Materials** Whey powder, skim milk powder, and Na-caseinate used in this study were purchased from Virak Co. (Korea). Flavourzyme 500MG (Novo Nordisk, Bagsvaerd, Denmark), Protamex (Novo Nordisk), Papain 30,000 (Oka Zone International, IN, USA), protease NP (Bioland, Asan, Korea), GC 106 (Oka zone international), Multifect nutral (Oka zone international), neutrase (Novo Nordisk), and Alcalase (Novo Nordisk) were used as enzymes for hydrolysis of whey powder, skim milk powder, and Na-caseinate.

**Measurement of enzymatic endopeptidase activity** Endopeptidase activity of all the enzymes used in this study was measured as the following method. Each of 1 mL of the 1% skim milk, whey, and casein solutions adjusted to pH 7.0-7.3 was put for 5 min at 50°C water bath and 1 mL of 100 mg/mL enzyme concentration was reacted for 30 min. The 2 mL of 0.4 M trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) was mixed to the hydrolytic solution and put for 25 min at the 50°C water bath. The sample solution was centrifuged at 1,935×g for 10 min and 1 mL of the upper part was obtained. In the upper part, 5 mL of 0.4 M sodium carbonate and 1 mL of 50% Folin & Ciocalteu's (Sigma) reagent were mixed and colorized for 5 min at 50°C. The absorbance of the sample solution was measured at 660 nm of a spectrometer (V-570; Jasco, Tokyo, Japan). For the control solution, 1 mL of 1% casein solution, 2 mL of 0.4 M TCA and 1 mL enzyme were mixed and the same method as the above procedure was carried out. One unit of endopeptidase activity was defined as the activity for the production of 100 μg L-cysteine in the 1 mL upper part of the reaction.

$$\text{Protease activity (unit/g)} = (At\text{-}Ao) \times F \times 1/100 \times n$$

Where At is the absorbance of sample treatment, Ao is the absorbance of control, F is the amount of L-cysteine obtained from a formula at 1.000 value of L-cysteine absorbance × dilution factor of enzyme (μg) and n is dilution times of sample solution.

**Enzymatic hydrolysis according to enzyme and casein concentrations** Based on the above results of enzymatic activity of endopeptidase, protamex, flavourzyme 500MG, and mixture (protamex/flavozyme 500MG = 1:1) were used for casein hydrolysis. Namely, to 1% casein solution, 0.5, 1, 1.5 and 2%(v/w) enzyme concentrations were hydrolyzed with agitation for 24 hr in a water bath of 50± 2°C. The 1 mL hydrolysis solution and 1 mL of 0.4 M TCA were mixed and centrifuged at 1,882×g for 20 min and the absorbance of the solution was measured at 280 nm. To the 1, 5, and 10% casein concentrations, 1% of each of protamex, flavourzyme 500MG, mixture (protamex, flavozyme 500MG, 1:1) was added. The sample solution of pH 6.8-7.0 was hydrolyzed with agitation for 24 hr at 50°C water bath. The degree of hydrolysis was measured as the dried weight after the reaction solution was centrifuged at 3,836×g for 30 min and the upper part was freeze-dried.

**Measurement of ACE inhibitory activity** ACE inhibitory activity was measured according to Cushman's method (2). Namely, 1 g/10 mL (w/v) of rabbit lung acetone powder (Sigma) was extracted within 0.1 M sodium borate buffer (pH 8.3) including 0.3 M NaCl for 24 hr at 4°C. The extraction solution was centrifuged for 40 min at 40,000×g and 4°C. The crude ACE was obtained. For the measure-ment of ACE inhibitory activity, 100 μL of 0.1 M sodium borate buffer (pH 8.3) and 50 μL of crude ACE solution was added to 50 μL of 10 mg/mL sample solution. The mixed solution was initially reacted for 5 min at 37°C and then reacted for 30 min at 37°C after adding 50 μL of the substrate which was made by adding 25 mg hippuryl-histidyl-leucine (HHL) to 5 mL of 0.1M sodium borate buffer (pH 8.3) including 0.3 M NaCl. After the reaction was stopped by adding 250 μL of 1 N HCl, 1.5 mL ethyl acetate was added and agitated for 15 sec. The 1 mL upper part was obtained by centrifugation (1,882×g /5 min, 4°C). The upper part was completely dried for 30 min at 120°C and 3 mL distilled water was added. The absorbance of the solubilized solution was measured at 228 nm and the value was used for ACE inhibitory activity. For the control treatment, 50 μL extraction solvent instead of extraction solution was used and the ACE inhibitory activity was calculated as the following formula.

