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Response Surface Methodology in Development of Oyster Hydrolysate

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Abstract

The optimal condition for hydrolysis of oyster was evaluated with proteases using response surface methodology (RSM). Among 11 commercial proteases, APL™ 440 was selected as the suitable protease for producing oyster hydrolysate on the basis of cost per unit enzyme activity. The effect of autolysis on degree of hydrolysis in oyster was negligible comparing to that of APL 440 protease treatment. From RSM and ridge analysis, the conditions favoring the highest degree of hydrolysis were pH 9.95, 61.1°C, 2.64 hr reaction time, 49.2% substrate, and 0.35% enzyme/substrate ratio. Oyster hydrolysate prepared under optimal conditions showed virtually 51.98% of hydrolysis.

Key words : oyster hydrolysate, response surface methodology, protease

INTRODUCTION

Oysters, having a unique taste and aroma, are favored throughout the world. However, in recent years consumption of fresh oysters has declined due to the potential health hazard associated with their consumption. An alternative use for unconsumed oysters may be the production of flavorants via enzymatic hydrolysis. A wide range of hydrolyzed shellfish protein products may be produced by choosing the enzyme type and hydrolysis conditions. Many studies have been conducted to produce hydrolysate and flavorants from seafoods by enzymatic hydrolysis (1,2). Rebeca *et al.* (3) reported about nutritional value on fish protein hydrolysates produced with bacterial proteases.

Nowadays, increased environmental awareness and strict regulations have prompted. The shellfish processing industry to seek a viable alternative utilization for byproducts of seafood processing. Many potentially recoverable compounds were produced from shellfish byproducts (4-7). Developing for natural seasoning agents from shellfish with commercial proteases was also attempted by Lee *et al.* (8) and Choi *et al.* (9). However, the parameters affecting to the hydrolysis of seafood have not been fully studied on the determination of optimal conditions for the hydrolysis except for a few reports (10,11).

The aim of this study was to determine optimal hydrolysis conditions for oyster using RSM.

MATERIALS AND METHODS

Materials

Fresh oysters (*Crassostrea gigas*) purchased from a fish market in Masan, Korea were packed in ice and transported within 1 hr to the Food Science & Nutrition Department, Changwon National University and stored at 4°C. Before analysis, oysters were homogenized using a Waring blender (Waring Products Co., Winsted, CT). Eleven commercial proteases were obtained from Amano International Enzyme Co. Inc. (Japan), Solvay Enzymes, Inc. (USA), Novo Nordisk Bioindustrials, Inc. (Denmark), and Pacific Chemical Co. LTD. (Korea).

Enzymatic hydrolysis of oyster

A mixture of oyster homogenate (50g) and distilled water (30ml) was placed into a 100-ml jacketed reaction vessel (Wheaton, Millville, NJ) and preincubated for 10min at each enzyme's optimal temperature. Freshly prepared enzyme solution was added to the vessel, and the mixture was vigorously stirred during reaction using a magnetic stirrer. After 1 hr reaction,

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The 0.3M trichloroacetic acid (TCA) soluble peptides were analyzed by a modification of Lowry method (12). Further details have been reported elsewhere (10,13).

Estimation of the degree of hydrolysis

The degree of hydrolysis was determined using a modification of the method described by Boudrant and Cheftel (14) and defined by the following ratio :

$$D_t - D_o / D_{max} - D_o$$

Where D_o is the blank (amount of 0.3M TCA soluble peptides of unhydrolyzed oyster as tyrosine), D_{max} corresponds to the maximum amount of 0.3M TCA soluble peptides as tyrosine, which was made after hydrolysis of 0.1g of oyster with 4ml of 6N HCl at 110°C for 24hr. D_t was the corrected amount of 0.3 M TCA soluble peptides at time t. This amount was determined by subtracting the amount of TCA soluble peptides of enzyme from that of hydrolyzed oyster at time t.

Protease selection

Protease selection was based on the method of Hale (15). Enzymes were evaluated based on the degree of hydrolysis per enzyme amount at 30% proteolysis. The mount of each enzyme required for 30% proteolysis was extrapolated from a plot of proteolysis after 1hr versus $\log_{10}$(enzyme amount). Enzyme activity was expressed as a inverse value of the amount of enzyme, and the most effective enzyme was determined by the highest enzyme activity per a cost basis.

