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High Temperature Cooking of Fish Protein Extracts for Plastein Reaction

Keun-Tai Lee†, Seong-Min Park, Sang-Ho Lee, Hong-Soo Ryu* and Ho-Dong Yoon**

Dept. of Food Science and Technology, Pukyong National University, Pusan 608-737, Korea
**Dept. of Food and Life Science, Pukyong National University, Pusan 608-737, Korea*
***National Fisheries Research and Development Agency, Pusan 619-900, Korea*

Abstract

High temperature-cooking conditions of cultured fishes(loach, crucian carp, bastard halibut, and jacopever) were optimized by response surface methodology(RSM), and plastein products were prepared using enzymatic hydrolysis. Four models were proposed with regard to effects of time(t), temperature(T), and water/fish meat (w/f) ratio on the amount of 0.3M TCA soluble fractions. The model coefficients were ranged from $p<0.0001$ for jacopever to $p<0.0433$ for bastard halibut. Cooking conditions for 60% hydrolysis were optimized at 1) 140°C except for crucian carp(136.7°C); 2) 10.08 hours(loach), 7.25 hours(crucian carp), 9.85 hours(ba-stard halibut), and 9.38 hours(iacopever); 3) 1:1(w/f) ratio except for the crucian carp(1.1:1). When protein hydrolyzates were employed for the plastein synthesis, optimum plastein-reaction conditions were determined to be pH 9.0 with chymotrypsin for the loach and crucian carp hydrolyzates, pH 9.0 with papain for the bastard halibut hydrolyzate, and pH 11.0 with trypsin for the jacopever hydrolyzate. Plastein reaction could be performed in water at concentration up to 20%(w/f).

Key words: high temperature cooking, fish protein hydrolyzate, response surface methodology, plastein reaction

INTRODUCTION

Extracts from cooked fish meat at high temperature have been favored by Korean people as a health food. Such home-made fish-meat extracts using fresh water fishes like crucian carp, eel, and snake-head fish etc have been served to pregnant women, old adults, patients, and undernourished persons. Conventionally cooked fish-meat extracts are processed at the boiling temperature for over 10 hours without considering energy cost or nutritional value. However, those conditions should be modified to increase nutritional value and cost-effectiveness. Most of cultured fishes are distributed as live fishes, and consumed in raw fish dishes or traditional hot taste soup. Recently health-food stores have designed an autoclave type extractor to increase yields, and manufactured the fish-meat extracts with some herbs(ginger and medicinal herbs) and spice(garlic and onions). Higher processing temperature than that used in the conventional methods may result in the formation of brown products as well as damage to proteins(1-4). Therefore, development of a technology is needed to modify the physicochemical properties of these proteins so that they may successfully perform specific functions and yet be nutritious.

A novel approach to alter the physicochemical, functional, and nutritional properties of those unstable proteins is application of the plastein reaction(5-7). The application of the plastein reaction to enzymatic hydrolyzates of fish proteins may give rise to products with enhanced functional and/or nutritional properties. But protein hydrolyzates are always accompanied by bitterness from bitter tasting peptides after extensive hydrolysis(8). The bitterness is a critical problem in the application of protein hydrolyzates for foods. Many methods with aim of debittering protein hydrolyzates have been studied using carboxypepetidases(9,10). On the other hand, incubation of a partially hydrolyzed protein with certain proteolytic enzyme could be resulted in the formation of a plastein, which has a high molecular weight protein- like substance with different properties from those of the original protein source and no longer has any special flavor. It is also expected to incorporate essential amino acid onto products and to produce peptide having certain functional properties during the plastein reaction.

The object of our study was to optimize high tem-

†Corresponding author

perature cooking conditions for 60% hydrolysis of fish-meat extracts using response surface methodology (RSM). The partial hydrolyzates from fish meats were used as a substrate for determining optimum plastein formation for the better utilization of cultured fishes and high economic returns from aquacultured resources.

