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ORIGINAL ARTICLE

Optimization of enzymatic hydrolysis of shrimp waste for recovery of antioxidant activity rich protein isolate

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Abstract Shrimp waste is an important source of astaxanthin, which occur as a complex with proteins, and protein isolates as well as carotenoids are known to possess antioxidant activity. Investigations were carried out to optimize hydrolysis of shrimp waste using a bacterial protease to obtain antioxidant activity rich protein isolate. The effect of three process variables namely enzyme concentration to waste, incubation temperature and time on carotenoid recovery, protein content, trichloro acetic acid (TCA) soluble peptide content and DiPhenyl Picryl Hydrazylchloride (DPPH) scavenging activity was evaluated using a fractionally factorial design. A high correlation coefficient (>0.90) between the observed and the predicted values indicated the appropriateness of the design employed. Maximum carotenoid recovery was obtained by hydrolysing the shrimp waste with 0.3 % enzyme for 4 h. DPPH radical scavenging activity of carotenoprotein isolate was markedly affected by enzyme concentration, temperature and time of hydrolysis. The study indicated that in order to obtain the carotenoprotein from shrimp waste with higher carotenoid content hydrolysing with an enzyme concentration of 0.2–0.4 %, at lower temperature of 25–30° upto 4 h is ideal. However, in order to obtain the protein isolate with increased antioxidant activity hydrolysing at higher temperature of 50 °C, with higher enzyme concentration of 0.5 % for shorter duration is more ideal.

Keywords Shrimp waste · Carotenoid · Carotenoprotein · Antioxidant · RSM · DPPH scavenging

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Introduction

Processing of crustaceans such as shrimps generates large quantities of solid wastes accounting for approximately 35–45 % of whole shrimp weight (Sachindra et al. 2005; 2006a). These waste spoils rapidly, thus causing environmental problems. Further, as shrimp waste being a rich source of protein, chitin, carotenoid and enzymes, considerable interest has been shown recently to recover these valuable components as marketable products.

Astaxanthin is the major carotenoid present in crustacean waste, and occurs as carotenoprotein complexes, where carotenoids are bound to proteins (Ghidalia 1985; Shahidi et al. 1998). Complexing of carotenoids to protein results in display of various colors in crustaceans and provides stability to carotenoids, which are otherwise very unstable (Zagalsky 1985; Zagalsky et al. 1990). Attempts have been made to recover carotenoids from shrimp waste either as carotenoids or as carotenprotein complex. Studies have been carried out on recovery of carotenoids and carotenoproteins from crustacean waste. Carotenoids from shrimp waste has been recovered using solvent extraction and oil extraction (Sachindra and Mahendrakar 2005; Sachindra et al. 2006b) and its stability under different storage conditions has been reported (Sachindra and Mahendrakar 2010). Enzymatic hydrolysis of shrimp waste was found to enhance the oil extractability of carotenoids (Sachindra and Mahendrakar 2011). Carotenoproteins from shrimp waste can be isolated by enzymatic and fermentation techniques. Chelating agents like EDTA and the proteolytic enzyme trypsin has been used to recover carotenoprotein from shrimp waste (Simpson and Haard 1985; Cano-Lopez et al. 1987). Trypsin hydrolysis of snow crab waste followed by ammonium sulphate precipitation yielded carotenoprotein with increased carotenoid content (Manu-Tawai and Haard 1987).

Fermentation ensilaging was found to be better option for stabilizing the carotenoids in shrimp waste without affecting

Table 1 Observed and predicted dependent variables at different combinations of independent variables

Run No.	X1 Enzyme concentration	X2 Temperature	X3 Incubation Time	Y1 Carotenoid recovery (%)		Y2 Protein recovered (mg/g waste)		Y3 TCA soluble peptide recovered (mg/g waste)		Y4 DPPH scavenging activity (%)	
				Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0.1	20	60	71.85	74.78	32.55	31.30	3.79	3.81	28.49	39.33
2	0.1	35	240	73.81	71.52	25.87	28.49	3.84	4.11	36.44	29.40
3	0.1	50	150	43.92	43.29	24.69	23.33	5.11	4.82	65.14	61.34
4	0.3	20	240	82.51	81.88	28.84	27.47	3.96	3.68	42.81	39.00
5	0.3	35	150	71.20	74.12	28.94	27.68	3.87	3.89	35.51	46.36
6	0.3	50	60	47.81	45.51	19.17	21.79	4.56	4.83	79.52	72.48
7	0.5	20	150	78.47	76.18	13.76	16.38	3.83	4.10	77.23	70.19
8	0.5	35	60	68.67	68.04	17.22	15.85	4.82	4.54	75.54	71.74
9	0.5	50	240	41.39	44.32	8.93	7.68	5.33	5.35	75.54	86.39
r				0.9890		0.9685		0.9208		0.9204	

its recovery (Sachindra et al. 2007a) and a fermentation process has been standardized for recovery of carotenoprotein rich in essential amino acids (Bhaskar et al. 2010). Lyophilised fermentation liquor from Indian shrimp waste was found to be rich in carotenoids and exhibited strong antioxidant activity (Sachindra and Bhaskar 2008).

