Enzymatic Hydrolysis of Grass Carp Myofibrillar Protein and Antioxidant Properties of Hydrolysates

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Abstract

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Myofibrillar protein was extracted from grass carp, a freshwater fish, and hydrolysed using five commercial proteases (papain, pancreatin 6.0, bromelain, Neutrase 1.5MG, and Alcalase 2.4 L). The antioxidant activities of the hydrolysates were determined. Pancreatin 6.0 proved to be the most efficient protease for hydrolysing myofibrillar protein with a very high protein recovery (90.20%), its hydrolysates exhibiting the highest hydroxyl radical (•OH) scavenging activity (IC_{50} = 349.89 ± 11.50 μg/ml) out of all five hydrolysates. Molecular weight distribution analysis revealed that pancreatin 6.0 hydrolysate rendered a higher proportion of the 6–10 kDa fraction and a lower proportion of the 3–6 kDa fraction as compared with other hydrolysates. The maximum •OH scavenging activity for pancreatin 6.0 hydrolysate (IC_{50} = 229.90 μg/ml) was obtained at the enzyme to substrate ratio of 0.52%, the incubation time of 7.03 h, and the incubation temperature of 50.56°C, as optimised by response surface methodology. *In vitro* antioxidant experiments proved that pancreatin 6.0 hydrolysates had obvious inhibitory effects on lipid peroxidation and low-density lipoproteins oxidation under optimised conditions.

Keywords: grass carp; myofibrillar protein; proteolysis; oxidation; inhibition; response surface methodology

Proteins can be divided into three groups: myofibrillar, sarcoplasmic, and connective tissue proteins (Tornberg 2005). Myofibrillar proteins are considered a major part, which constitutes around 55% to 60% of total protein content (Tornberg 2005). Myofibrillar proteins play an important role in the meat processing since it influences the quality of the meat products, such as tenderness, juiciness, and flavour (Xiong 1997).

There exist a lot of studies dealing with the functional properties of myofibrillar proteins such as viscosity, water- and oil- holding capacity, gelation properties, emulsion capability, and rheological properties (Xiong *et al.* 2008; Joo *et al*. 1999). Some studies have also indicated that myofibrillar proteins can be degraded by endogenous proteases such as cathepsins (An *et al.* 1994) or serine proteinases (Cao *et al.* 2000). The breakdown of myofibrillar proteins has drawn much attention because it is considered to be the main cause of thermal gel degradation of fish/meat jelly foods and leads to the deterioration of the food quality (Cao *et al.* 1999). However, the degradation of myofibrillar proteins by proteases could also create bioactive products. For example, antioxidant peptides have been formed from porcine myofibrillar proteins by protease treatment, as reported by Saiga (2003). This reinforces the trend to search for natural

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antioxidants, since people now start to realise that synthetic chemicals have potential health risks (Becker 1993). Myofibrillar protein present in fish species may have unique characteristics due to the special selective pressures exerted by the environment they live in. Hence, proteolysis of fish myofibrillar protein by exogenous proteases might create good protein-based antioxidants. There is to date, however, scanty work in this field.

Grass carp (*Ctenopharyngodon idella*) is a popular cultured freshwater fish species in Southeast Asian countries. It currently comprises approximately 40~50% of the total fresh water fish caught in China and its culture industry has contributed significantly to the national economic development (Ren *et al.* 2008). This has highlighted the need to maximise the use of this abundant and low price fish. So far, little information is available regarding the protein characteristics of grass carp.

In the present study, we investigated the proteolysis of grass carp myofibrillar protein by commercial proteases. The antioxidant properties of the hydrolysates obtained were evaluated by hydroxyl radical scavenging activity, *in vitro* rat liver lipid peroxidation, and *in vitro* rat serum low-density lipoproteins oxidation. Response surface methodology (RSM) was applied as well to optimise the hydrolysis conditions including enzyme to substrate (E/S) ratio, as well as the incubation time and temperature.

MATERIALS AND METHODS

Materials. The healthy grass carps (*Ctenopharyngodon idellus*, 822 ± 147 g in weight and 40.8 ± 2.8 cm in length) were obtained from a local market in Guangzhou, China. The fresh fish were instantly sacrificed, eviscerated, filleted, and minced twice in a Hobart mincer (model AE 200, Beijing, China) under cold conditions (4°C). The minced material was immediately used for the myofibrillar protein preparation. Five food-grade proteases (papain, pancreatin 6.0, bromelain, Neutrase 1.5MG, and Alcalase 2.4L) were provided by Novo Nordisk Co. (Beijing, China) and Mingyuan Co. (Guangzhou, China). Glutathione (GSH) was of chromatographic purity and was purchased from Sigma Chemical Co. (Beijing, China).

