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Optimization and Flavor Quality of Enzymatic Hydrolysate from Dark Muscle of Skipjack

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Abstract

Enzymatic hydrolysis of dark muscle of skipjack was optimized by using response surface methodology. Three factors of independent values were pH (4.2 to 9.8), time (0.6 to 3.4 hrs) and temperature (34°C to 76°C), and independent values were optical density and brix. The optimum conditions for enzymatic hydrolysis were pH 7.0 to 8.0, 55°C and 3 hrs. The headspace volatile compounds of reaction flavors using the enzymatic hydrolysate, cysteine and xylose were identified by using the combination of a canister system, gas chromatography and mass selective detector. Among 67 compounds, we identified 8 sulfur-containing compounds and 7 furans which were thought to be highly related to meat-like flavors.

Key words: skipjack, dark muscle, alcalase, enzymatic hydrolysis, optimization

INTRODUCTION

About 3 billion tons of skipjack (*Scomber japonicus* of *Lateolabrax*) are caught year worldwide (1). The skipjack has been mainly used in canned foods, and the use of high-quality raw fish is increasing. By-products of skipjack account for about 30% of materials during canning, and is increasing according to increased amount of processing of canning food. Therefore, efficient recovery and utilization are very important to reduce environmental problems and maximize economical benefits. Dark muscle produced as by-products during the processing of skipjack is mainly used for pet foods and feeds due to the unpleasant odor and bitter taste, even thought it contains many useful nitrogen compounds associated with peptides and proteins (2).

The main nitrogen source for Maillard reaction is vegetable protein hydrolysate from soybeans (3); fish protein hydrolysates (FPH) (2) have been studied for the replacement of soy protein. To maximize flavor intensity, model systems of Maillard reaction were studied by several investigators. Among them, the main studies were the formation of meat-like flavor with amino acid and sugar sources. For example, Maillard reaction of cysteine and ribose has been reported to give a meat-like flavor (4). Fish protein hydrolysates from capelin (5), sardine (6), lobster (7), cod (8) and Pacific whiting (9) were studied, but there have been few studies on the enzymatic hydrolysis of dark muscle of skipjack. The enzymes evaluated for enzymatic hydrolysis to increase the concentration of amino acids were neutrase 0.5L™, complex enzyme 2000, alcalase 2.4L™, protease A™, protease S™, protease P™, protease APL™ and optimase APL™ (10). Among them, optimase APL™ is not viable for enzymatic hydrolysis, because the price is very high, even if the reaction rate is high. Alcalase 2.4L™ is cheap and the reaction rate is fast under moderate conditions compared to neutral or acidic enzymes (9,11). Response surface methodology (RSM) has been used to optimize several phenomena for treatment of data. RSM in food products is increasingly used for optimizations of extraction (12), gelatin processing (13), concentration (14) and more. The objectives of this study were to investigate the optimum conditions for alcalase hydrolysis of the dark muscle and to study the reaction flavors from its hydrolysate for industrial application.

MATERIALS AND METHODS

Materials

Dark muscle of skipjack was obtained from Dongwon F&B Co., Ltd. (Changwon, Korea), freeze-dried and stored at 4°C. Alcalase 2.4L™ was from Novo Nordisk Bioindustrials Inc. (Danbury, CT, USA). Protein assay kit, L-cysteine and xylose were purchased from Sigma Co. (St. Louis, MO, USA).

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Reaction conditions of alcalase and measurement of brix and protein content

Thirty grams of freeze-dried dark muscle of skipjack was homogenized in a 150 mL buffer solution and 0.3 g alcalase 2.4L™ was reacted under each of the conditions. Hydrolysate obtained after alcalase reaction was filtrated and heated for the inactivation of the enzyme at 95°C for 10 min. After cooling, brix of the hydrolysate was measured by a refractometer N-1E (ATAGO Co., Ltd., Tokyo, Japan). Protein concentration of the hydrolysate was measured by the method of Lowry et al. (15) using bovine serum albumin as the standard.

Experimental design and analysis for the optimization of alcalase

An optimum condition for enzymatic hydrolysis of dark muscle of skipjack was determined by response surface methodology. Based on the results of preliminary studies, three important independent variables were pH, temperature and time which were coded to five levels of -1.4, -1, 0, +1, +1.4 (Table 1). Experimental design was carried out by two dimensional regression equation of central composite design as follows:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} b_{ij} X_i X_j \quad (i<j)$$

Where b_0 is an intercept, b_i, b_{ii}, and b_{ij} are regression coefficients of the model. X_i and X_j are coded independent variables and are linearly related to X_1, X_2 and X_3.