MATERIALS AND METHODS

Materials

Live loach(*Misgurnus anguillicaudatus*), crucian carp (*Carassius carassius*), bastard halibut(*Paralichthys olivaseus*), and jacopever(*Sebastes ineronis*) were purchased from a local market in Pusan, Korea. All of fishes were eviscerated and scaled. Loach skins were scrubbed in 5% salt water to separate foreign bodies. Pepsin, papain, trypsin, and chymotrypsin that were used for the production of a plastein were purchased from Sigma Chemical Co.(U. S. A). All other chemicals used were commercial sources of reagent grade.

Cooking of fish proteins

Condition of hydrolysis was established based on central composite design(CCD). Hydrolysis parameters such as temperature(T), time(t), and ratio of water(W) to fish-sample weight were selected as independent variables. A range of independent variables(Table 1) was based on information from preliminary experiments and capacity of heating system.

Table 1. Independent variables and experimental design levels expressed in coded and natural units for hydrolysis

Code units			Independent variables		
χ_1	χ_2	χ_3	Temp(°C)	Time(hr)	W(ratio)
-1	-1	-1	110	4	2
1	-1	-1	130	4	2
-1	1	-1	110	8	2
-1	-1	1	110	4	5
1	1	-1	130	8	2
1	-1	1	130	4	5
-1	1	1	110	8	5
1	1	1	130	8	5
0	0	0	120	6	3.5
0	0	0	120	6	3.5
0	0	0	120	6	3.5
1.5	0	0	135	6	3.5
-1.5	0	0	105	6	3.5
0	1.5	0	120	9	3.5
0	-1.5	0	120	3	3.5
0	0	1.5	120	6	5.75
0	0	-1.5	120	6	1.25

Each fish sample was cut into proper blocks(3cm×3cm×2cm) and whole rubbed off loaches were used in experiments for hydrolysis. 1kg of fish-sample blocks that was put into a cotton cloth pocket(50cm×50cm) were placed in a high pressure extractor(Oriental medicine extractor, Sea-Dong Co.) and cooked at desired conditions shown in Table 1.

Extracts from cooked fish meat were collected by filtering through Toyo No. 2 filter paper. Freeze-dried extracts were stored at 5°C and used for the experiment of plastein reaction.

Estimation of the degree of hydrolysis

The degree of hydrolysis(D.H.) was determined using modification of the method described by Bourant and Cheftel(11). An aliquot of extracts(1ml) was transferred into test tubes containing 2ml of 0.3M trichloroacetic acid (TCA) solution. The solution was allowed to stand at ambient temperature for 20min and filtered through Whatman No. 40 filter paper. A 25μl aliquot of filtrate was mixed with a solution containing 225μl distilled water, 1.25ml of 0.5N NaOH solution, and 0.2ml of 1.0N Folin & Ciocalteu's phenol reagent. The resulting solution was incubated at 30°C for 15min, and then filtered through Toyo No. 2 filter paper to remove turbid material. Absorbance of the filtrate was measured at 750nm. D.H. was defined as follows :

$$D.H = \frac{D_t - D_0}{D_{max} - D_0} \times 100$$

where the blank, D_0 was prepared by adding 0.3M TCA solution to cooked fishes; D_{max} is the maximum absorbance of 0.3M TCA soluble amino acids and was determined after hydrolysis of 0.1g fishes with 4ml of 6N HCl at 110°C for 24hr; D_t was determined from 0.3M TCA soluble peptides of hydrolyzed fishes at time t.

Optimization of cooking conditions

After hydrolysis of each fish-sample, DH was measured as dependent variable and parameters for hydrolysis were chosen as the independent variables.

The coded values were obtained by the following formula:

$$x = \frac{(X - X^0)}{\Delta X}$$

where x is the coded value; X is the corresponding natural value; X^0 is the natural value in the center of the domain; and ΔX is the increment of X corresponding to one unit of x.