Preparation of myofibrillar protein. Myofibrillar protein was extracted according to the method of Hashimoto *et al.* (1979) with some modifications. The flow chart is shown in Figure 1. Grass

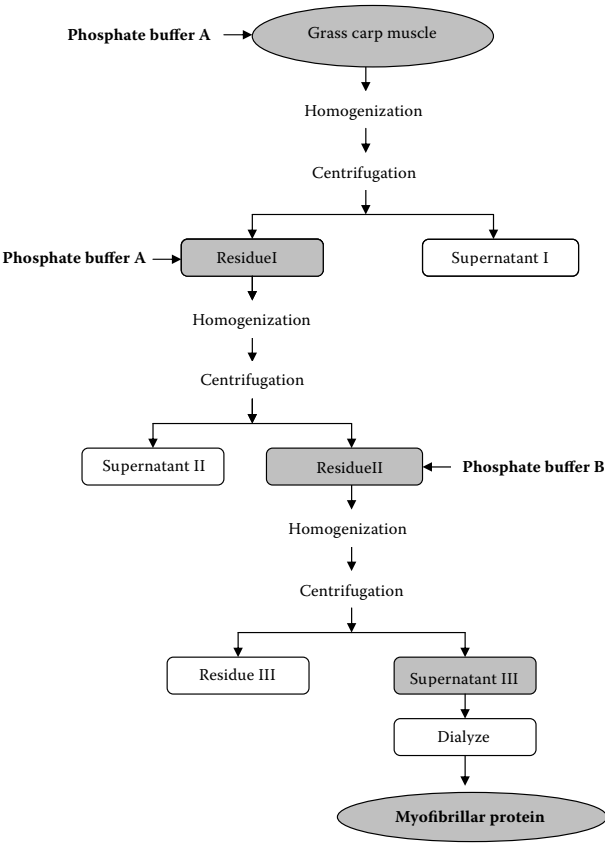

Figure 1. The process flow for the extraction of myofibrillar protein from grass carp muscle

carp muscle (20 g wet weight) was homogenised in 200 ml of ice-cold phosphate buffer, pH 7.5 (15.6 mmol/l Na_2HPO_4, 3.5 mmol/l KH_2PO_4) using a homogeniser (IKA T25 Basic, Staufen, Germany). The homogenisation process was carried out at 10 000 rpm for an interval of 1 min with 6 intermissions (10 s each time). The resulting homogenate was centrifuged at 5000 g at 4°C for 15 min using a CR22G refrigerated centrifuge (Hitachi, Japan). The supernatant was discarded, while the pellet was collected and resuspended in 200 ml of ice-cold phosphate buffer, homogenised, and centrifuged at 4°C again. After two repeated cycles of homogenisation and centrifugation, the resulting pellet was suspended in 10-fold volume of ice-cold phosphate buffer, pH 7.5 (0.05 mol/l NaH_2PO_4, 0.05 mol/l Na_2HPO_4) containing 1.1 mol/l KCl (pH value was adjusted with stock acid or base when necessary). The mixture was homogenised and centrifuged at 5000 g for 15 min at 4°C. The supernatant was collected and dialysed against deionised water at 4°C in the dark for 48 h using cellulose tubular membrane (molecule cut-off =

3.500 Da, Fisher Scientific, Shanghai, China). The dialysis water was changed at first every 2 h and after 12 h, it was changed every 8 h. The resulting pellet was regarded as myofibrillar protein.

Proteolysis of myofibrillar protein. The extracted myofibrillar protein was mixed with 3-fold volume of deionised water and homogenised for 1 min at 10,000 rpm with a homogeniser (IKA T25 Basic, Staufen, Germany) at 4°C. The mixture obtained was divided into five fractions. Each of the fractions was adjusted to the required pH with 0.01 mol/l NaOH and heated to the required temperature in a shaking incubator (New Brunswick Scientifics C24, Beijing, China) before protease was added in the proper proportion based on its activity (Table 1). The hydrolysis reaction was performed for 4 h and stopped by inactivating the respective protease at 100°C for 10 min. The mixtures were cooled down to room temperature and centrifuged in a CR22G refrigerated centrifuge (Hitachi, Tokyo, Japan) at 3000 g for 20 minutes. The supernatants were collected and lyophilised. Kjeldahl method (AOAC 1999) was applied to analyse the nitrogen contents in the supernatants ($N_{supernatant}$), and the nitrogen content in the original myofibrillar protein ($N_{myofibrillar}$) as well. Protein recovery (PR) was defined as the ratio of the hydrolysed protein to total protein, which could be calculated as:

$$PR\ (\%) = (N_{supernatant}/N_{myofibrillar}) \times 100$$

The degree of hydrolysis (DH), defined as the percentage of the peptide bonds cleaved, was based on the number of free amino groups determined by the TNBS method described by Alder-Nissen (1979).