All of the experimental data were statistically analyzed with SAS software (Version 8.01, SAS Institute Inc., USA), and three dimensional graphs were made by Maple software (Maple 6, Waterloo Maple Inc., Canada).

Conditions and composition for reaction flavors

The 5.6 mL of hydrolysate produced under the optimum condition was placed into a screw-cap test tube, and xylose and cysteine of 0.1% (w/v) were added. The final volume was adjusted to 7 mL. The pH was adjusted to 7.0 using 6 M HCl and NaOH. The test tube was reacted at 130°C for 3 hours in a heating block.

Table 1. Experimental domain and levels expressed in coded and natural units for optimizing the alcalase activity on dark muscle of skipjack

Code units	Independent variables		
	pH	Temperature (°C)	Time (hour)
-1.4	4.2	34	0.6
-1	5	40	1
0	7	55	2
1	9	70	3
1.4	9.8	76	3.4

Measurement of volatile compounds comprising reaction flavors

The headspace volatile compounds of the reaction flavors were identified by using the combination of a canister system (AUTOCan Canister Autosampler/Concentrator, Tekmar, USA), gas chromatography (5050, Shimatzu, Japan) and mass selective detector (QP-5050A, Shumatz, Japan). The column was an HP-innowax capillary column (30 m × 0.25 mm × 0.25 μm, 19091N-133, Supelco, USA). The operating condition for gas chromatography was 200°C injector temperature, 250°C detector temperature, 30°C initial temperature, 200°C final temperature and 3°C/min increasing rate of temperature in the splitless injection mode with 0.75-mm inlet liner (2-6375, Supelco, USA). Carrier gas was helium (purity 99.9999%) and the flow rate was 0.1 mL/min. Mass range was from 20 to 350 m/z.

RESULTS AND DISCUSSION

Optimization of enzymatic hydrolysis of dark muscle by alcalase

The experimental design for investigating the optimum conditions for enzymatic hydrolysis of dark muscle by alcalase was performed by central composite design which had three independent variables of pH, temperature and time, and dependent variables of optical density for protein concentration and brix for solid content. The results on the above central composite design are shown in Table 2. Response surface expression was obtained using SAS software for verification of significant differences. The results of multiple regression analysis are shown Table 3, showing very high values of 0.9895 in optical density and 0.9262 in brix for coefficients of determination. This experiment was properly designed based on the high R^2 values. In the case of optical density, pH and temperature were significantly more effective than any other terms ($p<0.01$), showing that time and other terms were not significantly effective. In the case of brix, pH, temperature and the square of the terms were significantly high effective ($p<0.01$). But time and other terms were significantly less ($p<0.05$) effective or not effective. Based on Table 3, the regression equations obtained from the response surface analysis for optical density and brix were as shown below.

$$OD = -0.4006 + 0.26129\ pH + 0.02730\ Temp. - 0.02115\ pH^2 - 0.000318\ Temp.^2 - 0.01443\ Time^2 + 0.000962\ pH \times Temp + 0.006062\ pH \times Time$$

$$Brix\ (\%) = -19.40 + 4.748\ pH + 0.3455\ Temp. + 2.797\ Time - 0.3492\ pH^2 - 0.003487\ Temp.^2 - 0.4530\ Time^2$$

Table 2. Responses of the dependent variables of optical density and brix to the hydrolysis condition of commercial alcalase on dark muscle of skipjack

Run number	Independent variables[1]			Dependent variables	
	pH	Temperature	Time	Optical density[2]	Brix[3] (%)
1	-1	-1	-1	1.171	7.1
2	-1	-1	1	1.139	8.2
3	-1	1	-1	1.100	6.9
4	-1	1	1	1.030	7.1
5	1	-1	-1	1.229	7.4
6	1	-1	1	1.214	7.7
7	1	1	-1	1.242	7.5
8	1	1	1	1.252	7.9
9	0	0	0	1.346	10.2
10	0	0	0	1.352	10.1
11	0	0	0	1.337	10.2
12	0	0	0	1.335	9.9
13	-1.4	0	0	1.102	7.0
14	1.4	0	0	1.274	8.7
15	0	-1.4	0	1.242	9.0
16	0	1.4	0	1.185	9.1
17	0	0	-1.4	1.326	8.9
18	0	0	1.4	1.325	10.5

[1]The codes of independent variables were the same as represented in Table1.
[2]The values were measured at 750 nm on a spectrophotometer.
[3]The values were measured by a refractometer.