$$\text{ACE inhibition (\%)} = 1 - \left(\frac{Sa-Sb}{Ca-Cb}\right) \times 100$$

Where Sa is sample absorbance, Sb is absorbance of sample blank, Ca is control absorbance, and Cb is absorbance of control blank.

**Response surface methodology** Enzyme hydrolysis from casein was optimized for maximum ACE inhibition by statistical response surface methodology (RSM). Experimental design for optimization analysis was central

**Table 1. Endopeptidase activities of enzymes used**

| Enzymes | Enzyme activity (unit/g) | | |
|---|---|---|---|
| | Whey | Skim milk | Casein |
| Flavourzyme 500MG | 22.25 | 36.5 | 65.08 |
| Protamex | 21.22 | 45.83 | 77.03 |
| Papain 3000 | 9.15 | 13.33 | 43.34 |
| Protease NP | 1.25 | 9.93 | 36.23 |
| GC 160 | 14.24 | 32.71 | 53.76 |
| Multifect Nutral | 5.52 | 17.14 | 41.55 |
| Neutrase | 5.31 | 16.41 | 41.62 |
| Alcalase | 15.79 | 25.16 | 42.81 |

composite design (15). Enzyme concentration ($X_1$, %), casein concentration ($X_2$, %), and enzymatic hydrolysis time ($X_3$, min) were selected as the independent variables. The ranges and center points of 3 independent variables were based on the results of preliminary experiments (Table 2). The coded values of independent variables for the central composite design on the dependent variables of the yield and ACE inhibitory activity of hydrolysate from casein were shown in Table 3. Experimental runs were randomized in order to minimize the effects of unexpected variability in the observed responses. The response surface regression (RSREG) procedure of SAS (Version 8.01, SAS Institute Inc., Cary, NC, USA) was used to fit the following quadratic polynomial equation (1):

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

Where Y is the dependent variable ($Y_1$, ACE inhibitory activity (%); $Y_2$, recovery rate of hydrolysate (%), $\beta_0$ is constant, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are regression coefficients and $X_i$, $X_j$ are levels of the independent variables.

The surface response plots were developed using Maple software (Maple 7, Waterloo Maple Inc., Maple, ONT, Canada). The relationships as a function of 2 independent variables were shown at the fixed value of the optimum condition for the other independent variable.

**Statistical treatment** Data were subjected to analysis of variance (ANOVA) ($p<0.05$). Means were separated using Duncan's multiple range test ($\alpha=0.05$). Analysis was performed using the REG procedure of SAS (Version 8.01, SAS Institute Inc.).