Gel permeation chromatography (GPC). The molecular weight distribution profile of each hydrolysate was determined using high-performance liquid chromatography (Waters 1525, Mildford, USA) on a GPC column (7.8 × 300 mm, Protein-Pak 60). The column was equilibrated and eluted with 0.05 mol/l Tris-HCI buffer (pH 7.2) at a flow rate of 0.6 ml/min in isocratic mode. Peptides were detected at 214 nm. Protein standard mixture including Globin III (2512 Da), Globin II (6214 Da), Globin I (8159 Da), Globin I+III (10 700 Da), Globin I + II (14 404 Da), and Globin (16 949 Da) supplemented with glutathione (307 Da) (Amersham Biotech, GE, CA, USA) was used to calibrate the column. The plot of log *MW* against the elution time was constructed and the molecular weight distribution for each hydrolysate was then calculated according to the plot.

Hydroxyl radical scavenging activity assay. Hydroxyl radical (•OH) scavenging activity was determined according to the method described by Chung *et al.* (1997) with some modifications. The reaction mixture including 0.9 ml of sodium phosphate buffer (pH 7.4), 0.1 ml of 10 mmol/l $FeSO_4$, 0.5 ml of 10 mmol/l α-deoxyribose, 0.1 ml of 10 mmol/l EDTA, and 0.2 ml of the sample, was thoroughly mixed. An aliquot of 0.2 ml H_2O_2 (10 mmol/l) was then added to initiate the reaction. The reaction mixture was incubated at 37°C for 1 hour. One milliliter of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 1.0% thiobarbituric acid (TBA) were added to the mixture which was then boiled for 15 minutes. After cooling, the absorbance of the mixture was measured at 532 nm. Sodium phosphate buffer (pH 7.4) instead of the sample was used as blank. The •OH scavenging activity was evaluated as follows:

$$\text{•OH scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where:

A_0 – absorbance of the blank

A_1 – absorbance in the presence of the test compound

Table 1. Parameters for enzymatic hydrolysis of grass carp myofibrillar muscle

Proteases	Activity (U/g)	Incubation		pH	E/S[a] (%, w/w)
		temperature (°C)	time (h)		
Papain	2.4×10^4	50.0	4.0	7.0	0.15
Pancreatin 6.0	6.0×10^4	50.0	4.0	8.0	0.10
Bromelain	3.0×10^4	50.0	4.0	7.0	0.20
Neutrase1.5MG	3.0×10^4	50.0	4.0	7.0	0.20
Alcalase 2.4L	6.9×10^4	55.0	4.0	8.0	0.15

[a]enzyme to substrate ratio

These values of scavenging activity were plotted against the concentrations of the individual samples, and the concentration needed to scavenge 50% of radical activity was defined as the IC_{50} value.

Differential scanning calorimetry (DSC) analysis. DSC analysis was performed on a DSC Q100 V9.0 Build 275 device (Universal V4.1D TA Instruments, Inc., New Castle, Germany). The DSC instrument was calibrated for the temperature and baseline using indium as standard. Ten milligrams of the sample was thoroughly mixed with 200 µl of distilled water. The paste obtained was weighed into a hermetically sealed aluminium pan and allowed to equilibrate at the initial scanning temperature (20°C) for 2 minutes. A sealed, empty pan was used as reference. A heating rate of 10°C/min was used to scan the samples over 20–120°C. The total enthalpy change (ΔH) associated with protein denaturation was estimated by measuring the area above the DSC transition curve with a straight baseline constructed from the start to the end of the endotherms. The temperature of the peak maximum was taken as the transition temperature.

Response surface methodology (RSM) analysis. Hydrolysis conditions including E/S ratio ($X1$), time ($X2$), and temperature ($X3$), were further optimised using a statistical model established by RSM (software Design Expert 6.0, Stat-Ease Inc., USA) to produce hydrolysates with a high hydroxyl radical scavenging activity (Y). The central design was applied based on three different E/S ratios (0.2%, 0.5%, and 0.8%, w/w), as well as three different time intervals (1.0, 6.5, and 12.0 h) and temperatures (45.0, 52.5, and 60.0°C). Totally, 20 runs containing five replicates at the center point were performed. The second order polynomial regression equation including the effects of the linear, quadratic, and interaction coefficients of the three variables ($X1$, $X2$, and $X3$) on the response value (Y) was given as:

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

where:

Y – response variable

b_0 – constant coefficient (intercept)

b_i – linear coefficient

b_{ii} – quadratic coefficient

b_{ij} – two factors interaction coefficient

e_i – random error (Cornelly *et al.* 2002)

Antioxidant activity evaluation of optimised myofibrillar protein hydrolysate (MPH). The freeze-dried MPH was dissolved in deionised water to obtain serial concentration solutions: 0.2, 0.5, 1.0, 1.5, and 2.0 mg/ml, respectively. GSH was used as a positive control. Male wistar rats weighing 200–250 g were obtained from the Department of Pharmaceutical Sciences of Southern Medical University (Guangzhou, China), who obtained the permission to perform the animal experiments. The rats were sacrificed with sodium pentobarbital (62 mg/kg). Rat liver tissues were rapidly dissected from the abdomen and then homogenised in Tris-HCl buffer (40 mmol/l, pH 7.0) to produce a 25% (w/v) homogenate, which was used in the lipid peroxidation experiment. Rat serums were also collected and used in the following low-density lipoprotein (LDL) oxidation evaluation.