Optimization of hydrolysis condition

Independent variables affecting oyster hydrolysis, e.g. pH, temperature (T), reaction time (t), substrate concentration (S), and enzyme/substrate (E/S) ratio, were optimized using response surface methodology (16). Experimental domain for each parameter is shown in Table 1. The central composite design consisted of 36 experiments including 16 fractional factorial design, 10 star points, and 10 central points (Table 2). The equation of second order model was finally defined as follows :

$$Y_i(\text{degree of hydrolysis}) = b_0 + b_1X_1 + b_2X_2 + b_3X_3 +$$

Table 1. Boundaries of the experimental domain and spacing of levels expressed in coded and natural units

Coded units	Experimental factor (abbreviation)				
	pH	Temp (T)	Time (t)	Substrate conc. (S) (% w/v)	E/S Ratio (E/S) (% v/w)
−2	8.0	40	0.5	15	0.1
−1	9.0	50	1.5	30	0.2
0	10.0	60	2.5	45	0.3
+1	11.0	70	3.5	60	0.4
+2	12.0	80	4.5	75	0.5
X*	1.0	10	1.0	15	0.1

*X is the increment of the experimental factor natural values corresponding to one unit of the coded variable

Table 2. Central composite design consisting of 36 experiments for the study of five experimental factors in coded units

Run No.	Factor					Coefficients assessed by :
	pH	T	t	S	E/S	
1	−1	−1	−1	−1	−1	
2	1	−1	−1	−1	1	
3	−1	1	−1	−1	1	
4	1	1	−1	−1	−1	
5	−1	−1	1	−1	1	
6	1	−1	1	−1	−1	Fractional 2^{5-1}
7	−1	1	1	−1	−1	factorial
8	1	1	1	−1	1	design
9	−1	−1	−1	1	1	(16 points)
10	1	−1	−1	1	−1	
11	−1	1	−1	1	−1	
12	1	1	−1	1	1	
13	−1	−1	1	1	−1	
14	1	−1	1	1	1	
15	−1	1	1	1	1	
16	1	1	1	1	1	
17	−2	0	0	0	0	
18	2	0	0	0	0	
19	0	−2	0	0	0	
20	0	2	0	0	0	Star points
21	0	0	−2	0	0	(10 points)
22	0	0	2	0	0	
23	0	0	0	−2	0	
24	0	0	0	2	0	
25	0	0	0	0	−2	
26	0	0	0	0	2	
27	0	0	0	0	0	
28	0	0	0	0	0	
29	0	0	0	0	0	
30	0	0	0	0	0	
31	0	0	0	0	0	Central
32	0	0	0	0	0	points
33	0	0	0	0	0	(10 points)
34	0	0	0	0	0	
35	0	0	0	0	0	
36	0	0	0	0	0	

$b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5$

Statistical analysis

Response surface equations were obtained using the RSREG procedure of the Statistical Analysis System (17). Response surface plots were drawn using PS-Plot (Polysoft, Salt Lake City, UT) to show the effect of two independent variables with all other factors at zero level.

RESULTS AND DISCUSSION

Protease selection for hydrolysis of oyster

The protein and moisture content of fresh oyster were 9.56 and 78.45%, respectively (Table 3). As shown in Table 4, enzyme activity was expressed as a inverse value of the amount of enzyme that required for 30% proteolysis from a plot of proteolysis after 1hr reaction. Among 5 commercial neutral proteases, Alcalase™ 2.4L showed the highest activity for the hydrolysis of oyster, followed by Protease A™, and Alcalase™ 0.6L. Among the 4 alkaline proteases evaluated, APL™ 440 showed the highest protease activity and followed by Protease P™, and Proleather™. Protease M™, which showed higher activity among acidic proteases, showed lower activity comparing to other proteases. However, APL 440 was the most suitable enzyme for enzymatic hydrolysis of oyster on the basis of cost per unit activity to enhance economic efficiency, and also showed similar results in evaluation commercial proteases to hydrolyze crawfish processing byproduct (10). Oyster hydrolysate produced with APL 440 did not give any bitter taste by author's sensory evaluation.

Effect of autolysis to degree of hydrolysis

The effect of autolysis on degree of hydrolysis was evaluated with and without APL 440 protease during hydrolysis of oyster at optimal condition (pH 10.0, 60 °C) (Fig. 1). Degree of hydrolysis in oyster with APL 440 protease increased sharply after 1 hr of hydrolysis, while control (without APL 440) showed below 3% in degree of hydrolysis during 4 hr of reaction. Lee *et al.* (18) reported that the effect of autolysis showed up to 70% proteolysis at 55° C in processing of sardine sauce. However, the level of proteolysis by autolysis in oyster was negligible compared with the enzyme treatment.