## Results and Discussion

**Endopeptidase activity of several enzymes on casein, skim milk, and whey** Enzymatic activities of the 7 preteases on casein, skim milk, and whey were shown in Table 1. The best substrate among 3 kinds of dairy products was casein for all enzymes. In the result of hydrolytic activities of the enzymes on casein, protamex showed the highest activity of 77.03 unit/g for casein. Enzyme activities of flavourzyme 500MG were 65.08 unit/g for casein. This result that protamex had the highest activity among endopeptidase activities of hydrolytic proteases showed the same tendency as Kim *et al.* (16). In the substrate of casein for other enzymes, GC 160 showed activity of 53.76 unit/g and alcalase showed 42.81 unit/g. Also, in the case of skim milk, protamex showed the highest activity of 45.83 unit/g, and flavourzyme 500MG showed 36.50 unit/g. In the substrate of skim milk, alcalase had the activities of 25.16 unit/g, and GC 160 was 32.71 unit/g, showing low activities. In the case of whey, all the enzymes showed low activities. In the result of Yoon *et al.* (17), proteolytic activity of kiwifruit was 196.95 unit/mg protein in the optimum condition of pH 3.0 and 60°C. Their hydrolysis yields were 73.5 and 89.3 % for 10 and 20 min reaction times, respectively.

**Table 2. Levels of independent variables for experimental design**

| Variables | Symbols | Coded variables | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | 1 | 2 |
| Enzyme concentration (%) | $X_1$ | 0.25 | 0.5 | 0.75 | 1 | 1.25 |
| Casein concentration (%) | $X_2$ | 2.5 | 5 | 7.5 | 10 | 12.5 |
| Hydrolysis time (min) | $X_3$ | 20 | 40 | 60 | 80 | 100 |

**Effect of enzyme mixture and casein concentrations on enzymatic hydrolysis** When protamex and flavourzyme 500MG were mixed to the ratio of 1:1, the degrees of enzymatic hydrolysis on the enzyme concentrations of 0.5, 1, 1.5, and 2% did not showed big difference. In the result of hydrolysis yields on casein concentration, hydrolysis yields of 1% casein had 85.5, 88.5, and 92.5% for flavourzyme 500MG, protamex, and mixture, respectively. In the case of casein 5%, they were 79.5, 86.0, and 90.5% for flavourzyme 500MG, protamex, and mixture, respectively. In the case of casein 10%, the lowest hydrolysis yields for flavourzyme 500MG, protamex, and mixture were shown with the values of 73.0, 76.6, and 88.1%, respectively.

**ACE inhibitory activities on hydrolysis yield of casein** ACE inhibitory activities on hydrolysis yield of casein for several enzymes were measured and the results of ACE inhibitory activities during hydrolysis times of 24 hr in casein were shown in Fig. 1-3. Casein hydrolysate by protamex generally showed good ACE inhibitory activity and the mixture and flavorzyme were the next following order. In the result of ACE inhibitory activities during hydrolysis time of 24 hr on casein, protamex showed the ACE inhibitory activities of 74.5 and 84.6% for 1 and 24 hr, respectively, and the mixture showed the 61.8 and 74.1% activities for 1 and 24 hr, respectively. The flavourzyme showed the 43.4 and 60.9% activities for 1 and 24 hr, respectively. Hydrolysis degree and ACE inhibitory activity were sharply increased within an hour and they were slowly increased with hydrolysis time of 1 to 24 hr, showing no big difference. Yoon *et al.* (18) enzymatically hydrolyzed casein from 8 to 24 hr with the