(1) *In vitro* liver lipid peroxidation inhibiting activity assay. The method of Anup *et al.* (2006) was slightly modified and used for the present assay. An aliquot of 0.1 ml rat liver homogenate was incubated with 0.2 ml MPH solution, 100 ml KCl solution (30 mmol/l), 100 ml $FeSO_4$ solution (0.16 mmol/l), and 100 ml ascorbic acid solution (0.06 mmol/l) at 37°C for 1 hour. Thiobarbituric acid reagent (1 ml of 0.67% TBA and 1 ml of 15% TCA) was then added. The resulting solution was heated in a boiling water bath for 15 min, cooled on ice for 10 min, and then centrifuged at 6000 g for 10 minutes. The absorbance at 532 nm was read in a UV-754 spectrophotometer (Analytic Instrumental, Shanghai, China). The blank was performed by substituting Tris-HCl buffer (40 mmol/l, pH 7.0) for MPH solution. The inhibition activity was calculated as $100 \times (A_{blank} - A_{sample})/A_{blank}$, where A_{blank} and A_{sample} are the absorbance values of the blank and sample, respectively. The plot of the inhibiting activity against the concentration of hydrolysate was made and IC_{50} (the concentration of the sample that inhibits 50% of the peroxidation) was obtained.

(2) *In vitro* serum LDL oxidation inhibiting activity assay. LDL was prepared from the rat serum according to the method of Wilkins and Leake (1994) and dialysed against a 200-fold volume of 20 mmol/l phosphate buffered saline (pH 7.4) containing 140 mmol/l NaCl at 4°C for 24 hours. The assay of the protein content was conducted by a modified Lowry assay (Schacterle & Pollack 1973). LDL was then diluted with phosphate buffer (50 mmol/l, pH 7.4) to the concentration of 0.15 mg protein/ml. An aliquot of 25 µl of $CuSO_4$ solution (80 µmol/l) was added to LDL solution to initiate the reaction, in the presence or absence

of 25 µl MPH solution, and incubated at 37°C for 8 hours. The oxidation was terminated by the addition of 25 µl EDTA (1% w/w). LDL oxidation was determined by TBA-reactive substance assay as previously described, the absorbance being read at 532 nm. The results were expressed as percentage inhibition of LDL oxidation.

Statistical analysis. All of the PR, DH, and antioxidant activity assays described in this study were conducted with three replicates and the data analysis was performed using SPSS 13.0 statistical software (SPSS Inc, Chicago, USA). A one-way analysis of variance (ANOVA) was conducted for the analysis of the response values obtained by RSM model.

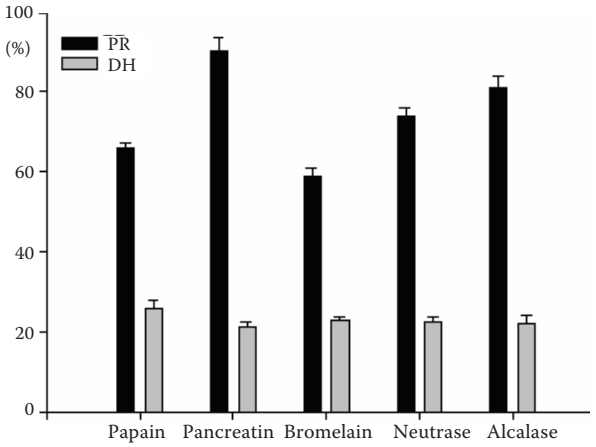

Figure 2. The protein recovery (PR) and degree of hydrolysis (DH) of grass carp myofibrillar protein hydrolysates prepared by five proteases (papain, pancreatin 6.0, bromelain, Neutrase 1.5 MG and Alcalase 2.4 L), respectively

RESULTS AND DISCUSSION

Myofibrillar protein extracted from grass carp muscle was hydrolysed using five proteases (papain, pancreatin 6.0, bromelain, Neutrase 1.5 MG, and Alcalase 2.4 L). The protein recovery (PR) and degree of hydrolysis (DH) were determined. As Figure 2 shows, PR of pancreatin 6.0 hydrolysate (90.20%) was the highest out of all five hydrolysates. Pancreatin 6.0 is a mixture containing trypsin-like and chymotrypsin-like proteinases which can split myosin, the most abundant fraction in myofibrillar protein, into two fractions including heavy meromyosin and light meromyosin (Hashimoto *et al.* 1979). Therefore, the hydrolysis of myosin by pancreatin 6.0 may be the major contributor to the high PR value observed. In terms of DH value, the differences between the individual hydrolysates were not statistically significant ($P > 0.05$). It can be seen that the hydrolysate with a higher PR value does not manifest a greater DH value. This might be due to that all the small proteins, peptides or free amino acids released by proteolysis contribute to the PR value while DH value is more related to the number of the released peptides.

