Chapter 4

COLLAGEN PEPTIDE DEVELOPMENT, OPTIMIZATION AND CHARACTERIZATION

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4.1 Introduction

Collagen peptide is the hydrolyzed form of collagen. After hydrolysis the product loses its gelling ability and makes it soluble in cold water. It differs greatly from other proteins as it contains amino acids glycine, proline and hydroxyl proline in a free form at a concentration that is around 10-20 times higher than in other proteins (Whitford, 2005). These amino acids play an important role in building fibrous tissues. An insufficient supply of these amino acids results in painful joints, brittle fingernails and hair. Many studies indicate that Collagen Peptide has a preventive and regenerative effect on bones, cartilage, and tendon etc. Collagen Peptide also contains all the essential amino acids except tryptophan (Nam *et al.,* 2008).

Skins, scales and bones are the major by-products of the fish processing industry. These by products are not regarded as ordinary saleable products and are usually discarded causing a heavy environmental impact. However, they are a good source of collagen. This collagen could be extracted and further enzymatically hydrolyzed to liberate physiologically active peptides. Specifically, some fish collagen derived peptides may exhibit interesting

antioxidant activity, potent antihypertensive activity, anti microbial activity against different strains of bacteria, protective effect on cartilage, or capacity to stimulate bone` formation. The bioactive properties of collagen-derived peptides, and also their resistance to protein digestion, make them potential ingredients of health promoting foods. (Nagai and Suzuki, 2000a)

Enzymatic hydrolyzing process can produce small fragments of collagen peptides. Furthermore, some of its bioactivity increased and the antigenicity decreased after hydrolysis (Suetsuna *et al.,* 2000). The functional properties of fish proteins may be improved by the use of specific enzymes and by choosing a defined set of hydrolysis conditions.

Collagen hydrolysate varies from each other with respect of peptides molecular weight, mostly their molecular weight range from 2 to 6 kDa. Its molecular weight is less than the average molecular weight of peptones. After purification, the product is concentrated and dried. The most common post dried procedures are related to the control of molecular size and the elimination or reduction of bitterness in the resulting hydrolysate. (Katarzyna and Piotr, 2009). The most efficient procedure to remove residual high molecular weight peptides and proteins or to reduce the antigen content of hypoallergenic formulas is ultrafiltration.

Bioactive peptides usually contain 2–20 amino acid residues per molecule (Pihlanto, 2000); the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Roberts, 1999). So, a three-step hydrolysis reaction was adopted, and orthogonal experiments were designed to optimize processing conditions which might result in high degree of hydrolysis. Depending on the specificity of the enzyme, environmental conditions, and the extent of hydrolysis, wide variety of peptides will be generated. The resultant protein hydrolysate will possess particular properties according to the new peptides generated (Zhang *et al.,* 2010). Several factors, like pH, time, enzyme activity,

and temperature, influence enzyme function, offering possibilities to control the process.

Present study aims to optimise the enzymatic process of collagen hydrolysate production using response surface methodology (RSM) and characterize collagen peptides from the skin of Malabar grouper with enzymatic hydrolysis methods using three different enzymes.

RSM has been a quite effective method of statistical and mathematical analysis for experiment data ever since it was first proposed by Box and Wilson (1951). It can evaluate the influence of all the variables in the multiple factor experiment design. The mutual interaction among factors can also be estimated simultaneously (Myers *et al.,* 2008). A polynomial regression equation can be given to predict the optimal condition of factors on the response. Here, we employed four main factors (pH, temperature, the ratio of enzyme to substrate (E/S) and time) as variables and five levels to optimize the hydrolysis of fish skin. The Degree of Hydrolysis (DH) was set as response to evaluate the efficiency of hydrolysis. Protein hydrolysate qualitative analysis used different techniques based on spectrophotometric, chromatographic and electrophoretic methods.

4.2 Materials and Methods

4.2.1 Raw materials

The species used for the study was Malabar grouper (*Epinephelus malabaricus*). The skin in iced condition was procured from Fort Cochin (9.9680°N, 76.2449°E), Kerala, India. Enzymes used for the hydrolyzation process were pepsin (624 U/mg), papain (3.0 U/mg), and protease from bovine pancreas (1.1 U/mg) from Sigma Chemical (St. Louis, MO, USA).

4.2.2 Optimization of enzymatic processing conditions by RSM

4.2.2.1 Experimental design

Box-Behnken design was applied to evaluate and optimize the effects of four controlled input parameters viz: pH (X_1), temperature (X_2), time (X_3), ES ratio (X_4) and their interaction on the measured response, DH (Y) for three different enzymes viz: pepsin, papain and protease. The input variables were coded at three levels (-1, 0, +1,) and the complete design consisted of 27 experimental points including 3 replications of the centre for each enzyme. The original and coded levels of the independent variables used in the RSM design for each enzyme are listed in Table 4.1.

Table 4.1 Uncoded and coded levels of independent variables used in the RSM design

		Pepsin			Papain			Protease		
Independent variable	***Coded variables***	***-1***	***0***	***1***	***-1***	***0***	***1***	***-1***	***0***	***1***
pH	X1	1.6	2	2.4	5	6	7	6	7	8
Temperature	X2	30	40	50	20	30	40	30	40	50
Time	X3	2	4	6	2	4	6	2	4	6
E/S	X4	0.5	2	8	0.5	2	8	0.05	0.2	0.8

4.2.2.2 Statistical analysis

The second-order polynomial regression model fitted to the DH (Y) as a function of input variables and the adequacy of the fitted model was assessed by using R^2 for each enzyme viz: pepsin, papain and protease. The functional form of the fitted model for each enzyme is given in the equation

$$Y = \beta_o + \sum_{i} \beta_i x_i + \sum_{ii} \beta_{ii} x_{ii}^2 + \sum_{i} \sum_{j,i<j} \beta_i \beta_j x_i x_j + e, i \neq j$$

Where, "Y" is response variable, "β_o" is intercept, "β_i" is linear regression coefficients, "β_{ii}" is quadratic regression coefficients, "$\beta_i\beta_j$" is interaction regression coefficients and "e" is error term. Ridge analysis was carried out to predict the response variable at different radius of the design region. The optimisation of response variables was done based on the ridge score and response surface plot of the response variables. The fitting of second-order polynomial regression model and numerical optimization was done by writing a SAS code in SAS 9.3.

Once the condition of pH (X_1), temperature (X_2), time(X_3), ES ratio (X_4) for maximum DH optimized based on the fitted model, the validation of the optimized condition was done for each enzyme with three replications for each enzyme.

4.2.3 Preparation of Collagen hydrolysate

The selected fish skin was thoroughly washed, minced and mixed with sodium hydroxide solution and kept stirred for 24 h over a magnetic stirrer. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water. The residue was homogenized with 30 volumes of dilute acetic acid and the same was stirred over a magnetic stirrer for 24 h. The acid solution containing collagen was centrifuged. To the supernatant crystalline sodium chloride was added to the level of 5% and stirred for appropriate time to precipitate collagen. The precipitated collagen was separated and suspended in Tris-glycine buffer and dialysed against the same buffer for 24 h to get pure fish skin collagen.

For hydrolysate preparation the extracted fish skin collagen in double distilled water was preheated to 60°C for 5 min, and then hydrolyzed enzymatically. For hydrolysis three different proteolytic enzymes, via pepsin, papain and protease from bovine pancreas (PP) were used. Pepsin was dissolved in 10mM HCl solution (pH 2), papain was dissolved in deionised water and the PP dissolved in 10

mM sodium acetate buffer, pH 7.5. The hydrolysis conditions of time, temperature, E/S ratio and pH were optimised for the three enzymes by RSM. After hydrolysis, inactivation of enzymes was accomplished by heating for 3 minutes in boiling water bath.