For each factor, five levels were given and a second order model was proposed. Data were analyzed by multiple regression to fit the following second order equation:

$$y=\beta_0+\sum_{i=1}^{3}\beta_i x_i+\sum_{i}^{3}\beta_{ii}x_i^2+\sum_{i=1}^{2}\sum_{j=2}^{3}\beta_{ij}x_i x_j \quad (i<j)$$

where β_0, β_i, β_{ii}, and β_{ij} represent regression coefficients of the model, and x_i and x_j represent the independent variables in coded values. Conditions of cooking, which produced minimum DH 60% for plastein reaction, were found from response surface equations.

Response surface equations were obtained using the RSREG procedure of the Statistical Analysis System(SAS Institute, Inc., 1994) program.

Plastein reaction

The method of Montecalvo et al.(6) was employed to modify freeze-dried protein extracts of cooked fish meats. An aliquot of freeze-dried extracts(4g) was dissolved in 10ml distilled water to make a 40%(w/v) substrate solution. The pH was adjusted with addition of 1N HCl or 1N NaOH. Then 40mg of a proteolytic enzymes among pepsin, papain, trypsin, and chymotrypsin was added to the substrate solution. The solution was incubated at 37°C(±1°C) without agitation for 24hr. Then 10ml of 20% TCA was added and mixed. The samples were centrifuged at 5000rpm for 15 min to separate the TCA insoluble fraction. TCA insoluble materials were removed and freeze-dried.

Determination of plastein productivity

The plastein productivity of each reaction mixture was defined as the ratio of the content of initial free amino acids in the mixture to incorporate free amino acids. To determine the content of free amino acids, OPDA method (o-phthaldialdehyde spectrophotometeric assay) was used (12). 45ml of 95% ethanol was added to 10ml of the reaction mixture and then kept overnight at 7°C. After filtering the mixture, 1ml of filtrate was added to 1ml of OPDA reagent and this solution was mixed briefly at room temperature. Absorbance of the solution was monitored at 340nm after 10 min.

The percent incorporation of free amino acids into the products was calculated from

$$\frac{[A]_0-[A]_t}{[A]_0}\times 100$$

where $[A]_0$ is initial free amino acid content in the hydrolysate, and $[A]_t$ is free amino acid content after plastein reaction.

RESULTS AND DISCUSSION

Optimization of hydrolysis condition

Responses of dependent variables to hydrolysis conditions were followed as Table 2. Estimated regression coefficients for each dependent variable were obtained from these responses by multiple linear regression(Table 3). And the model adequacy for each equation was tested by coefficient of determination(R^2) and lack of fit.

The best explanatory model equation for loach which shows relationship between independent variables such as temperature (T, x_1), time(t, x_2), and ratio of water(W, x_3) and dependent variable(DH) is as follows:

$$y=12.79+6.08x_1+2.79x_2-6.11x_3-0.84x_1^2+1.96x_2^2+5.86x_3^2+5.46x_1x_2-5.46x_1x_3-0.74x_2x_3$$

The coefficient of determination(R_2) and level of significance for this model equation were 0.9288 and 0.0029,

Table 2. Responses of dependent variables to the hydrolysis conditions for fish proteins(loach, crucian carp, bastard halibut, and jacopever)

Independent variable			Dependent variables (Degree of hydrolysis)			
T	t	W	Loach	Crucian carp	Bastard halibut	Jacopever
-1	-1	-1	13	16.27	12.0	6.4
1	-1	-1	23.2	32.0	24.0	16.8
-1	1	-1	12.0	22.93	10.29	14.27
-1	-1	1	7.4	10.93	4.57	7.07
1	1	-1	30.2	45.87	34.14	34.13
1	-1	1	11.8	19.6	14.29	9.6
-1	1	1	9.0	12.53	11.86	8.27
1	1	1	19.2	23.73	20.86	18.93
0	0	0	13.6	21.07	14.14	13.87
0	0	0	13.6	19.20	6.29	12.67
0	0	0	12.6	20.53	17.57	14.13
1.5	0	0	15.4	31.07	21.86	21.33
-1.5	0	0	6.6	15.47	12.29	5.33
0	1.5	0	18.4	18.8	16.86	15.2
0	-1.5	0	9.2	16.13	13.43	5.87
0	0	1.5	9.2	12.53	5.57	12.0
0	0	-1.5	26.2	32.13	26.0	26.4