Table 3. Proximate composition of raw oyster[a]

(%)

Moisture	Lipid	Protein	Ash	pH
78.45±0.15	3.23±0.15	9.56±0.31	1.51±0.12	5.73

[a] Average ± standard deviation (n=3)

Determination of optimized hydrolysis condition by RSM

Response surface equations were obtained using the RSREG procedure of Statistical Analysis System (17) to evaluate significant differences among the treatments. Response surface plots were drawn to show the effect of two independent variables with all other factors at zero level. Response surface plot for the

Table 4. Evaluation of eleven commercial proteases for the hydrolysis of raw oyster

Protease[a]	Opt. Temp (°C)[b]	Opt. pH[b]	Activity (A)[c]	Cost (C)[d]	A/C
Neutral					
Neutrase 0.5L	45	6.0	0.48	1.24	0.39
Complex enzyme 2000	50	7.0	0.73	1.96	0.37
Alcalase 2.4L	60	7.0	10.20	4.95	2.06
Alcalase 0.6L	60	7.0	4.98	1.00	4.98
Protease A	50	7.0	7.25	8.87	0.82
Alkaline					
Protease S	70	8.0	4.84	8.87	0.55
Protease P	45	8.0	9.70	8.87	1.09
APL 440	60	10.0	22.37	3.61	6.20
Proleather	60	10.0	5.10	8.87	0.57
Acidic					
Newlase F	45	3.0	3.08	8.87	0.35
Protease M	50	4.5	4.72	8.87	0.52

[a] Neutrase 0.5L, Alcalase 2.4L and Alcalase 0.6L were obtained from Novo Nordisk (Denmark) ; Protease P, Proleather, Newlase F and Protease M were obtained from Amano International Enzyme Co. (Japan) ; Complex enzyme 2000 and APL 440 were obtained from Pacific Chemical Co. (Korea) and Solvay Enzyme Inc. (USA), respectively

[b] Optimal temperature and pH of each protease were suggested from the company produced

[c] Inverse of the amount of protease required for 30% hydrolysis at optimal temperature and pH of each enzyme for 1 hr

[d] Least expensive enzyme was given a value of 1.0

effect of time and temperature on the hydrolysis of oyster were shown in Fig. 2. Stationary point, having 55% hydrolysis ratio, was set between 3.0 hr reaction time and 65~70°C range. As shown in Fig. 3 to Fig. 6, however, the results of response surfaces for the effect of independent variables were exhibited saddle points in all stationary points. Except the effects of time and temperature, crossproduct regression among the treatments showed no significant difference, while linear, quadratic and total regression were significant at 0.01% level from the result of multiple linear regression. Response surface for degree of hydrolysis was considered to be designed satisfyingly from the result of coefficient of determination (R^2= 0.9122) in this experiment.

From the results of RSM a model equation was estimated by multiple linear regression for degree of hydrolysis (DH) as follows : $\%DH=48.362+1.096T+1.796t-2.154S+2.871E/S-2.693pH^2-1.918T^2-1.080t^2+0.982E/S^2$(Table 5). Stationary point, however, was a saddle point, and showed 47.25% DH. Therefore, ridge analysis was executed to obtain ma-

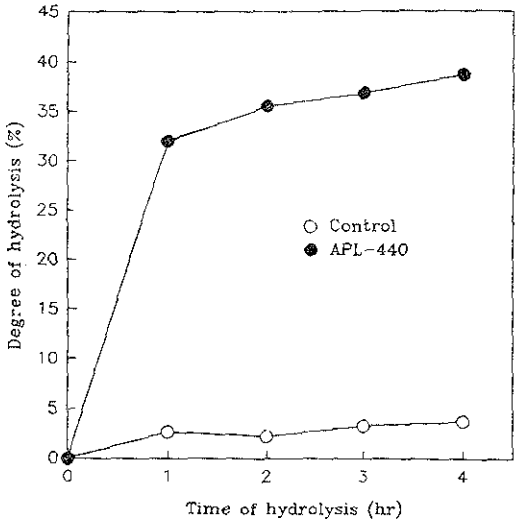

Fig. 1. Degree of hydrolysis in control with and without APL 440 protease during hydrolysis at 60°C, pH 10.0.

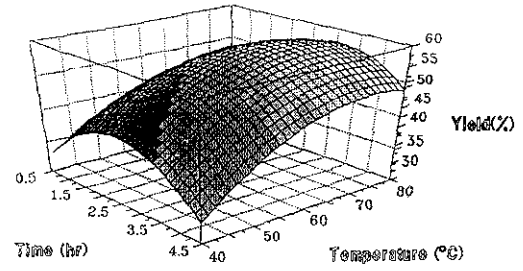

Fig. 2. Response surface plot for the effect of time and temperature on the hydrolysis of oyster.

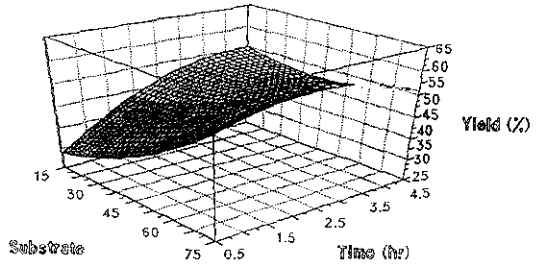

Fig. 3. Response surface plot for the effect of substrate and time on the hydrolysis of oyster.