Among five hydrolysates, the pancreatin 6.0 hydrolysate exhibited the highest •OH scavenging activity (IC_{50} = 349.89 µg/ml) (Table 2), which was not as potent as that of the standard antioxidant GSH (IC_{50} = 213.21 µg/ml), however, was close to it. Molecular weight distribution revealed that the pancreatin 6.0 hydrolysate had a relatively higher proportion of 6–10 kDa fraction and a lower proportion of 3–6 kDa fraction as compared with

Table 2. Molecular weight distributions and hydroxyl radical scavenging activities of grass carp myofibrillar protein hydrolysates

Hydrolysates	•OH scavenging activity (IC50)/(µg/ml)	Molecular weight distribution (%)			
		> 10 kDa	6–10 kDa	3–6 kDa	< 3 kDa
Papain	701.18 ± 17.63[a]	6.8 ± 0.6	42.9 ± 5.1	22.9 ± 2.4	27.4 ± 2.6
Pancreatin 6.0	349.89 ± 11.50[b]	7.9 ± 1.2	46.1 ± 5.3	20.6 ± 2.1	25.4 ± 3.1
Bromelain	931.88 ± 13.57[c]	11.6 ± 0.9	42.3 ± 4.2	26.1 ± 2.9	20.0 ± 1.5
Neutrase 1.5MG	710.78 ± 21.24[a]	10.1 ± 0.7	42.7 ± 4.6	25.6 ± 3.2	21.6 ± 2.2
Alcalase 2.4L	576.10 ± 14.23[d]	11.8 ± 2.1	40.8 ± 3.7	21.7 ± 2.5	25.7 ± 2.7
Glutathione (GSH)	213.21 ± 8.32[e]				

Results are presented as the means ± standard errors (n = 3); means sharing the same superscript in the columns were not significantly different at $P < 0.05$

Table 3. Code level design of the independent variables and experimental values for the optimisation of the hydrolysis conditions by RSM

Coded unit			Experimental values			
X_1: E/S ratio	X_2: time	X_3: temperature	E/S ratio (%, w/w)	time (h)	temperature (°C)	Y: IC_{50} (μg/ml)
–1	1	–1	0.2	12.0	45.0	250.32
1	–1	1	0.8	1.0	60.0	258.29
0	0	1	0.5	6.5	60.0	828.23
–1	–1	1	0.2	1.0	60.0	442.21
0	0	0	0.5	6.5	52.5	538.96
0	–1	0	0.5	1.0	52.5	266.31
–1	0	0	0.2	6.5	52.5	1002.33
0	0	0	0.5	6.5	52.5	805.28
1	–1	–1	0.8	1.0	45.0	846.24
0	0	0	0.5	6.5	52.5	396.72
1	1	1	0.8	12.0	60.0	516.33
0	0	0	0.5	6.5	52.5	460.02
0	0	0	0.5	6.5	52.5	256.32
–1	1	1	0.2	12.0	60.0	858.87
0	1	0	0.5	12.0	52.5	904.35
1	0	0	0.8	6.5	52.5	247.32
0	0	0	0.5	6.5	52.5	252.29
–1	–1	–1	0.2	1.0	45.0	398.11
0	0	–1	0.5	6.5	45.0	315.30
1	1	–1	0.8	12.0	45.0	408.29

the other hydrolysates, due to the degradation of myosin by trypsin/chymotrypsin being limited proteolysis rather than extended proteolysis (Hashimoto *et al.* 1979).