In most of the studies on isolation of carotenoprotein from shrimp byproducts by enzymatic technique, focus was on increased yield of protein and maximizing its recovery (Klomklao et al. 2009; Holanda and Netto 2006; Armenta and Guerrero-Legarreta 2009; Cao et al. 2008; 2009). Protein isolates as well as carotenoids are known to possess strong antioxidant activity. Hydrolyzed proteins from many animal and plant sources, individual peptides and amino acids have been found to possess antioxidant activity. Some amino acids were reported as having strong antioxidant activity in linoleic acid and methyl linoleate model systems (Marcuse 1962). Protein hydrolysate obtained during preparation of chitin from shrimp waste by *Bacillus* protease was found to have good antioxidant activity (Manni et al. 2010). An autolytic process to prepare antioxidant activity rich carotenoprotein from shrimp waste has been reported recently (Sowmya et al. 2011). The method for isolation of shrimp waste proteins by pH shift technique and the antioxidant activity of such protein isolate has been reported (Meenata et al. 2011). Huang et al. (2011) studied the effect of enzyme type and defatting on the antioxidant activity of shrimp byproduct hydrolysate. In the present study hydrolysis conditions were optimized using response surface methodologies (RSM) to recover, with focus on antioxidant activity of the protein hydrolysate.

Material and methods

Materials

Shrimp waste from *Penaeus indicus* comprising of head and carapace was collected from a local market, and transported to the laboratory under chilled condition. The material was homogenized in a table top vertical cutter (Robo-Coupe)

Table 2 Regression coefficients for predicting different dependent variables

	Carotenoid recovery	Extractable protein	TCA soluble peptide	DPPH radical scavenging
Mean/Interaction (βo)	44.972	21.431	4.6813	76.769
X1. Enzyme (L) (βi)	61.405	41.173	−3.807	−24.873
X2. Enzyme (Q) (βii)	−103.802	−128.631	8.074	177.923
X2. Temperature (L) (βi)	2.076	0.587	−0.0404	−3.028
X2. Temperature (Q) (βii)	−0.045	−0.012	0.0011	0.055
X3. Time (L) (βi)	0.024	0.004	−0.0044	0.054
X3. Time (Q) (βii)	−0.00002	−0.00005	0.0001	−0.00036