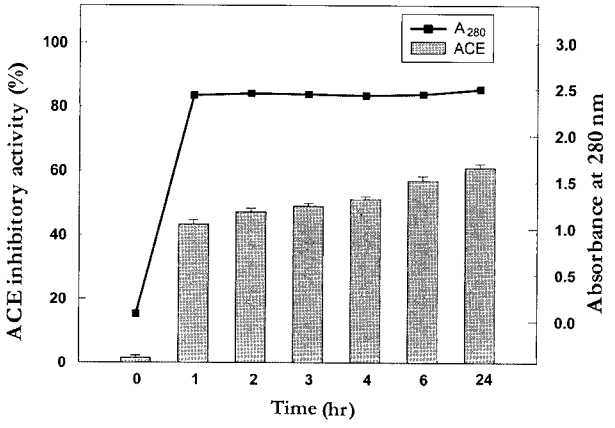



**Fig. 1. ACE inhibitory activity and hydrolysis rate of casein hydrolysate by Flavourzyme 500MG.**

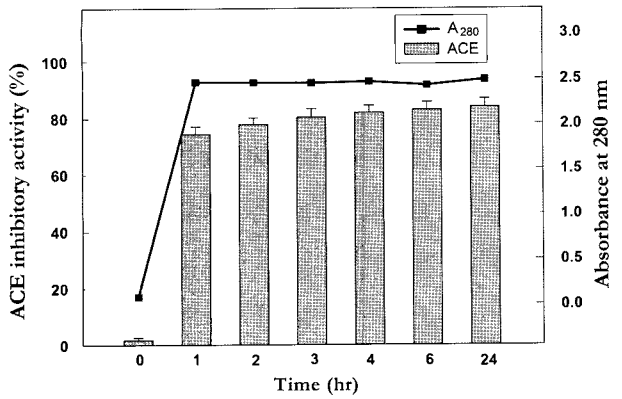



**Fig. 2. ACE inhibitory activity and hydrolysis rate of casein hydrolysate by Protamex.**

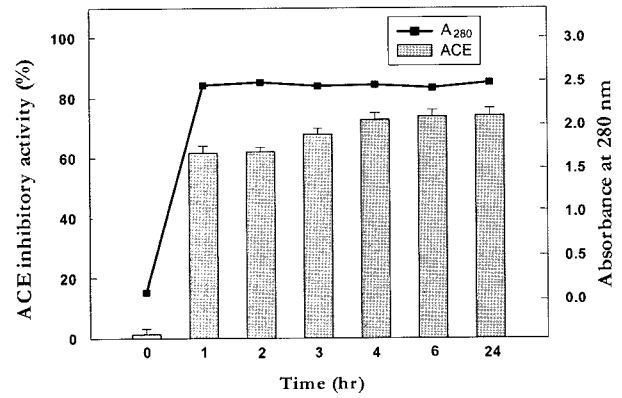



**Fig. 3. ACE inhibitory activity and hydrolysis rate of casein hydrolysate by mixture (Flavourzyme 500MG, Protamex).**

intervals of 8, 12, 24, and 48 hr. ACE inhibitory activities of hydrolysates of all the enzymes were increased with the reaction time.

**Surface response analysis on hydrolysis yield and ACE inhibitory activity** Surface response analysis (SRA), one of statistical analyses, has one or more independent variables which are changed by complicated functions of several independent variables on reaction changes. In this study, SRA was carried out for the purpose of producing milk-related ingredients with high anti-hypertension function. SRA was analyzed by using SAS program and its tried experimental design was central composite design. Based on the above experimental design, canonical correlation analysis showed not maximum and minimum values but saddle point for X. The procedure of canonical correlation analysis is explained in the below sentences.

**Table 3. Responses of dependent variables for the yield and ACE inhibitory activity of enzymatic hydrolysate from casein to independent variables[1)]**

| Experimental no. | Level of variable | | | Response | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $Y_1$ | $Y_2$ |
| 1 | -1 | -1 | -1 | 75.76 | 66.9 |
| 2 | -1 | -1 | 1 | 75.85 | 71.1 |
| 3 | -1 | 1 | -1 | 71.74 | 60.1 |
| 4 | -1 | 1 | 1 | 76.07 | 64.6 |
| 5 | 1 | -1 | -1 | 77.14 | 74.5 |
| 6 | 1 | -1 | 1 | 78.28 | 79.2 |
| 7 | 1 | 1 | -1 | 72.34 | 65.8 |
| 8 | 1 | 1 | 1 | 77.13 | 72.5 |
| 9 | 0 | 0 | 0 | 75.28 | 69.8 |
| 10 | 0 | 0 | 0 | 74.33 | 70.1 |
| 11 | 0 | 0 | 0 | 76.22 | 69.2 |
| 12 | -2 | 0 | 0 | 62.89 | 56.7 |
| 13 | 2 | 0 | 0 | 77.72 | 76.0 |
| 14 | 0 | -2 | 0 | 80.25 | 80.1 |
| 15 | 0 | 2 | 0 | 74.54 | 61.3 |
| 16 | 0 | 0 | -2 | 72.88 | 63.9 |
| 17 | 0 | 0 | 2 | 75.67 | 70.6 |

[1)]$X_1$, Enzyme concentration (%); $X_2$, casein concentration (%); $X_3$, hydrolysis time (min); $Y_1$, ACE inhibitory activity (%); $Y_2$, recovery rate of hydrolysate (%).