Table 3. Model coefficients[1] estimated by multiple lin-ear regression for hydrolysis of loach, crucian carp, bastard halibut, and jacopever

Factor	Coefficients			
	Loach	Crucian carp	Bastard halibut	Jacopever
Constant	12.79	19.55	12.82	13.60
Linear				
T	6.08	9.83	8 27	8.09
t	2.79**	3.63	3.92	5.97
W	-6.11	-9.56	-7.14	-5.92**
Quadratic				
T^2	-0.84	-4.49*	-3.96	-0.36
t^2	1.96	-1.31	2.03	-3.16**
W^2	5.86**	3.55	2 67	5.51***
Crossproduct				
T×t	5.46**	2.74	3.13	4.95***
T×W	-5.46**	-5.28**	-4.82	-4.80***
t×W	-0.74	-4.16*	1.53	-4.13***
R^2	0.9288	0.9692	0.8336	0.9874
Probability	0.0029	0.0007	0.0433	<0.0001

[1]Model on which x_1 = Temperature(T), x_2 = Hydrolysis time(t), x_3 = The ratio of water(W) is

$$y=\beta_0+\sum_{i=1}^{3}\beta_i x_i+\sum_{i}^{3}\beta_{ii}x_i^2+\sum_{i=1}^{2}\sum_{j=2}^{3}\beta_{ij}x_i x_j,\quad (i<j)$$

***Significant at 0.001 level
**Significant at 0.05 level
*Significant at 0.10 level

respectively. Accordingly, this assumed model was significant. The largest value of estimated regression coefficient for W(β_3 = -6.11) indicated that it was the most important linear variable influencing DH.

The best explanatory model equation for crucian carp is as follows:

$$y= 19.55+9.83x_1+3.63x_2-9.56x_3-4.49x_1^2-1.31x_2^2 +3.55x_3^2+2.74x_1x_2-5.28x_1x_3-4.16x_2x_3$$

The coefficient of determination for crucian carp was 0.9692, and lack of fit was significant(level of significance was 0.0007). This model showed exactly the change of DH in the hydrolysis of crucian carp. Temperature was the most important linear variable affecting DH and had the highest regression coefficient(β_1=9.83), but ratio of water(β_3=-9.56) was also important linear variable.

In case of the hydrolysis of bastard halibut, the best explanatorty model equation is as follows and the coefficient of determination(R_2) and level of significance were 0.8336 and 0.0433, respectively. It is the lowest value in accuracy of each surface response model equation. Namely, it indicated that only 17% of the total variation was not explained by the model. But this model was considered adequate with satisfactory R^2 value and significant F value.

$$y= 12.82+8.27x_1+3.92x_2-7.14x_3-3.96x_1^2+2.03x_2^2 +2.67x_3^2+3.13x_1x_2-4.82x_1x_3+1.53x_2x_3$$

Important linear variables affecting the hydrolysis of bastard halibut were temperature(β_1=8.27) and ratio of water(β_3=-7.14) like others.

The best explanatory model equation of cooked jacopever is as follows:

$$y= 13.60+8.09x_1+5.97x_2-5.92x_3-0.36x_1^2-3.16x_2^2 +5.51x_3^2+4.95x_1x_2-4.80x_1x_3-4.13x_2x_3$$

The coefficient of determination was 0.9874 and this is the highest R^2 value indicating that the model was adequate. Temperature(β_3=8.09) was the most important variable and followed by time(β_2=5.97) and ratio of water (β_3=-5.92).