The hydrolysis results demonstrated that pancreatin 6.0 was the most effective protease for hydrolysing grass carp myofibrillar protein. The hydrolysis conditions were further optimised by RSM. The influences of the E/S ratio, time, and temperature on the •OH scavenging activity of the hydrolysates are shown in Table 3. A response surface quadratic model was drawn and the statistic analysis for the linear, the quadratic, and the interaction coefficients of the three variables (X_1, X_2 and X_3) on the response values (Y) are presented in Table 4. The p value for the model was lower than 0.0001, which indicated that the model was significant and could be used to monitor the optimisation. The following empirical regression Eq. (2) represents the IC_{50} value of the hydroxyl radical scavenging activity (Y) as a function of E/S ratio (X_1), time (X_2), and temperature (X_3). As shown in Eq. (2), the three linear coefficients for E/S ratio, time, and temperature had negative signs, therefore, a proper increase of the three variables would result in the reduction of Y value (IC_{50} value) and thus favour the increase of the •OH scavenging activity.

$$Y = 13159.90 - 2990.80X_1 - 72.97X_2 - 464.40X_3 + 1282.80X_1^2 + 5.33X_2^2 + 4.43X_3^2 + 5.85X_1X_2 + 25.31X_1X_3 - 0.02X_2X_3 \quad (2)$$

The statistical analysis for the model showed R^2 (0.9817) was close to 1 and "Adj R^2" was 0.9652 (Table 4), indicating that the model explained 96.52% of the variation in the data and the experimental error was very small. "*Adeq* precision" measures the signal-to-noise ratio and, usually, a ratio greater than 4 is desirable (Canettieri *et al.* 2007). In the present study, the "*Adeq* precision" ratio was 20.146, which was an adequate signal-

Table 4. Statistic analysis for the response surface quadratic model obtained from RSM design

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	1.269×10^6	9	1.410×10^5	59.58	< 0.0001**
Linear					
X_1	1.047×10^5	1	1.047×10^5	44.27	< 0.0001**
X_2	8.885×10^2	1	8.885×10^2	0.38	0.5537
X_3	1.034×10^5	1	1.034×10^5	43.69	< 0.0001**
Quadratic					
$X_1 \times X_1$	3.666×10^5	1	3.666×10^5	15.49	0.0028**
$X_2 \times X_2$	7.151×10^5	1	7.151×10^5	30.22	0.0003**
$X_3 \times X_3$	1.710×10^5	1	1.710×10^5	72.25	< 0.0001**
Interaction					
$X_1 \times X_2$	7.460×10^2	1	7.460×10^2	0.32	0.5869
$X_1 \times X_3$	2.595×10^5	1	2.595×10^5	10.96	0.0079**
$X_2 \times X_3$	4.64	1	4.64	1.959×10^{-3}	0.9656
Statistic analysis for the model					
R^2		0.9817	Pred *R*-squared		0.8837
Adj R^2		0.9652	*Adeq* precision		20.146

**significant within a 99% confidence interval; *Significant whthin a 95% confidence interval

to-noise ratio. Therefore, this model proved to be powerful for navigating the design space.

The effect of E/S ratio on •OH scavenging activity is illustrated in Figure 3a. It was found that the increase of E/S ratio from 0.20% to 0.65% (w/w) caused a decrease in IC_{50} value, which corresponded to an increase in the antioxidant activity. This trend was in a reasonable agreement with the forecast of Eq. (2). When E/S ratio was further increased to 0.8% (w/w), the plateau was observed and the activity was not further changed.

The effect of temperature on •OH scavenging activity is displayed in Figure 3b. The IC_{50} value decreased first and then increased a little with the temperature increasing from 45°C to 60°C. The minimum IC_{50} value was observed at around 50°C. To explain this phenomenon, the thermal properties of raw grass carp myofibrillar protein were explored by DSC. As shown in Figure 4, two major endothermic transitions with the temperature maximums (*T*m) of 50.92°C (ΔH_I= 1.589 J/g) and 74.09°C (ΔH_{II} = 2.019 J/g) were observed. These were considered to correspond to the two main constituents of myofibrillar protein, myosin and actin, respectively, based on the information

Table 5. Test for validity of the RSM model

Items	Values
Independent variables:	
X_1: E/S ratio (%, w/w)	0.52
X_2: Time (h)	7.03
X_3:Temperature (°C)	50.56
Response values: IC_{50} value (μg/ml)	
Y: Estimated values based on RSM model	233.07 ± 9.97
Lower limit*	243.04
Higher limit*	223.10
Y': Experimental values	
Y_1	242.15
Y_2	213.57
Y_3	233.99
Average ± S.D.	229.90 ± 14.72
Lower limit*	244.62
Higher limit*	215.58