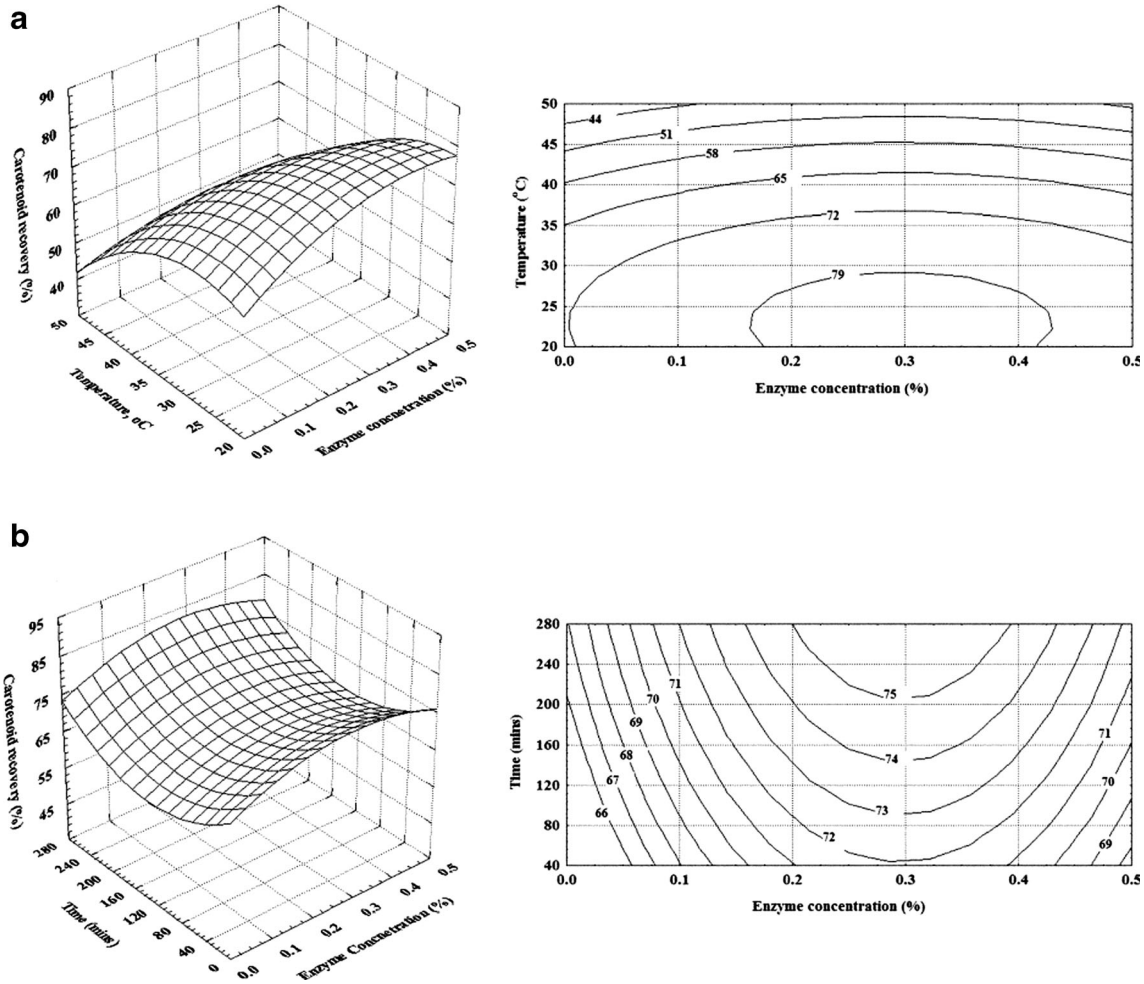

Fig. 1 Effect of enzyme concentration and temperature on carotenoid recovery (Time=150 mins) (**a**) and enzyme concentration and hydrolysis time on carotenoid recovery (Temperature=35 °C) (**b**)

before use. Alcalase, a bacterial protease, from M/s Genencor was used for hydrolysis.

Optimization of hydrolysis conditions

The effect of three process variables namely enzyme concentration to waste (X1), incubation temperature (X2) and time (X3) on recovery of carotenoid in the hydrolysate (Y1), protein content (Y2), TCA soluble peptide content (Y3) and DPPH scavenging activity (Y4) was evaluated using a fractionally factorial design . The homogenized shrimp waste was mixed with three different levels of enzyme (dissolved in buffer) and incubated at 3 different temperatures for 3 different periods. After specified period, the hydrolysate was recovered by centrifugation. The content of carotenoid, protein, TCA soluble peptide and DPPH scavenging activity was determined in the supernatant. The 9 combinations (Table 1) of the independent variables (X1, X2, X3) were determined with the aid of the software STATISTICA (Statsoft Inc 1999).

The factors, their levels and codes for the level were as follows.

Factors	Codes	Level		
		−1	0	+1
Enzyme concentration (% of wet waste)	X1	0.1	0.3	0.5
Incubation temperature (°C)	**X2**	20	35	50
Incubation time (mins)	X3	60	150	240

Statistical analysis

All the statistical analyses were carried out using the software STATISTICA (Statsoft Inc 1999). The optimization data was analyzed for determination of regression coefficients to arrive at the regression equation. Regression model containing 7 coefficients including linear and quadratic