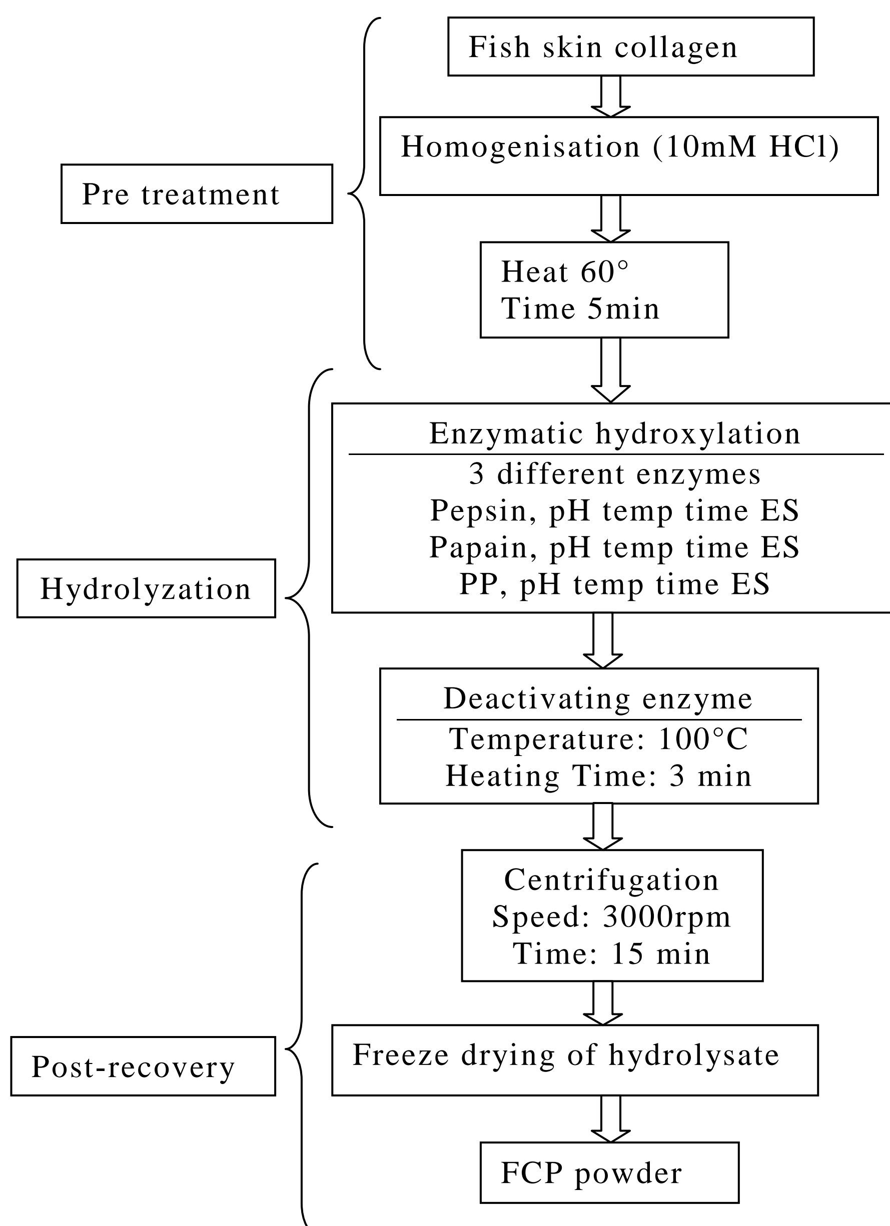
Filtration and lyophilisation: The extract was filtered to remove skin residues. Then fine residues were removed by centrifugation at 10,000 × g for 30 min at 4°C. The resulting solution was adjusted to pH 7 with saturated NaOH or HCl. Finally, the neutralised solution was filtered through Whatman No. 4 filter paper. The filtered solution was then freeze-dried in a lyophilizer. The freeze dried product was stored at 4°C until use.

4.2.4 Determination of Degree of Hydrolysis

Degree of hydrolysis (DH) is the percentage ratio between the numbers of peptide bonds cleaved to the total number of peptide bonds in the substrate studied. It was calculated by the determination of free amino group reaction with TNBS (according to TNBS method Alder-Nissen, 1979) with some modifications. Basically, this method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions and is terminated by lowering the pH. TNBS also reacts slowly with hydroxyl ions, whereby the blank reading increases; this increase is stimulated by light.

Properly diluted samples (125 µL) were mixed with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50 °C for 30 min in the dark. During incubation the test tubes must be covered with aluminum foil because the blank reaction is accelerated by exposure to light. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixtures were cooled down at ambient temperature for 15 min and the absorbance was measured at 340 nm.

Flow diagram of enzymatic processing method to produce FCP



A TNBS standard calibration curve was prepared using amino acid glycine (11.25 mg/ml) and was found to be linear (R^2 = 0.996). The amount of free amino group liberated was expressed in terms of glycine.

The DH was evaluated as

$$DH\ (\%) = \frac{C_0 - C_t}{C_0} \times 100\%$$

Where C_0 is glycine equivalent of sample at time = 0, C_t is glycine equivalent of sample at time t.

4.2.5 Characterization

4.2.5.1 Amino acid composition of hydrolysate

Spray dried collagen peptide was hydrolyzed in 6N HCl at 120°C for 24h. After cooling the test tubes the contents were filtered using Whatman No 1 filter paper. The tubes were rinsed with distilled water and filtered. The filtrate was evaporated in a vacuum flash evaporator. Then deionized water was added into the tubes and continued evaporation until the contents were acid free. The process was repeated for three times and the free amino acids were dissolved in 0.05M HCl and filtered using 0.45 micro syringe, then injected in to Shimadzu HPLC using the method (Ishida *et al.,* 1981).

4.2.5.2 SDS PAGE

The fish collagen peptides were analyzed by Tricine-SDS-PAGE according to Schagger *et al.* (1987) with slight modifications using 16.5% separating gel, 10% spacer gel and 4% stacking gel. The lyophilized hydrolysate was dissolved (20 mg/mL) in loading buffer (12% SDS, 6% β- mercaptoethanol, 30% glycerol, 0.05% Coomassie blue G-250, and 150 mmol/L Tris–HCl at pH 7.0), heated at 100°C for 5 min, and centrifuged at 12,000 g for 2 min.

The electrophoresis was performed in a mini electrophoresis apparatus (Bio-Rad, California, USA) at room temperature using the following procedure. The voltage was kept constant at 30 V until the samples completely left the stacking gel, then the voltage was kept constant at 90V - 100V until the tracking dye was close to the bottom of the gel. The loading volume of the samples and the standards was 5 µL-10 µL. After electrophoresis, gels were fixed with a mixture of 500 mL/L methanol and 100 mL/L acetic acid for 30 min, followed by staining with 0.5 mL/L Coomassie blue R-250 in 150 mL/L methanol and 50 mL/L acetic acid for 1 h. Finally, they were destained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1 h and destained again with the same solution for 30 min. The approximate molecular weights of the hydrolysate were determined using appropriate prestained protein molecular weight marker 2 kDa-71 kDa (SBS Genetech Co., Ltd, Beijing, China).

4.2.5.3 MALDI-TOF Mass Spectrometric Analysis

Using MALDI-TOF mass spectrometry, the molecular weight distributions of peptides from collagen hydrolysate was estimated. The analysis was done on positive ion mode MALDI TOF mass spectrometer. The mass spectrometer used for detection of analytes, was AB SCIEX TOF/TOF 5800, AB SCIEX Co., USA with Nd: YAG 1000-Hz laser with 355 nm wavelengths, 2.0m long TOF tube and a sample target plate with 96 wells. CentriVap -50° C cold trap, LABCONCO Ltd., USA was used for concentrating sample under speed vacuum.

Standard MALDI matrices like CHCA, Sinapinic acid were obtained from Sigma-Aldrich. Acetonitrile of LC-MS Grade was bought from Sigma-Aldrich. Trifluoroacetic acid obtained from Fluka. Deionized Water (18.2 MΩ/cm, 4ppb, and 25°C), Bradykinin, Angiotensin II, P14R and ACTH combinedly called Calmix, were procured from sigma as calibration mixture for mass spectrometer.

Operating mode – Reflector positive ion, linear positive ion
Laser intensity – 3600, 4400 (Reflector positive ion mode), 6000 (Linear positive ion mode) units
Laser type – Nd: YAG 1000-Hz laser with 355 nm wavelength
Mass range – 300 to 10000 m/z (Reflector positive ion mode), 300 to 20000 m/z (Linear positive ion mode)
Delayed extraction time – 400 ns
MALDI Plate velocity – 900, 1100 µm/sec
Laser shots: 4000 per spectrum

Sample preparation: A solution containing acetonitrile: 0.1% trifluoro acetic acid in deionised water in 1:1 v/v (diluent) was prepared for reconstituting these aliquots. Five aliquotes from the collagen peptide were evaporated to dryness by using CentriVap -50° C cold trap and reconstituted in diluent. These samples were further diluted to 10X and 100X concentration followed by aliquoting in 10µL volume each. There were two spotting methods used for mass spectrometric analysis as sandwich and dried droplet. For sandwich method, reconstituted samples spotted on MALDI target plate, sandwiched with matrices (CHCA, sinapinic acid) followed by drying. Each recontituted sample was mixed with each of matrices in 1:1 v/v, separately and vortexed. These resulted samples were spotted on MALDI target plate and dried. All the spotted samples were analyzed by AB SCIEX TOF/TOF 5800 system.Standard calmix peptide mixture was used for validating method for data acquisition.