As respects a marks of regression coefficients, the positive value implied that DH increased with increasing temperature and time. Net effect of the linear terms of ratio of water suggested that DH decreased as ratio of water further increased.

Optimum cooking conditions for each fish were investigated from these surface response model equations. The most important factor influencing the plastein reaction was the molecular size of the substrate. Hofsten and Lalasidis(13) reported that a whey protein hydrolysate fraction with an average molecular weight of less than 1000 can grow to form a plastein product with a molecular weight of 2000~3000. Montecalvo et al.(6) reported that plastein could be synthesized from a hydrolysate having over 60% DH. Considering that maximum temperature of heating system was 140°C and minimum ratio of water to meat was one(w/w), we found the condition of cooking which could produce a extract having 60% DH. The results are shown in Table 4. Extracts, which obtained

Table 4. Optimum cooking conditions of hydrolysis for the plastein reaction

Sample	Independent value		
	Temperature (T, °C)	Time (t, hr)	Ratio of water (W)
Loach	140	10.08	1
Crucian carp	136.7	7.25	1.1
Bastard halibut	140	9.85	1
Jacopever	140	9.38	1

from this cooking condition of cooking, were lyophilized and then were used in the following experiments.

Optimization of plastein production

At least three points are noted for the effective proceeding of plastein reaction. First, the substrate should be a low molecular weight peptide mixture. Second, the concentration of substrate should be high. Third, pH for the plastein reaction should be controlled to desired range (14). DH of hydrolysate was already controlled in the process of cooking as mentioned above, therefore, the effect of pH, kinds of enzyme, and substrate concentration on the plastein reaction was investigated using partial hydrolysates from cooked fish meat processed at high temperature and pressure.

Effect of enzyme and pH

Fig. 1~4 show the effect of pH on the plastein reaction for fish-meat hydrolyzates using four kinds of proteolytic enzymes(pepsin, papain, trypsin, and chymotrypsin). pH for the maximum plastein reaction was shown in the range of 9.0~10.0, and that of chymotrypsin was 9.0. But pepsin and papain did not show the pH dependency within the pH range of 2~12.

However, every curve indicated that a higher pH gave

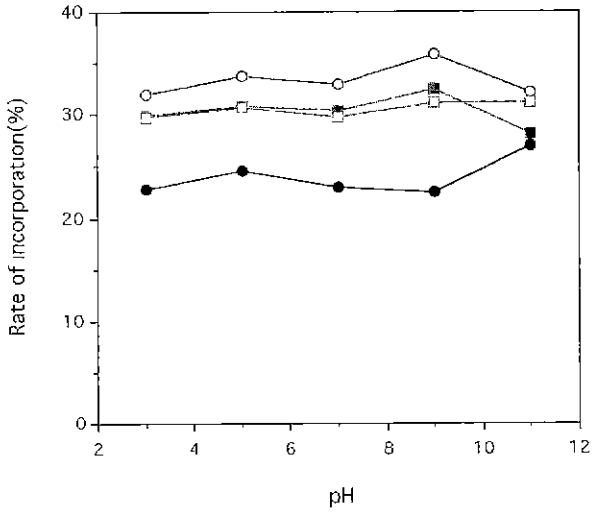

Fig. 1. Effect of pH on free amino acid incorporation into various fish protein hydrolysates using pepsin.
substrate concentration, 40%; enzyme/substrate, 1/100; reaction temperature, 37°C; reaction time, 24hr
-●- Loach -○- Crucian carp
-■- Bastard halibut -□- Jacopever