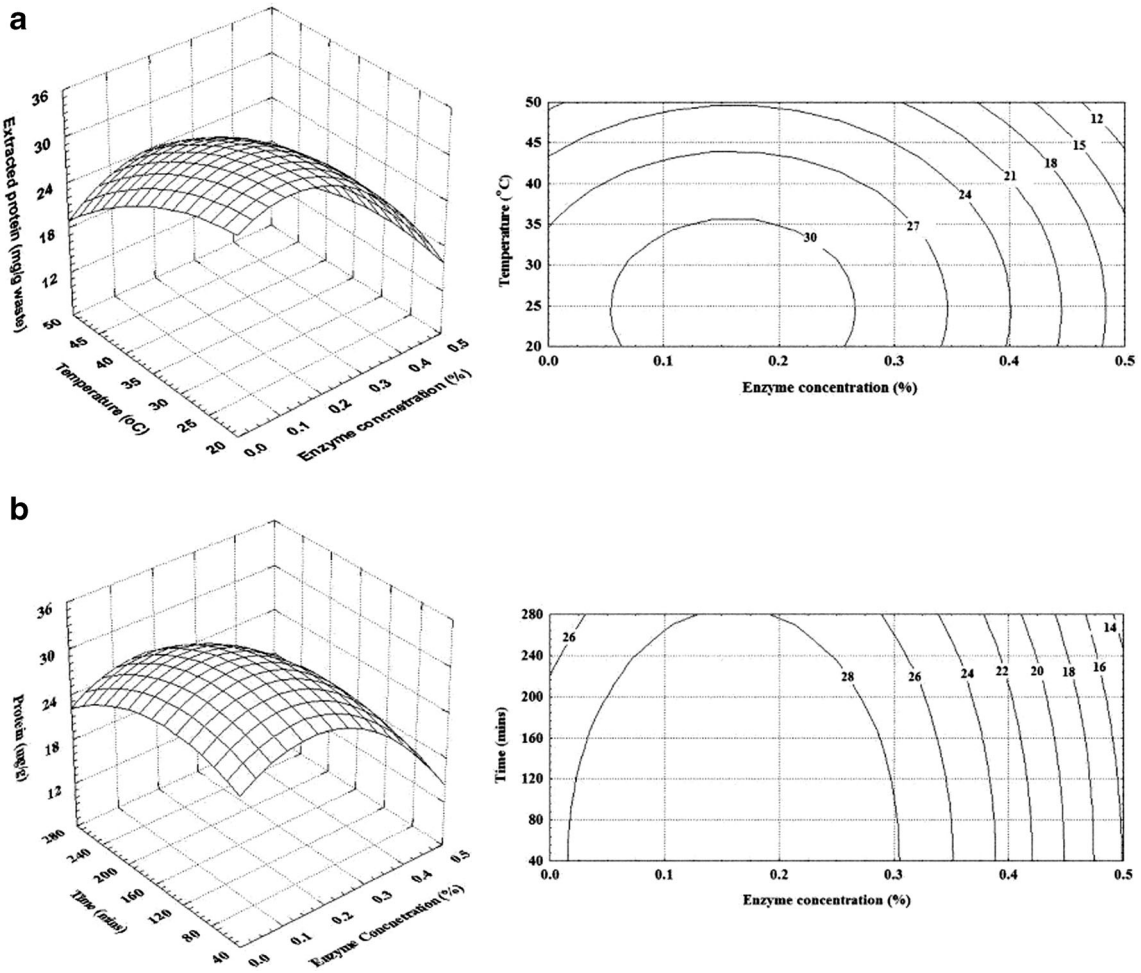

Fig. 2 Effect of enzyme concentration and temperature on extractable protein (Time=150 mins) (**a**) and enzyme concentration and hydrolysis time on extractable protein content (Temperature=35 °C) (**b**)

effect of factors was assumed to describe relationships between response (Y) and the experimental factors (X1, X2, X3) as follows,

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 \quad (1)$$

where β_0 is the constant coefficient, β_i is the linear coefficient of main factors, β_{ii} is the quadratic coefficient for main factors. The regression equation arrived was used to predict the different dependent variables in optimization and validation experiments. The 3D response graph and contour plots were plotted using the software (Statsoft Inc 1999). The regression model was further validated using different combinations of the independent variables and determining the regression coefficient between observed and predicted values of different dependent variables.

Determination of carotenoid content, protein and TCA soluble peptide content

Carotenoids in the samples (homogenized waste and the hydrolysate) were extracted by homogenizing the sample with 50 ml of acetone. The extract was filtered and the residue was repeatedly extracted with fresh solvent and filtrate collected till the filtrate is colorless. The solvent extracts were pooled together and were phase separated with equal quantity of hexane. The hexane extract was repeatedly washed with equal quantity of 0.1 % saline to remove traces of acetone if any, and dried with 25 g of sodium sulphate, filtered, flushed with nitrogen for 5 mins, and then evaporated under vacuum at 40 °C using a rotary flash evaporator. The resulting carotenoids concentrate was taken up in hexane and made up to 100 ml and the absorbance of the appropriately diluted extract was measured at 468 nm using spectrophotometer. The carotenoid content was