*within 95% confidence interval

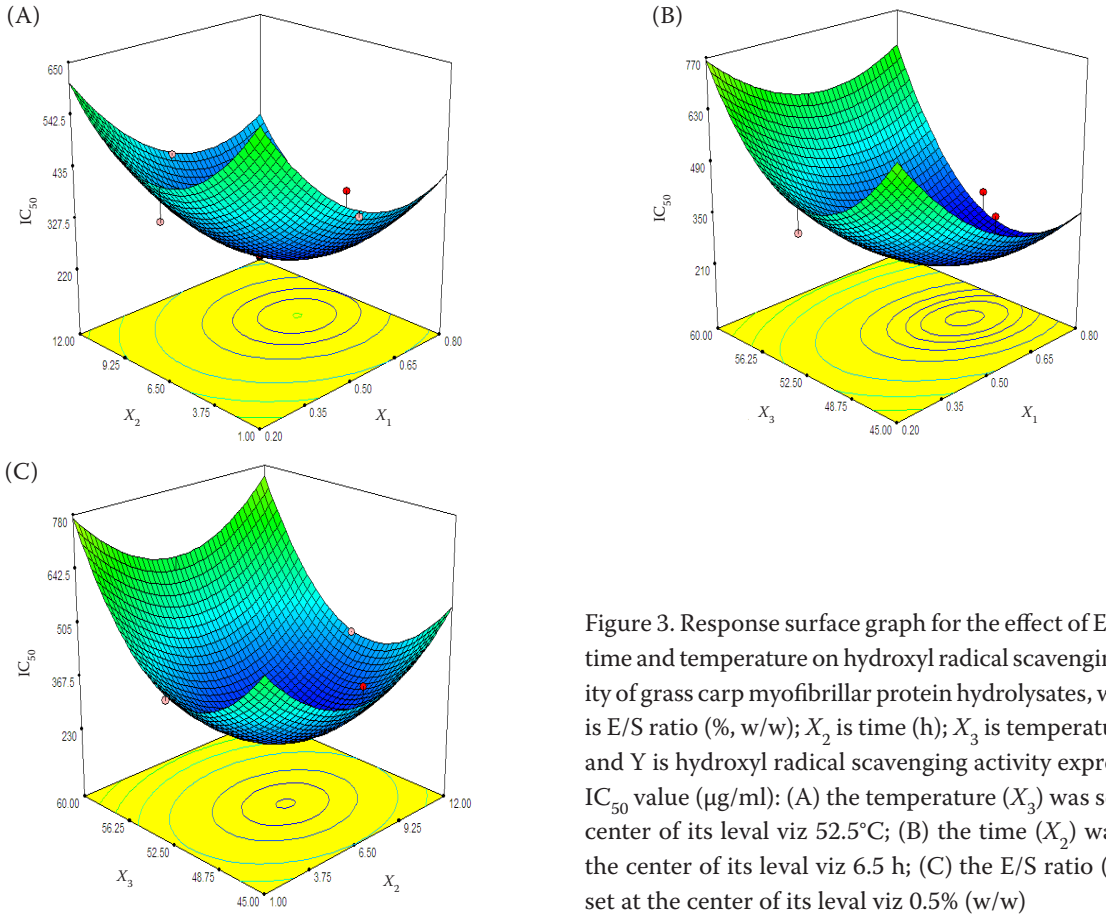

Figure 3. Response surface graph for the effect of E/S ratio, time and temperature on hydroxyl radical scavenging activity of grass carp myofibrillar protein hydrolysates, where X_1 is E/S ratio (%, w/w); X_2 is time (h); X_3 is temperature (°C); and Y is hydroxyl radical scavenging activity expressed as IC_{50} value (μg/ml): (A) the temperature (X_3) was set at the center of its leval viz 52.5°C; (B) the time (X_2) was set at the center of its leval viz 6.5 h; (C) the E/S ratio (X_1) was set at the center of its leval viz 0.5% (w/w)

obtained from the reports by Damodaran (1997) and Mochizuki *et al.* (1995). The transition at 111.85°C was not well resolved and, consequently, was not further analysed. The initiation of denaturation was automatically marked at 46.62°C by DSC instrument, therefore, myofibrillar protein was native at 45°C. As the temperature increased to around 50°C, myosin was gradually denatured and the IC_{50} value reached its minimum value, reflecting the maximum activity. Further increase to 60°C caused the decrease of •OH scavenging activity due to the overheating of myosin. Since the denaturation of actin was initiated at 61.72°C as shown in DSC thermogram, it was not much affected in the range of 45–60°C as designed in the RSM model. Therefore, it was reasonable to infer that proper denaturation of myosin favoured the increase of •OH scavenging activity, which might be induced by the exposing of hydrophobic or proton-donating residues in myosin.

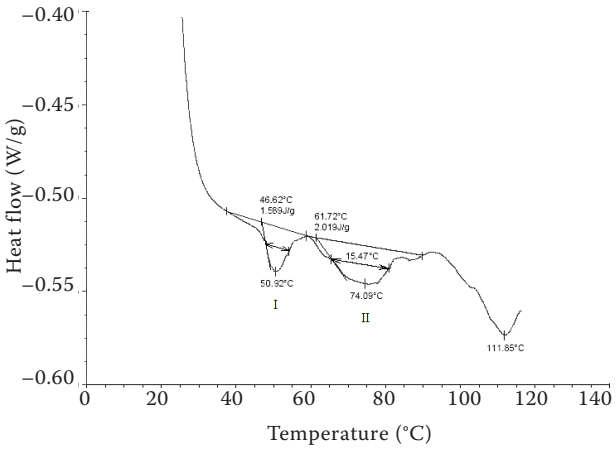

Figure 4. DSC thermogram of the grass carp myofibrillar protein heated from 20°C to 120°C