The formulas are shown in Table 5. The shapes of the formulas were shown in Fig. 4 and 5. In the result of canonical correlation analysis, when surface response is explained as canonical form, the shapes of surface response around maximum point can be predicted.

As shown in Table 5, because X values are existed as positive and negative, Y values can be higher or lower than $Y_0$ values. So $X_0$ can be saddle point.

Based on the above formula, the values of $Y_1$ are increased or decreased as X of independent variable is changed. The values of $X_1$, $X_3$, $X_1X_3$, $X_2X_3$, $X_2^2$ affected the increase of $Y_1$ value and the values of $X_2$, $X_1X_2$, $X_1^2$, $X_3^2$ affected the decrease of $Y_1$ value.

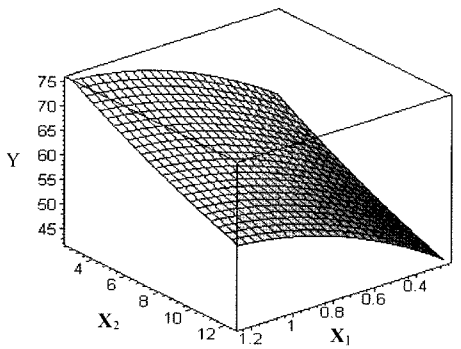
$Y_2$ showed the same tendency as $Y_1$. The values of $X_1$, $X_3$, $X_1X_3$, $X_2X_3$, $X_2^2$ affected the increase of $Y_2$ value and the values of $X_2$, $X_1X_2$, $X_1^2$, $X_3^2$ affected the decrease of $Y_2$ value.

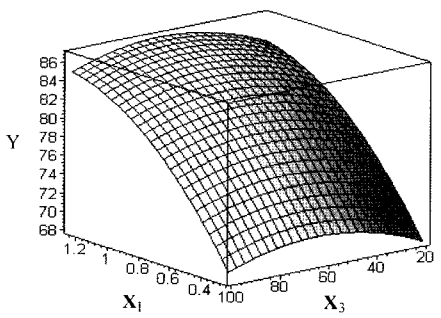
F-ratio is calculated by dividing to sum of square for error. If the calculated F value is higher than the

**Table 4. Predicted level of hydrolysis conditions for the optimum response of variables by the response surface methodology[1)]**

| Responses | $R^2$ | Pro>F | $X_1$ | $X_2$ | $X_3$ | Maximum | Morphology |
|---|---|---|---|---|---|---|---|
| Hydrolysis yield (g) | 0.9751 | 0.0001 | 0.6409 | 8.3870 | 55.81 | 3.9965 | Saddle point |
| ACE inhibitory activity (%) | 0.8398 | 0.0387 | 0.8698 | 5.9732 | 63.86 | 77.6647 | Saddle point |

[1)]$X_1$, Enzyme concentration (%); $X_2$, casein concentration (%); $X_3$, hydrolysis time (min).