For searching the optimum conditions for enzymatic hydrolysis, and how they correlated with independent values, response surfaces were plotted for two independent values with other independent values fixed, and the three dimensional graphs were made by Maple software (Maple 6, Waterloo Maple Inc., Canada), and critical values were shown in Table 3. In the case of optical density, response surface on the correlation of independent values was shown in Fig. 1. Direct correlation of optical density between pH and time was observed (Fig. 1(A)), the region of optimum response was localized at

Table 3. Effect of independent variables by multiple linear regression for producing hydrolysate from by product of skipjack processing

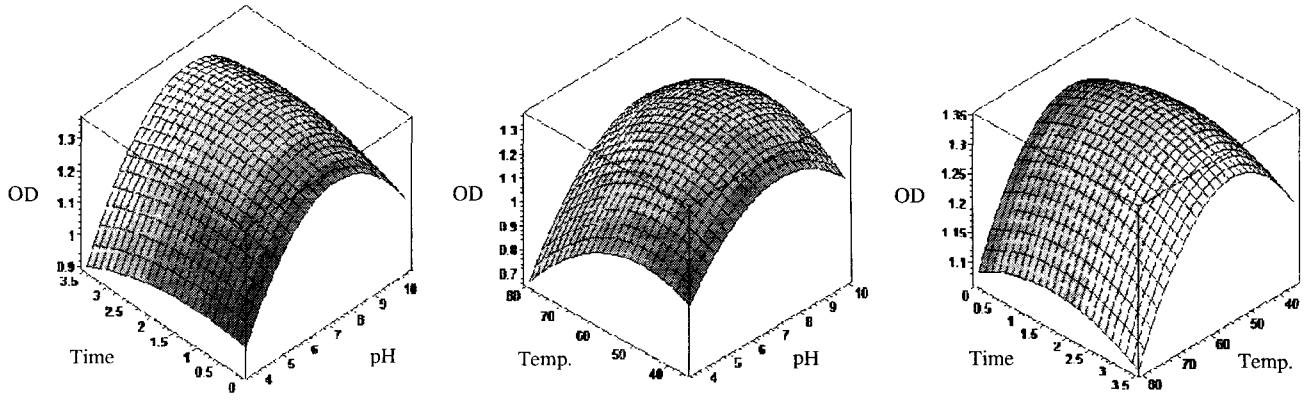
Factors	Coefficients	
	Optical density	Brix
Constant	-0.400574*	-19.396669**
pH	0.261940**	4.747713**
Temp.	0.027304**	0.345475**
Time	0.012163	2.797032*
pH^2	-0.021147**	-0.349229**
$Temp.^2$	-0.000318**	-0.003487**
$Time^2$	-0.014434*	-0.453040*
pH × Temp.	0.000962**	0.006667
pH × Time	0.006062*	-0.037500
Temp. × Time	-0.000108	-0.006667
R^2	0.9895	0.9262
probability > F	0.0001	0.0012

*,**Superscript letters are significantly different at the values of $p<0.05$, $p<0.01$, respectively.

pH 7.0 to 8.0, and at a temperature about 55°C. When optical density was plotted against pH and time at optimum temperature (55°C), it can be explained that optical density was increased with increasing time. In the case of brix, response surface on the correlation of independent values are shown in Fig. 2. Direct correlation between pH and temperature was similar to the case of optical density, but direct correlation between time and temperature had a saddle point, which was different from the case of optical density.

Measurement of volatile compounds of reaction flavors

Headspace volatile flavor compounds which resulted from the enzymatic hydrolysis of dark muscle, cysteine and xylose, were analyzed by the combination of purge and trap, a gas chromatography with a mass selective detector. The total ion chromatogram of the volatile



(A) (B) (C)

Fig. 1. Response surface of optical density on the effects of (A) pH and time, (B) pH and temperature and (C) temperature and time to determine the optimum condition for hydrolysate of skipjack by-product.