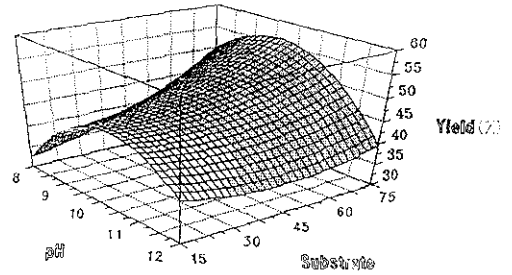

Fig. 4. Response surface plot for the effect of pH and substrate on the hydrolysis of oyster.

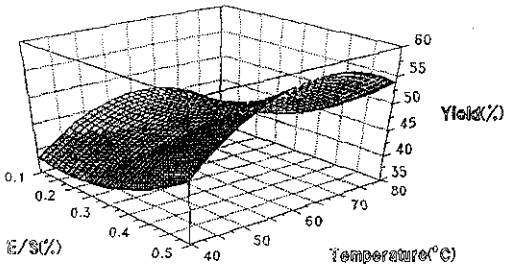

Fig. 5. Response surface plot for the effect of E/S and temperature on the hydrolysis of oyster.

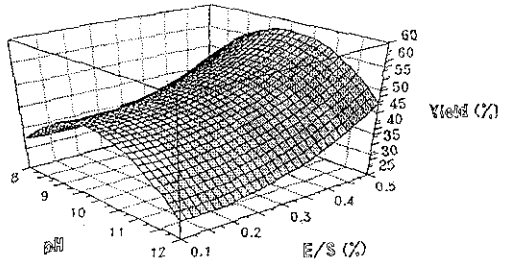

Fig. 6. Response surface plot for the effect of pH and E/S on the hydrolysis of oyster.

Table 5. Model coefficients estimated by multiple linear regression

Factor	Coefficient	Student's t-test
Constant	48.362	
pH	−0.388	−0.780
T	1.096[c]	2.205
t	1.796[b]	3.613
S	2.154[a]	4.334
E/S	2.871	5.776
pH^2	−2.693[a]	−6.256
T^2	−1.918[a]	−4.455
t^2	−1.080[b]	−2.509
S^2	0.720	1.672
$[E/S]^2$	0.982[c]	2.282
pH×T	−0.581	−0.955
pH×t	−0.144	−0.236
pH×S	−1.106	−1.817
pH×(E/S)	0.181	0.298
T×t	0.456	0.749
T×S	−0.531	−0.873
T×(E/S)	0.331	0.544
t×S	−1.144	−1.879
t×(E/S)	−0.506	−0.832
S×(E/S)	−0.794	−1.304

Superscript letters are significantly different : [a] $p<0.001$, [b] <0.01, [c] $p<0.05$

The final explanatory model is ;

$Y\%=48.362+1.096T+1.796t-2.154S+2.871E/S-2.693pH^2-1.918T^2-1.080t^2+0.982E/S^2$

ximum degree of hydrolysis. The ranges of estimated response were from 48.36 to 58.35% DH in between 0.0 and 1.0 coded radius. In order to minimize standard error (S.E.), nevertheless, optimal condition was set at 0.3 coded radius (S.E.=0.77) comparing to experimental DH in this study. Optimal hydrolysis conditions were determined to be pH 9.95, 61.1°C, 2.64 hr reaction time, 49.2% S and 0.35% E/S. Oyster hydrolysate produced under optimal condition showed 51.98% DH, while 50.87% in estimated SAS analysis.

The oyster hydrolysate produced under this condition could be a valuable seasoning agents, having economic benefits in seafood industry.

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굴 가수분해물 제조를 위한 반응표면분석

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요 약

반응표면분석법을 이용하여 굴 가수분해물을 제조하기 위한 최적조건 설정을 시도하였다. 11종의 상업용 단백질분해효소 가운데서 판매가격을 기준으로 효소활성도를 평가한 결과 APL 440이 가장 우수한 효소로 선정되었으며, 굴 가수분해과정에서 자가소화에 의한 영향은 무시할 정도로 적었다. 반응표면 분석 결과 정상점은 안장점 (saddle point)을 나타내었고 정준분석에 의한 최대점은 반경 0.3 범위에서 만족하였다. 이점을 만족하는 각 독립변수의 조건은 pH 9.95, 온도 61.1°C, 기질농도 49.2%, 기질에 대한 효소농도 0.35%에서 2.64시간 가수분해할 적에 가수분해율은 50.87%이었으며, 실제 이 조건에서 운전한 결과 51.98%의 분해율을 나타내었다.