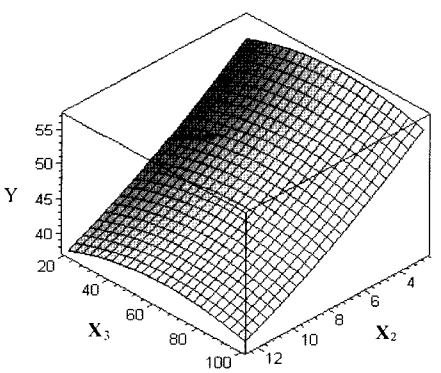
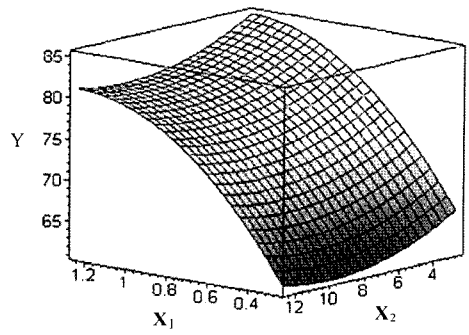
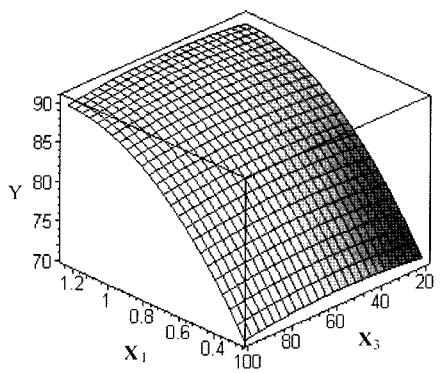




**Fig. 4. Response surface plots for optimization of recovery rate of hydrolysate from casein.** $X_1$, Enzyme concentration (%); $X_2$, casein concentration (%); $X_3$, hydrolysis time (min); Y, degree of hydrolysis (%).



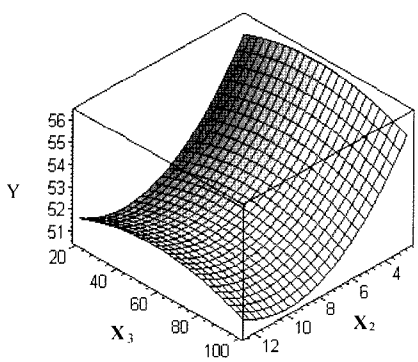




**Fig. 5. Response surface plots for optimization of ACE inhibitory activity of hydrolysate from casein.** $X_1$, Enzyme concentration (%); $X_2$, casein concentration (%); $X_3$, hydrolysis time (min); Y, ACE inhibitory activity (%).

probability value for the significant level, the model equation is significant. Namely, in the case of 95% confidence level, the predicted reaction model is adequate, if the probability is less than 0.05.

As shown in Table 6 and 7, the values of probability >F were 0.001 and 0.0387, respectively, the reaction models for Fig. 4 were statistically significant. Optimum hydrolysis yield was investigated by studying the relationship between enzyme concentration and substrate concentration.

As higher enzyme concentration and lower substrate concentration were, higher hydrolysis yield was obtained. This result was thought to be due to high water-holding capacity of the substrate. When casein concentration was close to 10%, enzyme activity was sharply decreased, so the result was thought to be occurred. In the case of high enzyme concentration and low substrate concentration, high hydrolysis yield was confirmed in Fig. 4. Different from the above result, hydrolysis yield on hydrolysis time was not changed a lot. Low hydrolysis yield was observed in initial and final times (Fig. 4).

Based on the experimental results, response surface regression of hydrolysis yield on 3 independent variables of enzyme concentration (0.25-1.25%), casein concentration (2.5-12.5%), hydrolytic time (20-100 min) was shown in the below formula, having a saddle point for optimum reaction. The optimum hydrolysis yield of 63.86% was shown in the reaction condition of 0.64% enzyme concentration, 8.38% casein concentration and 55.81 min hydrolysis time. $R^2$ for hydrolysis yield was 0.9751, showing significance in the confidence level of 99% (Table 4, Fig. 4).