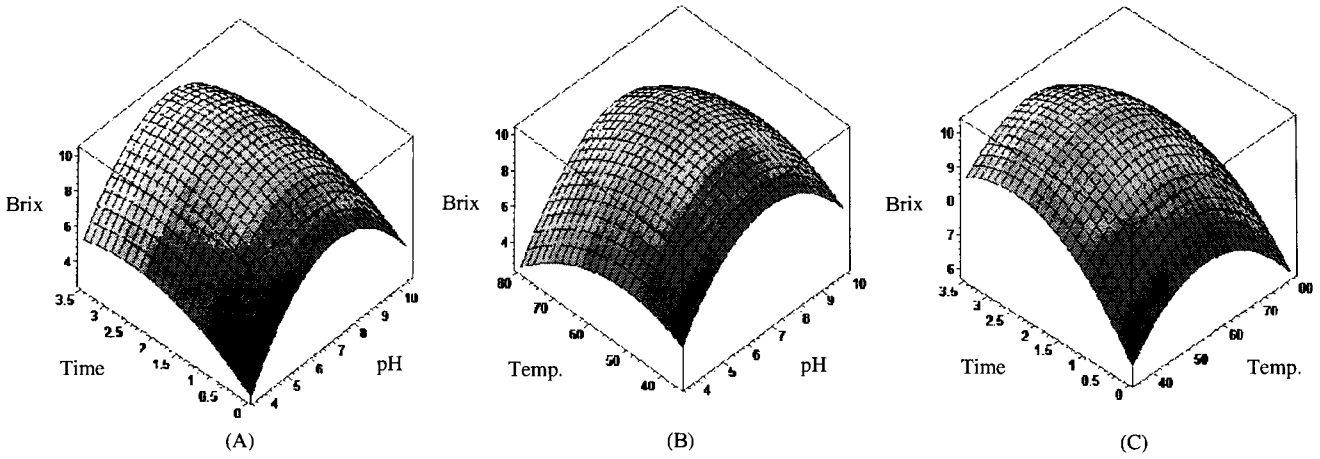


Fig. 2. Response surface of brix on the effects of (A) pH and time, (B) pH and temperature and (C) temperature and time to determine the optimum condition for hydrolysate of skipjack by-product.

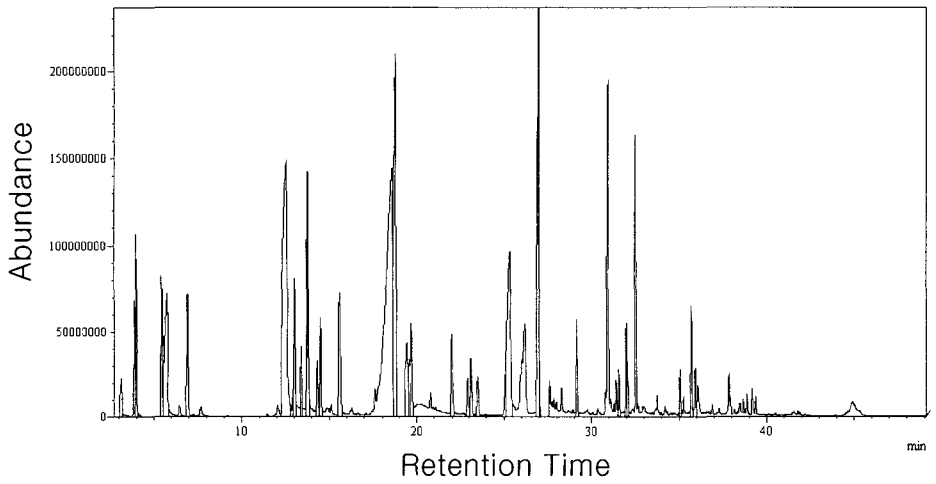


Fig. 3. Total ion chromatogram of volatile compounds separated from enzymatically hydrolyzed dark muscle by Maillard reaction.

compounds is shown in Fig. 3, and the identified volatile compounds are shown in Table 4. Among the 67 compounds, 14 hydrocarbons, 13 aldehydes, 11 surfur-containing compounds, 11 ketones, 7 furans, 3 alcohols, 8 others were identified. The hydrocarbons, straight chain aldehydes, ketones and alcohols were known to be produced by the oxidation of unsaturated fatty acids (16). The hydrocarbons were isolated in cooked beef, but the content was quite small. Hexanal, heptanal, nonanal and decanal and the others, which were known to be formed from straight chain fatty acids, were identified in both dry fermented sausages (17,18) and dry-cured hams (19,20). The flavor of aldehydes was a characteristic unpleasant rancid pungent odor (16,21). The identified benzaldehyde had an almond and nutty flavor, and it can progress to a precursor of heterocyclic compounds (22). According to Person's report (23), ketones were identified in canned beef, and most of the ketones were produced a roasted and boiled meat flavor. Sulfur-containing compounds which were produced from Maillard reaction of sulfur-containing amino acids and reducing sugars were known to be important compounds producing a meat-flavor (24-26). The aroma characteristics of these sulfur-containing compounds were pungent for methanethiol and thiophene; sulfury or cabbage-like for carbonsulfide, dimethilsulfide and dimethyldisulfide; sweet for 2-methylthiophene; styrene-like for 2-ethylthiophene; and cooked meat for 2,5-dimethylthiophene (27). Furans are very important compounds in meat-like flavor with sulfur-containing compounds (24). In this study, the identified furans were furan, 2-methylfuran, 2-ethylfuran, 2,5-dimethylfuran, 2-propylfuran and acetylfuran. They were identified in sausages (18,28) and hams (29). Most of the furans are known to be produced from the interaction between free amino group and carbonyl compound, showing the aroma characteristics of cooked meat flavor (30). Further studies on the precise contributions of these compounds to final products are required to