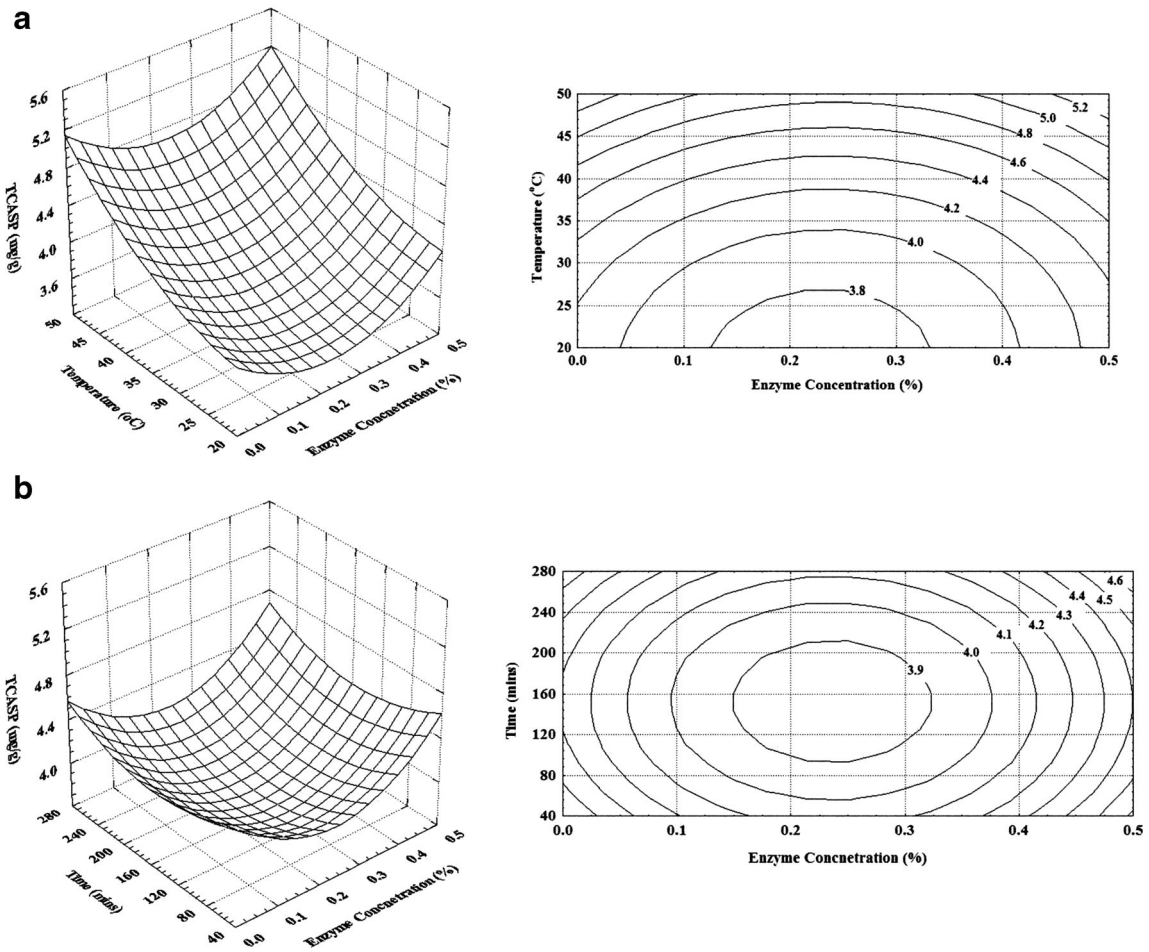

Fig. 3 Effect of enzyme concentration and temperature on TCA soluble peptide (TCASP) (Time=150 mins) (**a**) and enzyme concentration and hydrolysis time on TCA soluble peptide (Temperature=35 °C) (**b**)

calculated as astaxanthin (Simpson and Haard 1985) using the equation,

$$\text{Carotenoid content}(\mu\text{g astaxanthin/g sample}) = \frac{A_{468\text{nm}} \times V_{\text{extract}} \times \text{Dilution factor}}{0.2 \times W_{\text{sample}}}$$

Where, A is absorbance, V is volume of extract and 0.2 is the A_{468} of 1 μg/ml of standard astaxanthin and W is weight of sample in grams.

Protein content in the filtrate was determined by Lowry's method (Lowry et al. 1951). Total protein content in hydrolysate was calculated and the total protein recovered was determined per gram of wet waste. To determine TCA soluble peptide content in the filtrate, 1 ml of the sample was mixed with 1 ml of 5 % TCA, mixed well using cyclomixer and then the contents were filtered. Protein content in the TCA filtrate was determined by Lowry's method.