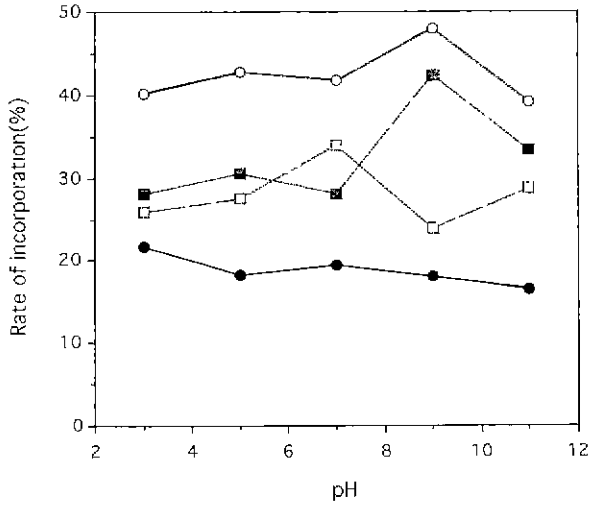

Fig. 2. Changes in free amino acid incorporation by papain at different pH.
substrate concentration, 40%; enzyme/substrate, 1/100; reaction temperature, 37°C; reaction time, 24hr
-●- Loach -○- Crucian carp
-■- Bastard halibut -□- Jacopever

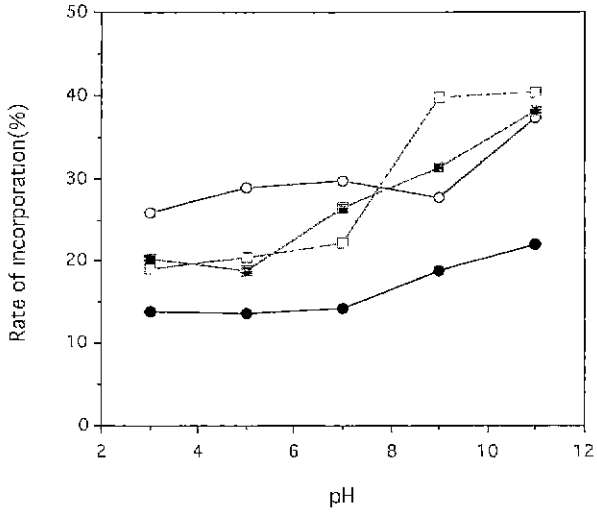

Fig. 3. Changes in free amino acid incorporation by trypsin at different pH.
substrate concentration, 40%; enzyme/substrate, 1/100; reaction temperature, 37°C; reaction time, 24hr
-●- Loach -○- Crucian carp
-■- Bastard halibut -□- Jacopever

better plastein reaction due to one-step plastein reaction of amino acid incorporation into hydrolyzed protein(15). Yamashita et al.(15) suggested that the one-step plastein reaction could occur above pH 9. In alkaline pH, hydrolysis was retarded while one-step plastein reaction was accelerated, possibly due to partially hydrolyzed fish protein at high temperature.

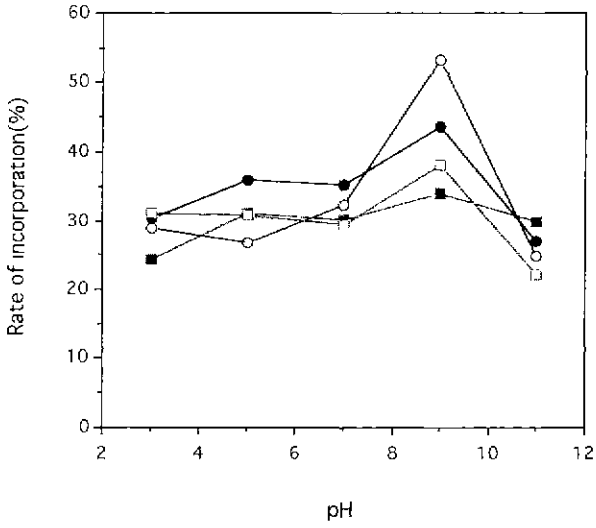

Fig. 4. Effect of pH on free amino acid incorporation into various fish protein hydrolysates using chymotrypsin.
substrate concentration, 40%; enzyme/substrate, 1/100; reaction temperature, 37°C, reaction time, 24hr
-●- Loach -○- Crucian carp
-■- Bastard halibut -□- Jacopever