As shown in Fig. 4, the results of Fig. 5 which is

**Table 5. Response surface model equations for ACE inhibitory activity from hydrolysate and hydrolysis yield from casein[1)]**

| Responses | Quadratic polynomial model |
|---|---|
| $Y_1$ | $Y_1 = 56.855707 + 35.301087X_1 - 2.374891X_2 + 0.183764X_3 - 0.420000X_1X_2 + 0.067500X_1X_3 + 0.005750X_2X_3 - 12.817391X_1^2 + 0.045826X_2^2 - 0.001440X_3^2$ |
| $Y_2$ | $Y_2 = 58.616603 + 48.455543X_1 - 1.772946X_2 + 0.059694X_3 - 1.030000X_1X_2 + 0.037250X_1X_3 + 0.012225X_2X_3 - 19.308696X_1^2 + 0.090513X_2^2 - 0.000536X_3^2$ |

[1)]$X_1$, Enzyme concentration (%); $X_2$, casein concentration (%); $X_3$, hydrolysis time (min); $Y_1$, ACE inhibitory activity (%); $Y_2$, recovery rate of hydrolysate (%).

**Table 6. Table of analysis of variance for surface response on hydrolysis yield by central composite design**

| Regression | Type DF | Sum of squares | $R^2$ | F Value | Pr > F |
|---|---|---|---|---|---|
| Linear | 3 | 633.021875 | 0.9345 | 87.55 | < .0001 |
| Quadratic | 3 | 25.382465 | 0.0375 | 3.51 | 0.0777 |
| Crossproduct | 3 | 2.123750 | 0.0031 | 0.29 | 0.8290 |
| Total model | 9 | 66.528090 | 0.9751 | 30.45 | < .0001 |

**Table 7. Table of analysis of variance for surface response on ACE inhibitory activity by central composite design**

| Regression | Type DF | Sum of squares | $R^2$ | F value | Pr > F |
|---|---|---|---|---|---|
| Linear | 3 | 133.899149 | 0.5807 | 8.46 | 0.0100 |
| Quadratic | 3 | 53.148704 | 0.2305 | 3.36 | 0.0847 |
| Crossproduct | 3 | 6.581838 | 0.0285 | 0.42 | 0.7472 |
| Total model | 9 | 193.629961 | 0.8398 | 4.08 | 0.0387 |

obtained from the relationship between enzyme concentration and substrate concentration, high ACE inhibitory activity was shown in the condition of low substrate concentration and high enzyme concentration.

This result can be shown in the condition of high enzyme activity. One of the important factors which affect such result can be thought to be water activity. The result was thought to be the cause of controlling ACE inhibitory activity system.

The result of Fig. 5 showed the relationship between hydrolysis time and enzyme concentration on antihypertension activity. As enzyme concentration increased, the ACE inhibitory activity increased. There was no big difference in hydrolysis yield on hydrolysis time. The result of Fig. 5 showed the relationship between hydrolysis time and substrate concentration on ACE inhibitory activity. As substrate concentration was decreased from 10 to 4% and increased from 10 to 12%, the ACE inhibitory activity was increased.

The reason why maximum point of all the graphs showed a saddle point was thought that central composite design on enzyme concentration, casein concentration, and hydrolysis time could not recognize the maximum value on its maximum ACE inhibitory activity.

In the case of casein concentration, more than 10% casein concentration was the cause of too much viscous solution, showing very low enzyme activity.

Because too much hydrolysis time reduces economical value, this experimental design was made. Considering the above aspect, saddle points of all the graphs were obtained.

Response surface regression of ACE inhibitory activity on 3 independent variables of enzyme concentration (0.25-1.25%), casein concentration (2.5-12.5%), and hydrolysis time (20-100 min) was shown in the below formula, having a saddle point for optimum reaction. The reaction condition of 0.86% enzyme concentration, 5.97% casein concentration 63.86 min in hydrolysis time was best for an optimum ACE inhibitory activity. $R^2$ for surface response regression of ACE inhibitory activity was 0.8398, showing significance in the confidence level of 95% (Table 5, Fig. 5).

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