4.2.6 Functional properties of fish collagen peptide

4.2.6.1 Solubility

To determine protein solubility, 250 mg of protein hydrolysate was dispersed in 5 mL of deionized water. The mixture was stirred at room temperature for 2 h and centrifuged at 2000g for 10 min. Protein content of the supernatant was determined by AOAC (2005) method. Solubility was calculated as protein content in supernatant over total protein content in the initial dispersion. Protein solubility was calculated as follows

% Solubility = protein content in the supernatant/ total protein content in the hydrolysate x 100

4.2.6.2 Change in viscosity

Viscosity of collagen solution and collagen hydrolysate solution was determined by rotary viscometer test method (Brookfield Digital Viscometer, Model DV-E). In this test method, 3mg/ml of test solution is placed in a glass tube, housed in an insulated block at a fixed temperature (37°C). A metal spindle is then rotated in the solution at 100 rpm, and the torque required to rotate the spindle is measured. Based on the internal resistance to rotation provided by the shear stress of the solution, the solution's absolute viscosity can be determined. Absolute viscosity is represented in centipoise (cP).

4.3 Results and Discussion

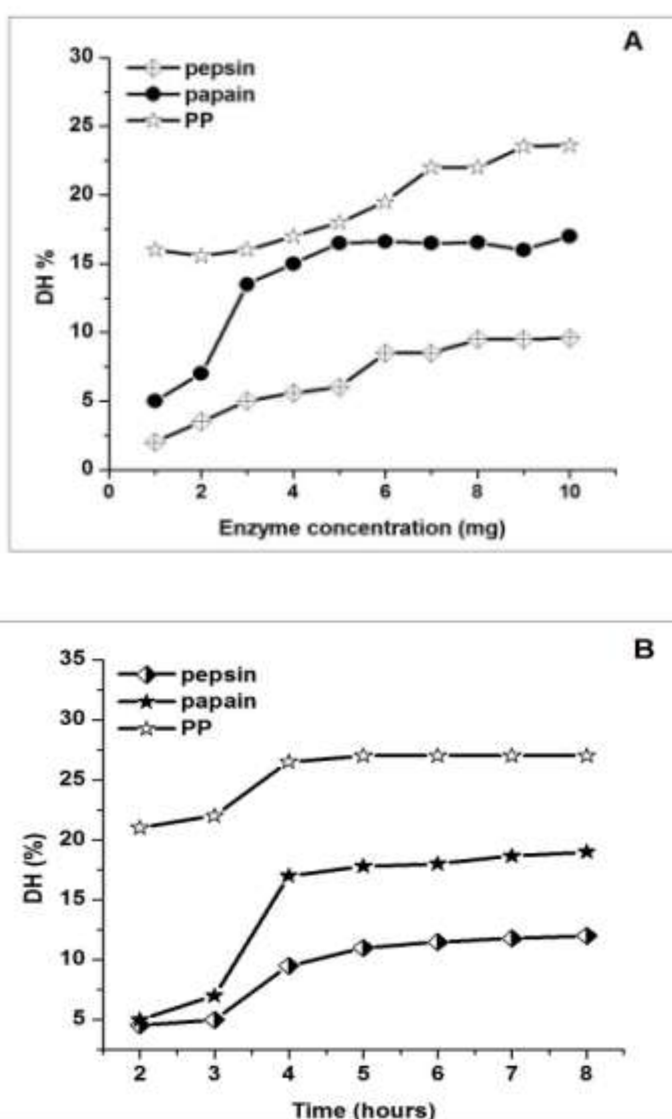
Bioactive peptides usually contain 2–20 amino acid residues per molecule (Pihlanto-Leppala, 2000); the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Roberts *et al.,* 1999). In the study, a three step hydrolysis reaction was adopted, and experimental points were designed to optimize processing conditions to obtain smaller peptides, which might possess potent bio activity.

The degree of hydrolysis, DH (i.e. percentage of peptide bond cleaved) is a true reflection of the progress of hydrolysis and thus it is selected as the primary indicator for the control of hydrolysis. This study used RSM to evaluate the effects of pH, temperature, enzyme concentration and time on the DH of the skin of grouper with three different enzymes pepsin, papain and protease from bovine pancreas. A second-order polynomial regression equation was given to describe the model and predicted an optimal condition of factors to maximize the DH.

4.3.1. Single factor experiments

In single factor experiments, three of four factors (pH, temperature, and time and enzyme substrate concentration) were fixed while the other factor was ranged to evaluate its effect on hydrolysis. This was done to fix the range of time and enzyme substrate ratio in the hydrolysis process. The DH of the hydrolysate could be measured by TNBS method and the results were shown in Fig. 4.1 A and 4.1 B.

Figure 4.1 The effect of enzyme concentration (4.1 A) and reaction time (4.1 B) on the degree of hydrolysis.



As can be seen from Fig.4.1 A, there was an increase in the DH with the increase of enzyme concentration. Despite of the aim to maximise the DH, the cost and the economy should be taken into consideration as well. It is a waste to pursue higher DH with excessive enzyme. Thus, 6.0mg of enzyme concentration was adequate in the case of pepsin and papain. In the case of protease from bovine pancreas, even at low concentrations itself it gives high degree of hydrolysis and so its concentration controlled at 2.0mg.

It can be seen from Fig.4.1B that the tendency for the DH increases with increasing incubation time. Too long time means too much expenditure of energy and so on. For the same reason, time of hydrolysis should be restricted to 6.0 h.

4.3.2 Optimization of enzymatic hydrolysis using RSM

4.3.2.1 Response surface analysis

The experiment was carried out based on the Box-Behnken design of experiment set up. The results of different combinations of input variables along with the response variable on degree of hydrolysis is given in the table 4.2, table 4.3, and table 4.4 for pepsin, papain and PP respectively.

Table 4.2 Factors and levels in the RSM and experimental results for the enzyme pepsin

Pattern	*pH*	*Temperature*	*Time*	*E/S Ratio*	*DH*
0−0+	2	30	4	8	5.5
+0−0	2.4	40	2	4.25	5.6
−00+	1.6	40	4	8	7.6
+00+	2.4	40	4	8	8.2
0000	2	40	4	4.25	8.5
−0+0	1.6	40	6	4.25	2.6
−00−	1.6	40	4	0.5	3.25
0++0	2	50	6	4.25	2
00––	2	40	2	0.5	3.25
+0+0	2.4	40	6	4.25	9.55
0000	2	40	4	4.25	8.36
00−+	2	40	2	8	7.65
0—0	2	30	2	4.25	3.25

0−0−	2	30	4	0.5	3.15
0+0+	2	50	4	8	1.95
+00−	2.4	40	4	0.5	1.14
−+00	1.6	50	4	4.25	1.17
0+−0	2	50	2	4.25	1.66
0−+0	2	30	6	4.25	6.5
00++	2	40	6	8	9.86
+−00	2.4	30	4	4.25	1.86
—00	1.6	30	4	4.25	3.14
0+0−	2	50	4	0.5	2.65
0000	2	40	4	4.25	8.75
00+−	2	40	6	0.5	4.15
−0−0	1.6	40	2	4.25	5.76
++00	2.4	50	4	4.25	1.55

Table 4.3 Factors and levels in the RSM and experimental results for the enzyme papain

Pattern	*pH*	*Temperature*	*Time*	*E/S ratio*	*DH*
0000	6	30	4	4.25	17
0+0−	6	40	4	0.5	11
+00−	7	30	4	0.5	7.6
0000	6	30	4	4.25	18.5
00—	6	30	2	0.5	8.75
00−+	6	30	2	8	14.5
0−+0	6	20	6	4.25	18
+0+0	7	30	6	4.25	17
−+00	5	40	4	4.25	0
0−0−	6	20	4	0.5	6.55
−00+	5	30	4	8	0
00++	6	30	6	8	19.43
—00	5	20	4	4.25	0
+−00	7	20	4	4.25	19
−0+0	5	30	6	4.25	0
+0−0	7	30	2	4.25	12
−00−	5	30	4	0.5	0
+00+	7	30	4	8	17.78
0−0+	6	20	4	8	16
0+−0	6	40	2	4.25	2
0+0+	6	40	4	8	2.5
00+−	6	30	6	0.5	12.65
++00	7	40	4	4.25	5.01
−0−0	5	30	2	4.25	0
0—0	6	20	2	4.25	9
0000	6	30	4	4.25	19.8
0++0	6	40	6	4.25	6.88

Table 4.4 Factors and levels in the RSM and experimental results for the enzyme PP