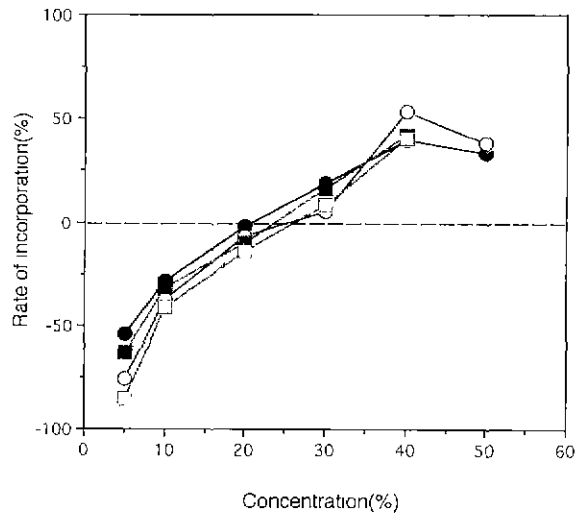

Fig. 5. Effect of the substrate concentration on free amino acid incorporation at maximum plastein reaction condition.
substrate concentration, 40%; enzyme/substrate, 1/100; reaction temperature, 37°C; reaction time, 24hr
-●- Loach(chymotrypsin, pH 9)
-○- Crucian carp(chymotrypsin, pH 9)
-■- Bastard halibut(papain, pH 9)
-□- Jacopever(trypsin, pH 11)

Montecalvo et al.(6) reported that the direction of reaction shifted toward plastein when the degree of hydrolysis exceeded 60%. Therefore, we controlled the degree of hydrolysis of hydrolyzates to 60% which is minimum level of hydrolysis for plastein.

Chymotrypsin gave a higher percent plastein yields in the hydrolysates of loach and crucian carp than in other hydrolysates, and trypsin was effective in plastein formation using the hydrolysate of jacopever. The higher (approximately 50%) plastein yields were obtained in crucian carp and jacopever with papain and chymotrypsin compared with other plastein(40% below). In case of pepsin which is known as an acid protease, DH was not enough to form plastein at acidic pH range and the activity was inactivated at alkaline pH range. Therefore, plastein formation did not change remarkably regardless of pH, and was maintained 30% level of incorporation.

Based on the results above, the optimum plastein productivity for the hydrolysates of loach and crucian carp was determined as chymotrypsin at pH 9.0; for the hydrolysate of bastard halibut was papain at pH 9.0; and hydrolysate of jacopever was trypsin at pH 11.0.

Effect of the substrate concentration

The effect of the substrate concentration upon the rate of plastein production is shown in Fig. 5. To check the effect, pH was adjusted to the pH mentioned previously for the maximum plastein reaction and reaction was performed at 37°C for 24 hours with 1% enzyme solution. As substrate concentration exceeded 20%, the plastein reaction was predominant over the hydrolysis reaction. When the substrate concentration decreased below 20%, hydrolysis reaction was more favorable than the plastein reaction. Tasi et al.(16) reported that the substrate concentration of reaction medium should be in the range of 20~40%(w/v) and no apparent reaction occurred when it was incubated at a concentration of 7.5%(13). Our results were in agreement with the results of Montecalvo et al.(6). Yamashita et al.(17) suggested that the superiority of plastein reaction with increased substrate concentration was due to decreased water proportion in the reaction system. Hydrolysis could be usually carried out at a substrate concentration of 1% or less, but hydrolysis retarded with increasing substrate concentration. When a substrate concentration was increased up to 50%, plastein formation did not change.

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