DPPH radical scavenging activity

DPPH radical scavenging was measured by the method of Duan et al. (2006). For determination of DPPH radical scavenging activity an aliquot of sample equivalent to 250 mg protein was made upto 2 ml and mixed with 2 ml of 0.16 mM DPPH in methanol and incubated at 37 °C for 30 min in dark. Sample blank was prepared by replacing the DPPH with methanol. The absorbance of the sample after incubation was measured at 517 nm and the scavenging activity was calculated.

$$\text{Scavenging\%} = (1 - (\text{A sample} - \text{A sample blank})/\text{A control}) \times 100$$

Results and discussion

Shrimp waste, especially the head is known to be rich in digestive enzymes such as proteases (Heu et al. 2003; Aoki

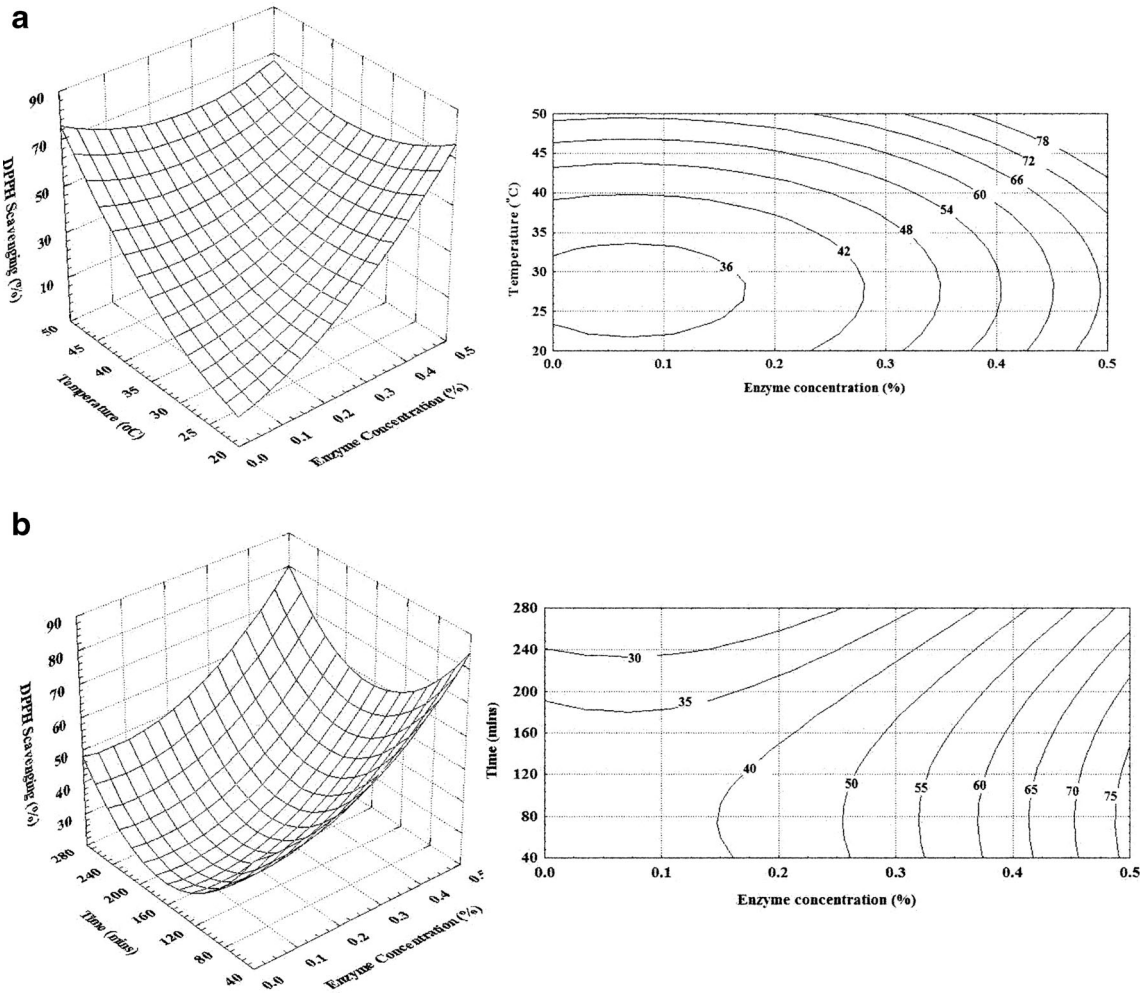

Fig. 4 Effect of enzyme concentration and temperature of hydrolysis on DPPH scavenging activity (Time=150 mins) (**a**) and enzyme concentration and hydrolysis time on DPPH scavenging activity of carotenoprotein isolate (Temperature=35 °C) (**b**)