Pattern	*pH*	*Temperature*	*Time*	*E/S Ratio*	*DH*
0+0+	7	50	4	0.8	2
+00+	8	40	4	0.8	14.76
0000	7	40	4	0.425	27
+−00	8	30	4	0.425	0
0—0	7	30	2	0.425	0
0−+0	7	30	6	0.425	0
0++0	7	50	6	0.425	0
−0−0	6	40	2	0.425	24
++00	8	50	4	0.425	0
—00	6	30	4	0.425	0
+0−0	8	40	2	0.425	12
−+00	6	50	4	0.425	0
00—	7	40	2	0.05	14.55
00−+	7	40	2	0.8	25.88
0−0+	7	30	4	0.8	0
00++	7	40	6	0.8	26
−00+	6	40	4	0.8	26
00+−	7	40	6	0.05	15.56
+00−	8	40	4	0.05	14.9
0000	7	40	4	0.425	26.53
0+0−	7	50	4	0.05	0
0+−0	7	50	2	0.425	0
0−0−	7	30	4	0.05	0
0000	7	40	4	0.425	27.55
−0+0	6	40	6	0.425	27.9
+0+0	8	40	6	0.425	12
−00−	6	40	4	0.05	15

The adequacy of the model was justified through analysis of variance (ANOVA). The examination of the fitted model was necessary to ensure that it provided an adequate approximation to the true system. Regression analysis was employed to fit a full response surface model for every response investigated, including all linear (X_1,X_2,X_3,X_4) and quadratic/interaction terms (X_1^2, X_2^2, X_3^2, X_4^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4,). The regression coefficients for the 2nd order response surface models in terms of coded units are shown in Table 4.5.

Table 4.5 Linear, Quadratic and Interaction regression coefficients of independent Variables where X_1: pH, X_2: temperature, X_3: time, X_4: enzyme concentration.

	Pepsin	***Papain***	***Protease***
Intercept	-97.65**	-423.62**	-608.62**
X_1	39.26	107.48**	69.08*
X_2	3.33**	5.95	18.26**
X_3	-1.2	4.32	10.5
X_4	1.02	1.83	78.2
X_1^2	-13.3**	-7.9**	-4.8*
X_2^2	-0.04**	-0.05**	-0.23**
X_3^2	-0.2	-0.68*	-0.84
X_4^2	-0.09	-0.23**	-26.87*
X_1X_2	-0.84	-0.35*	0
X_1X_3	2.2*	0.63	-0.49
X_1X_4	0.45	0.68	-7.4
X_2X_3	-0.04	-0.05	0
X_2X_4	-0.02	-0.12**	0.13
X_3X_4	0.04	0.03	-0.3
R^2	0.86	0.95	0.95

*-Indicates regression coefficients significant at 5% level of significance

**- Indicates regression coefficients significant at 1% level of significance

The reliability of fitted second order polynomial model was assessed using coefficient of determination (R^2) and Root mean square error (RMSE). The R^2 and RMSE value of fitted model was 0.86 and 1.55 for pepsin, 0.95 and 2.39 for papain and 0.95 and 3.63 for PP respectively. Based on the evaluation of R^2 value which is close to 1 and minimum RMSE value, it is inferred second order response model fitted well to the experimental data (Chauhan and Gupta, 2004). As shown in the table 6, 7 and 8 it is suggested that this quadratic model was appropriate to represent the real relationships among the chosen hydrolysis parameter and the model can explain a high percentage of the variability in the observed data.

Also the significance of linear, quadratic and interaction effect of pH, temperature, and time and enzyme concentration on DH for each enzyme was evaluated using ANOVA. The linear, quadratic and interaction effect of input factors was found to be significant at 5%

level of significance for all three enzymes except for some two way interaction effect for papain and protease. The results of ANOVA are given in table 4.9, 4.10 and 4.11.

Table 4.9 shows the linear, quadratic and interaction effect of four factors on DH in the case of pepsin. The linear effect of temperature, quadratic effect of pH and temperature was found to be significant at 1% level of significance. The interaction effect of pH and time was found to be significant at 5 % level of significance where as other effects were insignificant.

Table 4.10 shows the linear, quadratic and interaction effect of four factors on DH in the case of papain. The linear and quadratic effect of pH and temperature was found to be significant at 1% level of significance whereas time and ES ratio was found to be non-significant. The quadratic effect of time and ES ratio was significant at 5% level of significance. The interaction effect of temperature and pH, ES ratio and pH, ES ratio and temperature were found to be significant at 5% level of significance, where as other interactions were insignificant.

Table 4.11 shows the linear, quadratic and interaction effect of four factors on DH in the case of PP. The linear and quadratic effect of temperature was found to be significant at 1% level of significance. The linear effects of pH, quadratic effect of ES ratio were found to be significant at 5% level of significance where as all other effects were insignificant.

Three-dimensional response surface plots presenting the different combinations of input factors on the DH are given in Fig. 4.2, Fig 4.3 and Fig 4.4 respectively for pepsin, papain and protease. Each of the plots was drawn to predict the value of DH by varying the levels of two input factors at a time and by fixing the levels of other two factors constant. As shown in Figure, if the selected variable’s value was in the optimum range, the DH increased until combination of the time, temperature, and E/S ratio reached a maximum yield of the product. But if the conditions that selected for

the hydrolyzation were out of this range, even though it has higher value for each of the variables, the DH cannot reach the high value, and it will stay at the lower point of DH. This confirmed that extremes of pH and temperature are affecting the enzyme activity. The shape of the hydrolysis curve has been associated with enzyme inactivation, product inhibition by hydrolysis products formed at high degrees of hydrolysis, a low Km value for the soluble peptides that act as effective substrate competitors to the unhydrolyzed protein, and possibly auto digestion of the enzyme (Rebeca *et al.,* 1991; Mullally *et al.,* 1995).

Fig. 4.2A shows that at lower and higher level of pH and temperature, DH was less as compared to the centre points. Fig 4.2B and 4.2C shows that lower levels of pH, DH was less irrespective of the levels of time and ES ratio and DH was higher near pH 2 irrespective of levels of time, ES ratio. Fig 4.2D & 4.2E shows that the maximum predicted DH noticed at centre points of time and temperature, ES ratio and temperature than lower and higher levels. Fig. 4.2F shows that minimum and maximum DH was observed at lower and higher levels of ES ratio and time respectively.

As shown in Fig. 4.3A and 4.3B, the increase of pH affected the DH significantly up to a certain point irrespective of the changes in time or enzyme concentration, and the mutual interactions between pH and the other two factors were not so obvious. Fig. 4.3C showed noticeable quadratic effect of interaction between temperature and pH. Fig. 4.3E showed the degree of hydrolysis is very less at higher and lower levels of temperature and enzyme concentration.

In each of the plots in Fig.4.4B, 4.4C and 4.4F, there was a peak of the curved surface that revealed an optimal condition of the factors on the response, which mean that any level of the condition lower or higher than that would produce less influence on hydrolyzing process with the result of diminishing the DH.

These graphs met well with the results of ANOVA and described the statistical data visually. In each of the plots, there was a peak of the curved surface that revealed an optimal condition of the factors on the response, which mean that any level of the condition lower or higher than that would produce a negative influence on hydrolyzing process with the result of diminishing the DH.

4.3.2.2 Optimisation and Validation Study

To maximize the DH, the conditions of four factors in this study were optimized with Numerical optimisation of Design Expert software. According to the ridge analysis the recommended desirable values are: 2.1 of pH; 36.62°C of temperature; 3.6% of E/S ratio; and 5.47 h of time for pepsin. The recommended values for papain are 6.38 of pH; 26.22°C of temperature; 4.5% of E/S ratio; and 4.25 h of time. And the recommended values for protease are 6.3 of pH; 39.86°C of temperature; 1.8% of E/S ratio; and 4.25 h of time. Under these conditions, the predicted response of DH was estimated to be 10.0049%, 20.7234% and 28.0552% pepsin, papain and protease respectively.

Once an empirical model has been developed and optimized the process parameters using the developed model, it is very much important to validate the model by extrapolating the optimized conditions by conducting the actual experiment. Accordingly to confirm the validity of the developed model, three assays were performed under the recommended conditions given above. The experimental values of DH obtained for papsin, papain and protease were 11.5 ± 0.73% and 21 ± 1.03% and 28.6 ± 0.68% respectively. The experimental values agreed with the value predicted by the model within a 95% confidence interval. The above results confirmed that the model was powerful and suitable for the estimation for experimental values.