The effect of time on the antioxidant activity is demonstrated in Figure 3c. The IC_{50} value decreased steadily during the first 7 h of hydrolysis, indicating an increase of •OH scavenging activity, while further treatment up to 12 h caused a decrease of activity. This might be due to the initial hydrolysis resulting in the release of antioxidant oligo- or poly- peptides, while further treatment caused either proteolysis of the antioxidant peptides or physical aggregation of the peptides, thus weakening the antioxidant activity.

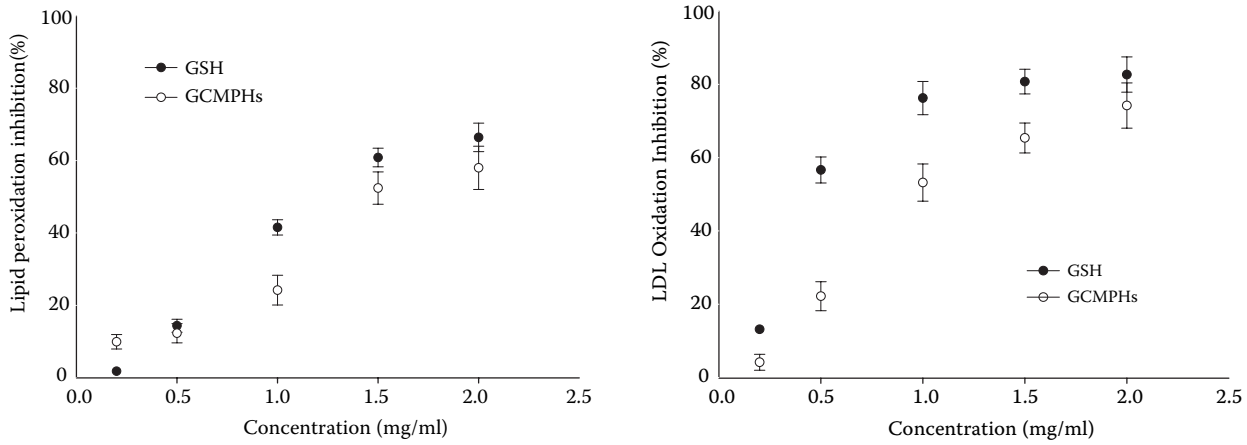

Figure 5. The inhibitory effect of optimised grass carp myofibrillar protein hydrolysate on *in vitro* rat liver lipid peroxidation and *in vitro* rat serum LDL oxidation . The data were displayed with mean ± SD (n = 3). Results were analysed by ANOVA ($P < 0.05$).

Finally, the optimisation of the model was performed using RSM auto-analysis software. The resulting minimum IC_{50} value was obtained with the following conditions: E/S ratio was 0.52% (w/w); time was 7.03 h and temperature was 50.56°C. To confirm the validity of the model, three assays were performed under the optimal conditions given above. As shown in Table 5, the experimental IC_{50} value was 229.90 ± 14.72 μg/ml, which was in a reasonable accordance with the value predicted by the model (233.07 ± 9.97 μg/ml).

The antioxiant activities of the myofibrillar protein hydrolysate (MPH) optimised by RSM were further evaluated by an assay with rat liver oxidation system or with rat serum LDL oxidation system, respectively. Figure 5a displays the inhibitory activity of MPH on *in vitro* rat liver lipid peroxidation while GSH was used as positive control. The results showed that the inhibitory effect of MPHs was comparable to the activity found with the same concentration of GSH. At a lower concentration (0.2 mg/ml), MPH (9.68 ± 1.99%) even demonstrated a higher antioxidant acitvity than GSH (1.61 ± 0.90%). The lipid oxidation analysis model applied in the present study was induced by the ferrous ion, therefore, the capability of MPH to inhibit lipid peroxidation might stem from its ferrous ion chelating ability. Figure 5b demonstrates the ability of MPH to inhibit LDL oxidation initiated by copper (II) ions. The protective capacity of MPH on LDL oxidation was inferior to that of GSH, but MPH at 2.0 mg/ml (74.18 ± 6.19%) showed an inhibitory activity comparable to that of GSH (82.93 ± 4.82%). This result implies that MPH has a potential capacity to prohibit LDL oxidation from occurring.

CONCLUSIONS

Grass carp myofibrillar protein can be effectively hydrolysed by pancreatin 6.0. The hydrolysate optimised by RSM had a distinct inhibitory effect on lipid peroxidation and low-density lipoproteins oxidation.

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