et al. 2004) and autolysis of shrimp waste utilizing the insitu proteases for recovery of carotenoprotein has been reported (Cao et al. 2008, 2009; Sowmya et al. 2011). Addition of commercial proteases for recovery of shrimp waste carotenoprotein also has been attempted to enhance the yield of carotenoid and protein in hydrolysate (Klomklao et al. 2009). The antioxidant activity of protein hydrolysates is known to be influenced by degree of hydrolysis and enzyme type (Klompong et al. 2006). Further, the functional properties of protein hydrolysates are influenced by several factors such as type and concentration of enzyme, temperature, time and substrate concentration (Kristinsson and Rasco 2000). This study focussed on optimization of hydrolysis condition for recovery of carotenoprotein from shrimp waste with special reference to enhanced antioxidant activity.

Hydrolysis of homogenised shrimp waste was carried out at with different level of enzyme (Alcalase) at different temperatures for different period of time. The resultant hydrolysate was analysed for carotenoid content, protein, TCA soluble peptide and DPPH scavenging activity. The observed values were compared with the predicted value (Table 1) obtained by the regression coefficients derived using the software (Table 2). A high correlation coefficient (>0.90) between the observed and the predicted values indicate the appropriateness of the design employed.

With increase in enzyme concentration the recovery yield of carotenoid in carotenoprotein isolate increased at lower temperature of hydrolysis (Fig. 1a). However, hydrolysis at higher temperature resulted in decreased carotenoid yield. An enzyme concentration of 0.2 to 0.4 % and temperature around 25 °C was found to be optimum for higher carotenoid recovery in carotenoprotein isolate. The combined effect of enzyme concentration and time of hydrolysis at a temperature of 35 °C (Fig. 1b) showed that carotenoid recovery slightly increased with increase in hydrolysis time, and lower concentration of enzyme is sufficient to recover higher carotenoids by hydrolysing for longer time. Maximum carotenoid recovery was obtained by hydrolysing the shrimp waste with 0.3 % enzyme for 4 h.

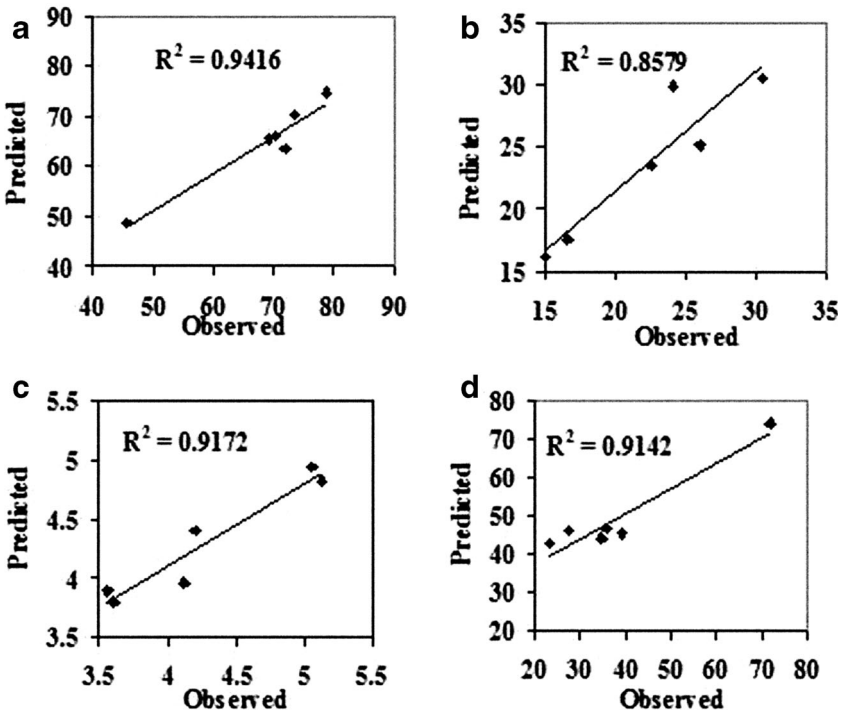

Fig. 5 Predicted V/s observed carotenoid yield (**a**), extractable protein content (**b**), TCA soluble peptide content (**c**) and DPPH scavenging activity (**d**) in validation experiment

Hydrolysis of shrimp waste with proteases was found to enhance the carotenoid recovery (Simpson and Haard 1985; Cano-Lopez et al. 1987; Sachindra and Mahendrakar 2011). Carotenoids occur as a complex with protein in crustaceans (Shahidi et al. 1998) and proteases disrupt the protein-carotenoid bond, thus increasing the carotenoid recovery. Protease treatment for long time enhanced carotenoid recovery when the target is to recover carotenoid either by oil or solvent extraction (Sachindra and Mahendrakar 2011). However, if the target is to extract carotenoprotein, extreme hydrolysis may completely disrupt this bond resulting in lower carotenoid content in the protein isolate. Thus controlled hydrolysis of shrimp waste with proteases with lower enzyme level at lower temperature will help in obtaining the protein isolate rich in carotenoid content.