Figure 4.2 Pepsin: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots

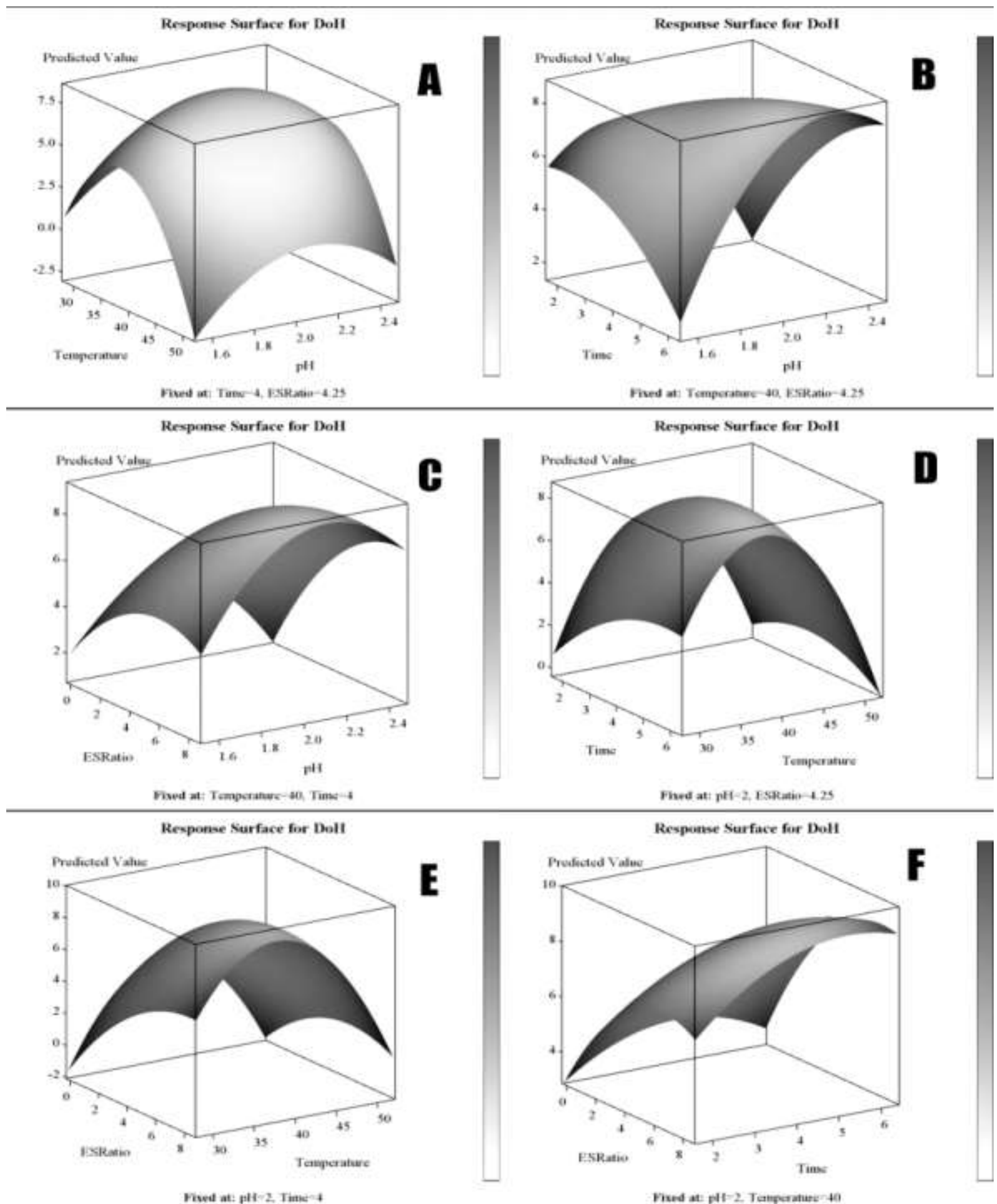


Figure 4.3 Papain: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots

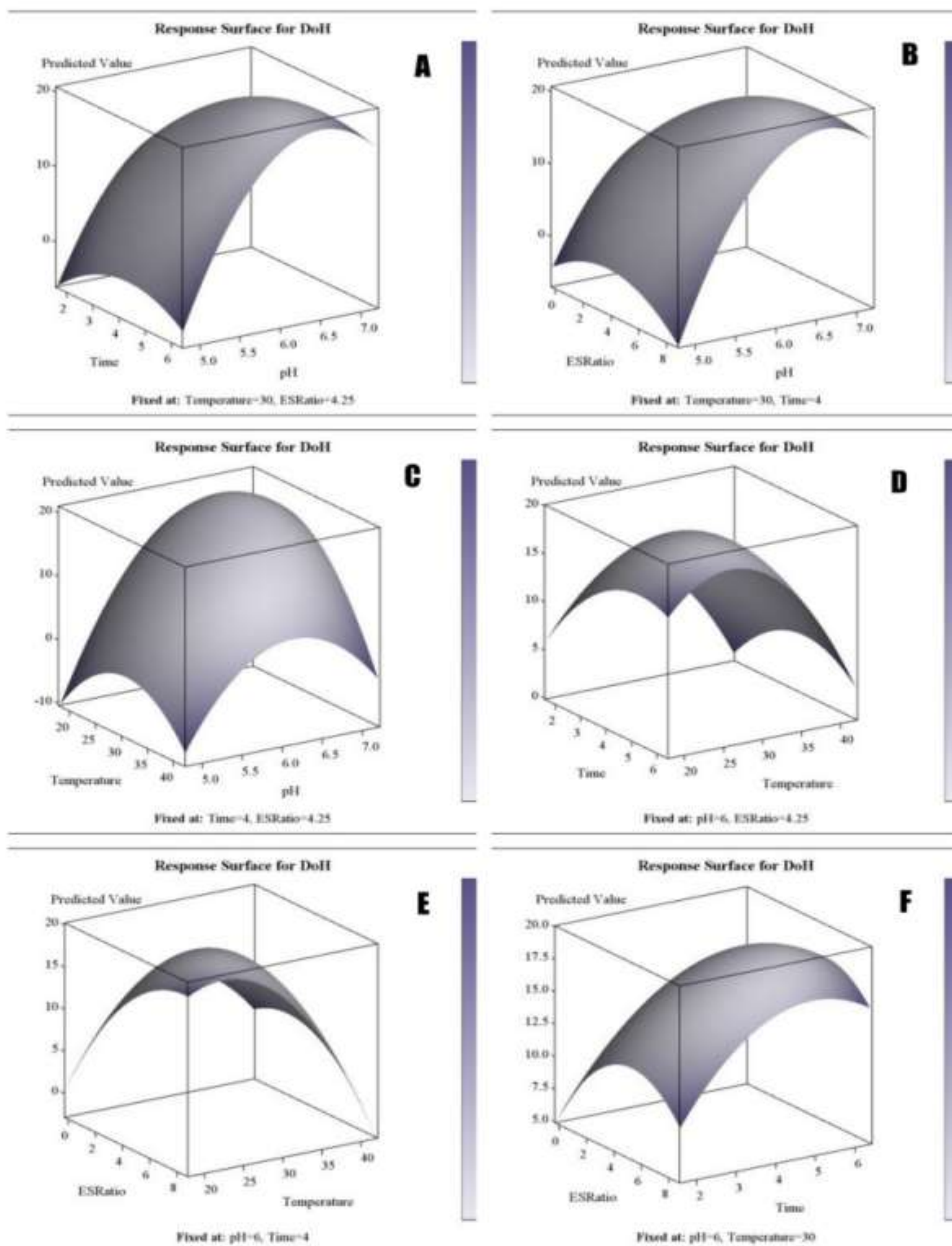
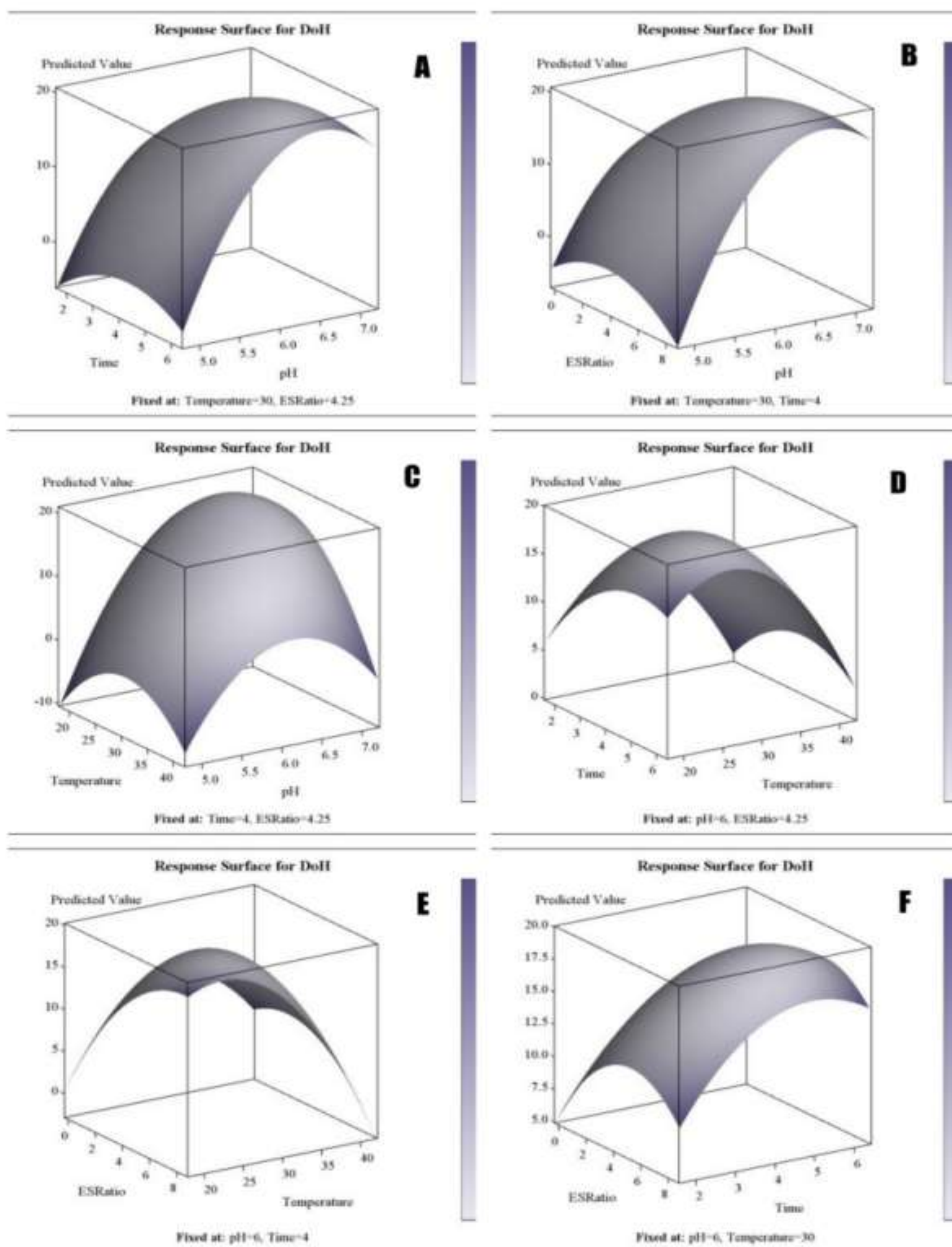