Table 4. Volatile compounds identified from the reaction flavor of the enzymatically hydrolyzed dark muscle by Maillard reaction

Chemical compounds	RT[1] (min)	RI[2]	Peak area ($\times 10^8$)
Aldehydes (13)			
Acetaldehyde	5.8	<500	9.27
Isobutanal	15.6	532	6.30
2-Methylbutanal	22.0	606	3.45
3-Methylbutanal	22.9	620	1.51
2,2-Dimethylpropanal*	26.2	665	5.41
2,4-Hexadienal*	27.0	675	11.96
(E)-2-Pentenal	28.9	698	3.16
Hexanal	32.0	775	2.23
(E)-2-Hexenal	33.6	816	0.25
Heptanal	36.1	884	1.42
Benzaldehyde	37.9	941	1.81
Nonanal	41.9	1086	0.04
Decanal	43.9	1157	0.12
Furans (7)			
Furan	13.0	<500	5.05
2,3-Dihydrofuran*	16.3	541	0.68
2-Methylfuran	19.6	580	14.99
2-Ethylfuran	27.8	685	0.50
2,5-Dimethylfuran	27.9	686	1.28
2-Propylfuran	32.1	777	1.39
Acetylfuran*	32.7	792	0.44
S-containing compounds (11)			
Methanethiol*	7.7	<500	0.63
Dimethylsulfide	13.8	506	9.98
Carbondisulfide*	14.3	514	1.71
Methylthioethane*	19.5	579	3.90
Thiophene	23.5	629	1.60
Isobuthylmercaptan	27.6	682	1.42
Dimethyldisulfide	29.2	703	2.59
2-Methylthiophene	31.0	750	8.59
2-Ethylthiophene	35.1	857	1.33
2,5-Dimethylthiophene	35.3	863	0.76
3(2H)-Dihydro-2-methyl-thiophenone*	38.5	962	0.86
Alcohols (3)			
Ethanol	12.1	<500	0.58
3-Buten-1-ol	12.6	<500	24.91
Pentanol	12.9	<500	0.61
Ketone (11)			
Acetone	14.9	522	0.72
2-Butanone	18.6	569	38.99
2-Pentanone	25.4	654	14.72
2,3-Pentanedione	26.0	662	3.86
2-Hexanone	31.4	760	0.99
3-Hexanone	31.6	765	1.32
3-Octanone	35.7	873	2.82
2,4-Dimethyl-3-hexanone	38.2	952	0.31
7-Octen-2-one	38.7	969	0.45
2-Octanone	38.9	976	0.70
2-Nonanone	41.6	1075	0.56
Hydrocarbons (14)			
2-Methylpropene	6.5	<500	0.42
1,3-Butadiene	6.6	<500	0.03
Trimethyloxirane	6.9	<500	4.05
Pentane	13.4	500	1.87
(Z)-1,3-Pentadiene	14.5	517	2.49
(E)-1,3-Pentadiene	15.1	525	0.58
1,4-Hexadiene	20.8	592	0.98
Methylcyclopentane	21.1	595	0.52
(E)-2-Hexene	31.3	757	0.40
(E),(Z)-3,4-Dimethyl-2,4-hexadiene	32.6	789	6.05
Octane	33.0	800	0.44
1,3-Octadiene	33.8	822	0.66
Nonane	36.7	900	0.17
(E)-1,3-Nonadiene	37.3	920	0.25
Others (8)			
Urea	3.9	<500	3.13
Acetylchloride	4.0	<500	5.10
Benzene	23.1	623	2.12
Phenol	28.3	691	1.25
Pyrrole	29.8	719	0.69
Toluene	30.8	745	0.83
3-Ethylphenol	36.9	906	0.37
Limonene	39.4	992	0.49

[1]RT means retention time. [2]RI means retention index.
*Tentative identification was done by only comparison with the reference mass spectrum.

know the optimum reaction condition for producing the best meat-like flavors and their mechanisms.

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