The functional properties of protein isolates obtained by hydrolysis is influenced by degree of hydrolysis (Kristinsson and Rasco 2000) and hence it is important to determine the amount of protein and TCA soluble peptide content in the protein isolates. Extractable protein content decreased with increase in temperature (Fig. 2a). At lower temperature the increase in enzyme concentration did not increase the extractable protein content. Highest extractable protein content was observed at an enzyme concentration of 0.25 % and a temperature of 35 °C. With increase in hydrolysis time not much difference was observed in extractable protein content (Fig. 2b). Enzyme concentration did not show marked effect on the TCA soluble peptide content (Fig. 3a). However, with increase in temperature the TCA soluble peptide content increased considerably, indicating higher degree of protein hydrolysis. TCA soluble peptide content also increased with increase in hydrolysis time (Fig. 3b). TCA soluble peptide content indicates the extent of hydrolysis, increasing with increase in degree of hydrolysis (Sowmya et al. 2011).

Degree of hydrolysis affects the antioxidant activity of resultant protein hydrolysate (Klompong et al. 2006) with peptides of higher molecular weight possessing higher antioxidant activity (Wu et al. 2003). DPPH scavenging activity is commonly used to evaluate the antioxidant activity. In the present study also DPPH scavenging activity of hydrolysates obtained was determined to arrive at an optimized hydrolysis condition for obtaining antioxidant activity rich protein isolate from shrimp waste. The results showed that DPPH radical scavenging activity of carotenoprotein isolate is markedly affected by enzyme concentration, temperature and time of hydrolysis (Fig. 4a and b). With increase in enzyme concentration to 0.5 % and a temperature of 50 °C more than 78 % scavenging was observed. However time of hydrolysis did not had a marked effect on DPPH scavenging activity of the protein isolate. At lower enzyme concentration, increase in hydrolysis time did not result in higher scavenging activity, but with increased enzyme concentration, shorter time was sufficient to obtain higher antioxidant activity in the hydrolysate.

Carotenoprotein from shrimp comprises of carotenoids and hydrolysed protein, and both exhibit antioxidant activity (Suetsuna 2000; Sachindra et al. 2007b). Shrimp protein hydrolysates exhibit strong antioxidant activity and the peptides present in shrimp cephalothorax were found to be responsible for antioxidant activity (Binsan et al. 2008).

Shrimp waste also contains other natural antioxidants such as phenolic compounds (Seymour et al. 1996). Hence several factors influence the antioxidant activity of protein isolates from shrimp waste. In the present study the DPPH scavenging activity was determined at a protein concentration of 250 mg. When the DPPH scavenging activity was compared with the carotenoid content and TCA soluble peptide content of the sample equivalent to 250 mg protein, it was observed that the correlation coefficient was 0.65 and 0.67 respectively. This indicates that the antioxidant activity of the isolates does not depend on any one component of the hydrolysate.

By optimization experiments regression coefficients for different independent factors were obtained (Table 2). By substituting these regression coefficients in the regression equation (Eq. 1) the values for different dependent variables can be predicted at different combinations of independent variables. For validation of the regression model 6 different combinations of independent variables (enzyme concentration, temperature and time) were used and different dependent variables (carotenoid yield, extractable protein content and DPPH scavenging activity) were determined. The observed and predicted values were plotted to obtain regression coefficient (R^2) between the two (Fig. 5). It was observed that the regression coefficient was above 0.9 for all the three variables indicating the fit of the regression model for prediction.

Conclusion

The study indicated that in order to obtain the carotenoprotein from shrimp waste with higher carotenoid content hydrolysis using the bacterial enzyme Alcalase, an enzyme concentration of 0.2–0.4 % and hydrolysing at lower temperature of 25–30° upto 4 h is ideal. However, in order to obtain the protein isolate with increased antioxidant activity hydrolysing at higher temperature of 50 °C, with higher enzyme concentration of 0.5 % for shorter duration is more ideal. The resultant carotenoprotein isolate, due to its high antioxidant activity would find use in food and feed applications. The efficient use of the shrimp waste would benefit not only the industry but also results in reduction in the pollution potential of the waste.

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