Figure 4.4 Protease: Effects of different variables on the degree of hydrolysis presented in response surface (3D) plots



4.3.3 Development of collagen hydrolysate

A three step hydrolysis of grouper skin collagen was done using the three enzymes consecutively under the optimised conditions. The process resulted in a fine powder of collagen hydrolysate (shown in fig 4.5A) having degree of hydrolysis and nitrogen recovery respectively of 56 ±0.73% and 76.11±1.03%.

4.3.3.1 Viscosity and solubility of collagen hydrolysate

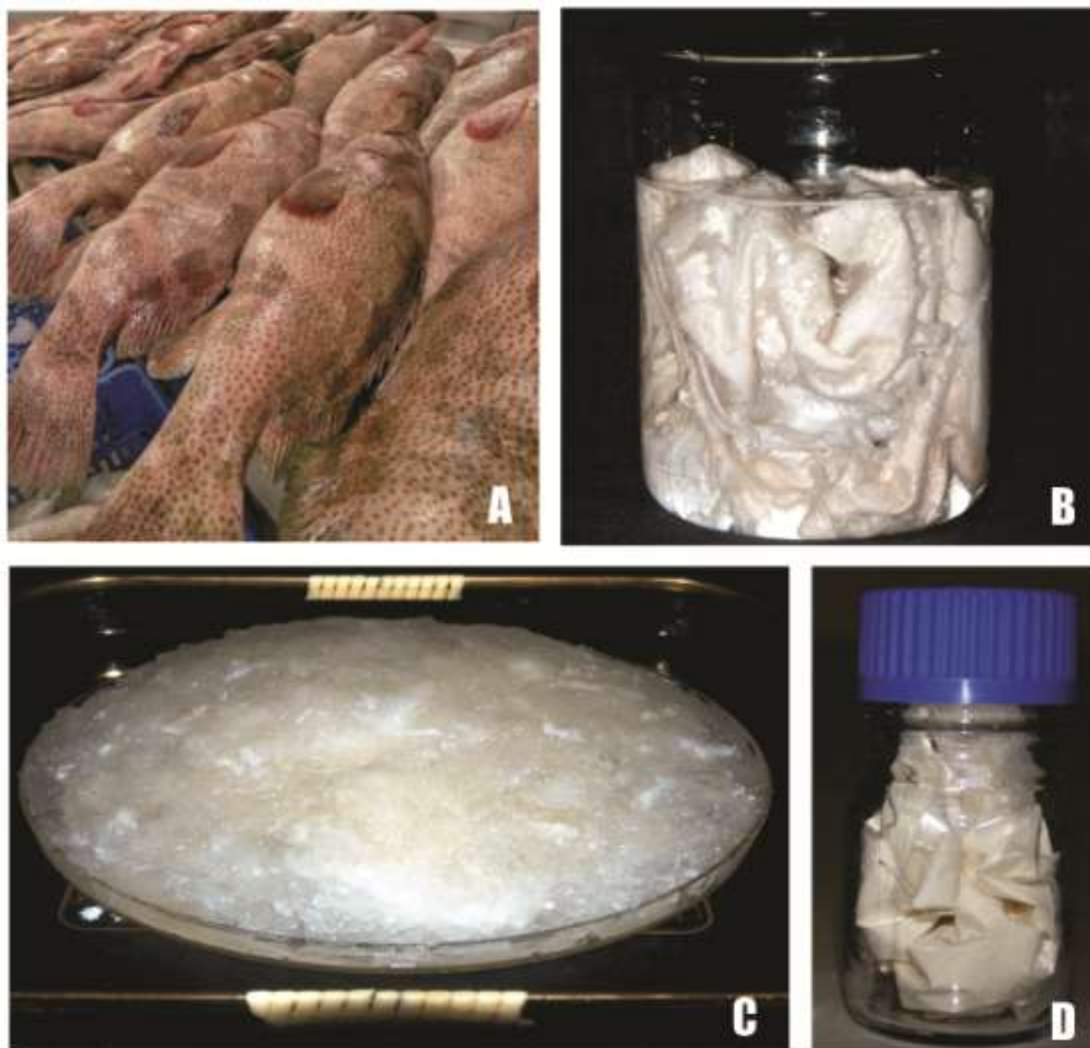
Lyophilized hydrolysate was almost 100% soluble over a wide range of pH values (3–9). The viscosity of collagen solution was 96 cP and the viscosity of collagen hydrolysate was 1.422 cP.

Hydrolysate is known to have excellent solubility at a high degree of hydrolysis (Gbogouri *et al.,* 2004). Hydrolysis potentially influenced the molecular structure, hydrophobic nature and polar groups of the hydrolysate (Kristinsson and Rasco*,* 2000). Higher DH means smaller peptides, which were expected to have proportionally more polar residues and the ability to form hydrogen bonds with water, thereby improving the solubility (Gbogouri *et al.,* 2004). The fish collagen hydrolysate had a DH of approximately 56 ±0.73%, and hydrolysate consisted of low molecular weight peptides that were mostly in the range of 2 kDa, which may support the findings of high solubility.

4.3.4 Characterization

Enzymatically hydrolyzed fish peptides exhibit different physicochemical properties and biological activities depending on their molecular weight and amino acid sequence. Therefore the molecular weight of the bioactive peptide is one of the most important factors in producing bioactive peptides with the desired biological activities (Kim and Mendis, 2006; Kim and Wijesekara, 2010)

Figure 4.5A Whole fish from landing centre B. Purified skin C. extracted collagen D. freeze dried collagen peptide



4.3.4.1 Amino acid composition analysis

The amino acid compositions of grouper skin collagen and its hydrolysate product are compared in table 4.12. As shown in the table, amino acid profile of the hydrolysate is comparable to the corresponding parent protein. When the amino acid composition of the collagen hydrolysate was analyzed, it was rich in proline, glycine, alanine and hydroxyproline residues and small amounts of tyrosine, histidine, and methionine, residues and complete absence

of tryptophan. The results are in agreement with those reported by Vivian Zague *et al.,* 2011; Wang *et al.,* 2008.

The amino acid composition of the porcine skin cocktail hydrolysate as reported by Moskowitz (2000) was different from that of the fish skin gelatine hydrolysate, although the major amino acid components of the two collagen hydrolysates were same. Glycine was the most abundant amino acid in all collagens and the amount was approximately 21% to 23%, which was one of the characteristics of collagens isolated from living organisms. Prabjeet *et al.* (2011) reported that the major amino acid in fish collagen is glycine, followed by proline, alanine, and hydroxyproline. Vivian Zague *et al.* (2011) reported that collagen hydrolysate had high contents of glycine (24.5%), glutamic acid (10.1%), arginine (8.1%), proline (13.8%), and hydroxyproline (7.4%) residues and small amounts of tyrosine, cysteine, histidine, and methionine residues.

Amino acid composition of the lyophilized hydrolysate was analyzed in order to determine the possible effect of the amino acid profile on biological activity. A previous report shows that the bioactivity of the lyophilized hydrolysates depended upon the amino acid sequence of the peptides. High content of hydrophobic amino acids could increase the solubility of collagen peptides in lipid and then enhance their antioxidant activities (Kim *et al.,* 2001). Rajapakse *et al.* 2005 found that fish skin gelatin peptides possessed higher antioxidant activity than peptides derived from other proteins because of the high percentage of Glycine and Proline. Chen *et al.,* (2006) reported that histidine and proline played important roles in the antioxidant activity of synthetic peptides. Therefore, we can predict one factor responsible for the bio activities of collagen hydrolysates were inherent to their characteristic amino acid sequences.

Table 4.12 Amino acid composition (g/100g protein) of grouper skin collagen (GC) and grouper skin collagen hydrolysate (GCH)

Amino acids	*GC*	*GCH*
Aspartic acid	4.03 ± 0.42	2.35± 0.75
Threonine	2.17 ± 0.25	1.43± 0.05
Serine	3.80 ± 0.36	2.65± 0.92
Glutamic acid	4.90 ± 0.26	2.21± 0.23
Proline	12.27 ± 0.60	9.59± 0.11
Glycine	31.23 ± 1.33	26.81± 0.76
Alanine	11.37 ± 0.47	10.80± 0.65
Cystine	ND	ND
Valine	2.73 ± 0.31	1.21± 0.45
Methionine	0.90 ± 0.10	0.43± 0.62
Isoleucine	1.03 ± 0.21	0.84± 0.40
Leucine	2.33 ± 0.32	1.50± 0.72
Tyrosine	0.37 ± 0.06	0.21± 0.44
Phenylalanine	1.47 ± 0.21	0.67± 0.72
Histidine	1.43 ± 0.42	0.28± 0.22
Lysine	3.80 ± 0.44	2.47± 0.55
Arginine	5.13 ± 0.45	3.55± 0.11
H. Proline	9.77 ± 0.35	5.99± 0.32

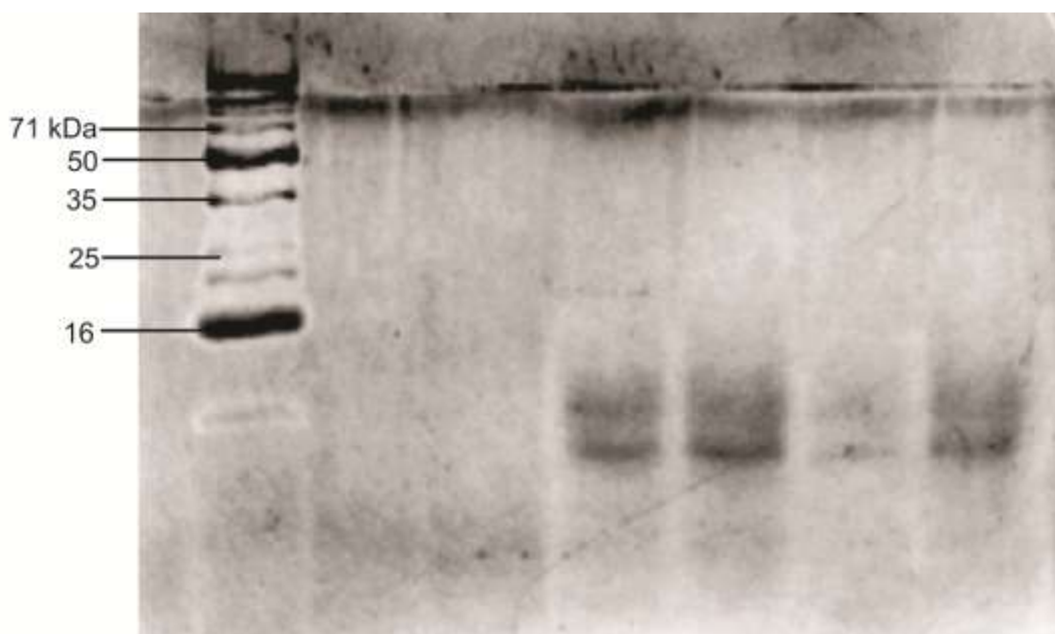
4.3.4.2 SDS PAGE

The electrophoretic pattern of the fractionated hydrolysate presented in Fig 4.6 indicated that the peptides formed due to hydrolysis were less than 16kDa in molecular weights, corresponding well with the higher DH values observed in the study.

This result indicated that the hydrolysis process had successfully cleaved the peptide bonds, resulting in lower molecular weight and higher DH. It can be predicted that most of the triple helices of collagen hydrolysate had been destroyed and parts of their peptide bonds were also broken out. Therefore, there were wide distributions and lower molecular weights for gelatin and collagen hydrolysate (Zhongkai Zhang *et al.,* 2005). In order to obtain protein hydrolysates of high nutraceutical value, the dietary protein in it

should rich in low molecular weight species, with the amounts of free amino acids as low as possible (Vijayalakshmi *et al.,* 1986).

Figure 4.6 Tricine SDS PAGE pattern of collagen hydrolysate. First lane shows molecular markers. Lane 4,5,6,7 are 1.5mg/ml, 2mg/ml, 2.5mg/ml, 3mg/ml concentrations of hydrolysate respectively.



4.3.4.3 MALDI TOF mass spectrum analysis

Molecular weight distribution of the hydrolysate was obtained from MALDI TOF mass spectrometer with resolving capabilities in the order of 400-1000 and accuracy ranging from ± 0.2% to 0.005%. The mass spectra (shown in fig.4.8) indicated the molecular weight of the most active peptides in the range below 2000 kDa. No signals were obtained above 2000m/z. Standard Calmix peptide mixture was used for calibrating the instrument (spectra shown in fig.4.7).

Figure 4.7 Mass spectra obtained for standard calmix peptide mixture (Bradykinin, Angiotensin II, P14R, ACTH 18-39 clip. This mixture was used for calibration of instrument).

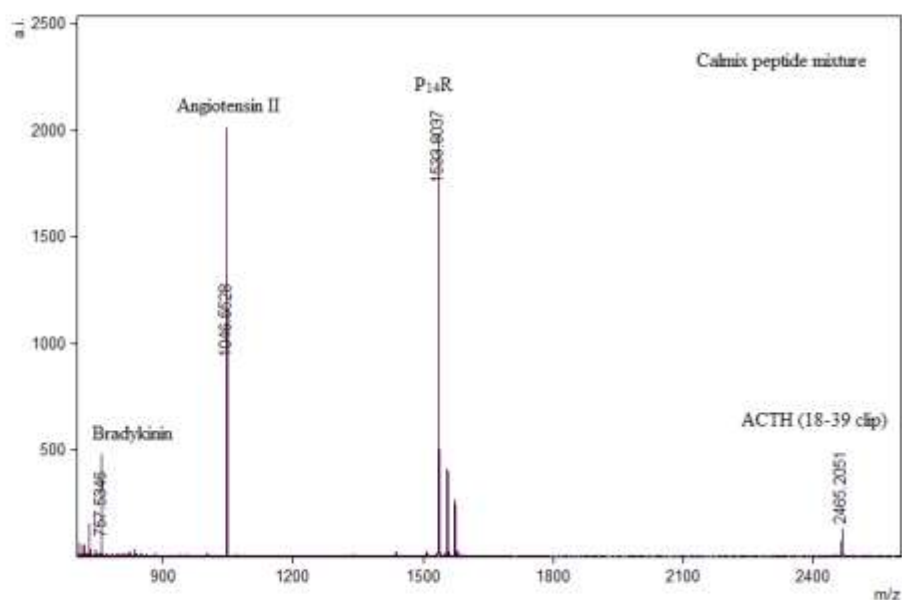
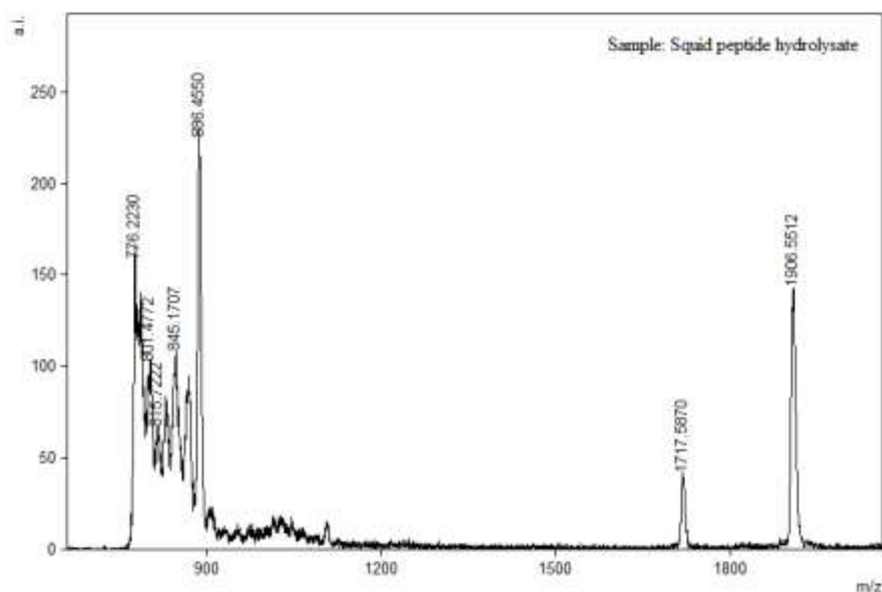


Figure 4.8 Mass spectra obtained for Collagen hydrolysate. Spectra acquired till 20000 m/z. However, no signals were obtained above 2000 m/z



4.4 Conclusion

In order to develop bioactive peptides, the grouper fish skin collagen was hydrolysed enzymatically using three different enzymes; pepsin, papain and protease consecutively. Since degree of hydrolysis depends on the bioactivity of peptides, we optimised the hydrolysis parameters of each enzyme using RSM with Box Behkenn model in order to get maximum degree of hydrolysis. The optimum hydrolysis conditions are: 2.1 of pH; 36.62°C of temperature; 3.6% of E/S ratio; and 5.47 h of time for pepsin. The optimum hydrolysis conditions for papain are 6.38 of pH; 26.22°C of temperature; 4.5% of E/S ratio; and 4.25 h of time. And the optimum hydrolysis conditions for protease are 6.3 of pH; 39.86°C of temperature; 1.8% of E/S ratio; and 4.25 h of time. Under these conditions the selected enzymes give maximum activity.

The three step hydrolysis process using the enzymes pepsin, papain and protease resulted in degree of hydrolysis and nitrogen recovery respectively of 56 ±0.73% and 76.11±1.03% under the optimised conditions. Lyophilized hydrolysate was almost 100% soluble over a wide range of pH values.

Several analyses were done for checking the quality control of the product; Filtration, analysis of hydrolysis degree, the molecular weight distribution, the total nitrogen, amino acid composition and the presence of toxic compounds (heavy metal contamination or pathogens).

SDS-PAGE combined with MALDI TOF method was successfully applied to determine the molecular weight distribution of the hydrolysate. The electrophoretic pattern indicated that the peptides formed due to hydrolysis were less than 16kDa in molecular weights. MALDI TOF spectral analysis showed that the molecular weight of most of the active peptides is in the range below 2000 kDa. The spray dried product is a pure mixture of peptides and can be recommended for further bioactivity studies.

Interest in nutraceuticals is growing rapidly worldwide, as they are a safe alternative to pharmaceutical drugs, which use is sometimes limited by toxicity or intolerance reactions. Collagen and collagen hydrolysates could be attractive nutraceuticals for their interesting bioactive properties. The beneficial effect of collagen or gelatine hydrolysates on different diseases has been reported in animal or clinical studies, and actually several supplements including collagen-derived peptides have been patented and are currently commercialised in USA, Japan and Europe. Moreover, hydrolysed collagen products have received GRAS status (Generally Recognized as Safe) from the US Food and Drug Administration (FDA).

Although mammalian gelatines are widely used in the field of nutraceuticals, the use of gelatines from marine-discarded sources for preparing protein hydrolysates is nowadays increasing, as they are not associated with the risk of outbreaks of bovine spongiform encephalopathy and also meet certain religious requirements of Jewish and Muslim markets. The resistance of some collagen-derived peptides to protein digestion is one of the most interesting properties of collagen hydrolysates. Several studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of Hyp-containing peptides. Some of these collagen-derived peptides have revealed biological activity in vivo after absorption from the digestive tract (Moskowitz, 2000).

4.5 Appendix

Table 4.6 ANOVA table for pepsin

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	63.865817	0.2947	6.62	0.0047
Quadratic	4	103.904346	0.4794	10.78	0.0006
Cross product	6	20.034625	0.0924	1.39	0.2965
Total Model	14	187.804788	0.8665	5.57	0.0025

Table 4.7 ANOVA table for papain

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	763.898117	0.5452	33.24	<.0001
Quadratic	4	402.105207	0.2870	17.50	<.0001
Cross product	6	166.147575	0.1186	4.82	0.0100
Total Model	14	1332.150899	0.9508	16.56	<.0001

Table 4.8 ANOVA table for PP

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	4	230.233333	0.0680	4.35	0.0210
Quadratic	4	2959.308241	0.8745	55.93	<.0001
Cross product	6	35.807500	0.0106	0.45	0.8309
Total Model	14	3225.349074	0.9531	17.42	<.0001

Table 4.9 ANOVA for the second order polynomial model in case of pepsin

Pepsin Parameter	DF	Standard Error	t Value	Pr > |t|
Intercept	1	30.677907	-3.18	0.0079
pH	1	19.076136	2.06	0.0620
Temperature	1	0.688307	4.84	0.0004

Time	1	2.868527	-0.42	0.6829
ES Ratio	1	1.451825	0.70	0.4977
pH*pH	1	4.201772	-3.17	0.0080
Temperature*pH	1	0.194072	0.53	0.6027
Temperature*Temperature	1	0.006723	-6.34	<.0001
Time*pH	1	0.970358	2.29	0.0410
Time*Temperature	1	0.038814	-0.94	0.3672
Time*Time	1	0.168071	-1.24	0.2402
ESRatio*pH	1	0.517524	0.87	0.3999
ESRatio*Temperature	1	0.020701	-0.98	0.3454
ESRatio*Time	1	0.103505	0.42	0.6806
ESRatio*ESRatio	1	0.047807	-1.88	0.0842

Table 4.10 ANOVA for the second order polynomial model in case of papain

Papain Parameter	DF	Standard Error	t Value	Pr > |t|
Intercept	1	51.511430	-8.22	<.0001
pH	1	13.270730	8.10	<.0001
Temperature	1	0.992742	5.99	<.0001
Time	1	4.587782	0.94	0.3647
ES Ratio	1	2.330710	0.79	0.4464
pH*pH	1	1.037884	-7.70	<.0001
Temperature*pH	1	0.119844	-2.92	0.0129
Temperature*Temperature	1	0.010379	-5.58	0.0001
Time*pH	1	0.599222	1.04	0.3175
Time*Temperature	1	0.059922	-0.86	0.4069
Time*Time	1	0.259471	-2.66	0.0209
ES Ratio*pH	1	0.319585	2.12	0.0552
ES Ratio*Temperature	1	0.031959	-3.74	0.0028
ES Ratio*Time	1	0.159793	0.21	0.8335
ES Ratio*ES Ratio	1	0.073805	-3.07	0.0096

Table 4.11 ANOVA for the second order polynomial model in case of PP

Protease Parameter	DF	Standard Error	t Valu e	Pr > |t|
Intercept	1	109.655598	-5.55	0.0001
pH	1	23.614131	2.93	0.0127
Temperature	1	1.842178	9.91	<.0001
Time	1	8.062146	1.30	0.2170
ES Ratio	1	41.486329	1.88	0.0839
pH*pH	1	1.574893	-3.05	0.0101
Temperature*pH	1	0.181853	0.00	1.0000
Temperature*Temperature	1	0.015749	-14.53	<.0001
Time*pH	1	0.909265	-0.54	0.6017
Time*Temperature	1	0.090926	0.00	1.0000
Time*Time	1	0.393723	-2.15	0.0531
ES Ratio*pH	1	4.849411	-1.53	0.1529
ES Ratio*Temperature	1	0.484941	0.27	0.7880
ES Ratio*Time	1	2.424706	-0.12	0.9036
ES Ratio*ES Ratio	1	11.199236	-2.40	0.0335

……………………………….