

Bioactive *L acidissima* protein hydrolysates using Box–Behnken design

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Abstract This study examines the extraction and hydrolysis of proteins using single factor and Box–Behnken Design (BBD). From single factor tests, optimised extraction parameters were 1% alkali concentration, 40 °C temperature, 60 min time, and 1:20 solid to alkali ratio. Under these conditions; 924.31 mg/g of total protein was obtained from *Limonia acidissima* (*L acidissima*). The maximum degree of hydrolysis was 39.82% at pH 2, enzyme to substrate ratio 2.5% (w/w), and hydrolysis time was 42.41 min using BBD design. *L acidissima* seed protein hydrolysate showed 32.94% DPPH and 88.18% of ABTS activity at concentration of 100 µg/ml and 1 mg/ml, respectively. Reducing power of 0.16 and metal chelating activity of 87.39% was obtained from 5 mg/ml protein hydrolysates. This implied that *L acidissima* seed protein hydrolysate could be utilised in protein rich product or as protein supplements.

Keywords *L acidissima* seed · Protein hydrolysate · BBD · Antioxidant activity

Introduction

The origin for *L acidissima* is India and Ceylon. Fruit is used in the preparation of chutneys and jelly and jam (Morton 1987). It has got various medicinal properties such as anti-tumor (Saima et al. 2000), antimicrobial activity

(Rahman and Gray 2002), anti-inflammatory, antipyretic and analgesic activity (Ahamed et al. 2008), antioxidant activity (Sonawane and Arya 2013, 2015) and anti-diabetic (Patel et al. 2012). There are recent reports which indicate that defatted *L acidissima* seed is rich in protein (49.51%) and also like Na, Mn, Mg, K, Cu, Fe, and Zn (Sonawane et al. 2016).

The digestion of various proteins benefits in liberation of bio-functional peptides (including animal and plant sources). The bio-functional peptides which are present in an inactive form in their parent proteins are released during gastrointestinal digestion or food processing (Matsui et al. 2002). The bio-functional activities of peptides depend on structure, composition, and sequence. In recent years, an extensive literature has also encouraged on bioactive peptides, with a view as functional ingredients pointed at fitness (Ryan et al. 2011).

The ambitions of present study were to boost extraction and hydrolysis using response surface methodology (RSM)—BBD which examine as high degree of protein hydrolysate, as well as to evaluate its potential as an antioxidant agent.

Materials and methods

L acidissima was acquired from a local market in one lot (APMC market, Navi Mumbai, India). The outer hard shell of *L acidissima* fruit was removed followed by scooping of pulp from shell was carried out. The seeds were detached from pulp by washing under running water. Furthermore, overnight drying was carried at 45 ± 2 °C and stored at -20 °C to prevent spoilage and to maintain uniformity in the entire research. The analytical grade chemicals were used in experiment.

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Preparation of defatted *L acidissima* seed flours

The flour mill was cleaned and *L acidissima* seeds were ground to obtain a fine powder. The fat from the seed flour sample was removed by soaking flour in petroleum ether with stirring for 6 h at 37 ± 1 °C. The solvent was decanted and the process was repeated thrice with fresh solvent. After removal of solvent, flour was air-dried at room temperature and ground to pass through 40 mesh sieve. Further, these flours were packed into airtight polyethylene bags and stored at 4 °C until the extractions were done (at least 2 weeks) (Kanbargi et al. 2016).

Optimization of extraction parameter for defatted *L acidissima* seed protein

The defatted *L acidissima* seed flour was suspended in alkali extraction. Further, this was incubated at elected time, temperature and solid-to-alkali ratio to enhance protein extraction. The resulting slurry was centrifuged (12,000 rpm for 60 min at 4 °C) after incubation. The supernatant was analysed for protein content using Bradford assay (1976) and expressed as mg protein per gram of flour sample. Optimization parameters obtained by single factor experiments are reported in Table 1. The supernatant was further precipitated at pH 4 and washed thrice with distilled water, followed by drying in a vacuum oven at 40 °C for 16 h.

Enzymatic hydrolysis of *L acidissima* seed protein using one factor

Six proteases (papain, pepsin, protease, pancreatin, trypsin, and chymotrypsin) were screened (data is not shown) was selected to boost optimal condition for hydrolysis to improve degree of hydrolysis. *L acidissima* seed proteins 1% (w/v) were suspended in distilled water and subjected to enzymatic hydrolysis with pepsin were optimized. The pH for hydrolysis ranged between 2 and 3, enzyme to substrate ratio (Es) 0.5–3% (w/w) and hydrolysis time

varied from 30 to 180 min. During hydrolysis, pH was maintained and the reaction was carried out at 37 °C. The hydrolysate kept in a water bath at 100 °C for 10 min to deactivate enzymes. The supernatant centrifuged at 10,000 rpm for 20 min and subjected to degree of hydrolysis (DH) as mentioned below.

Degree of hydrolysis

The DH was determined following TCA method (Hoyle and Merritt, 1994) with slight modifications. In the first part, 500 µl of hydrolyzed protein samples were mixed with the equal amount, i.e. 500 µl of 20% TCA solution. After incubation of 30 min, supernatant were centrifuged at 10,000 rpm and protein content was determined by the method of Lowry, Rosebrough, Farr, and Randal (1951). Finally, DH was determined using following equation:

$$\% \text{Degree of hydrolysis} = 100 \times \frac{\text{Soluble protein content in } 10\% \text{ TCA (mg)}}{\text{Total protein (mg)}}$$

Experimental design

A three-level-three-factor BBD (Design-Expert 16 software, Stat-Ease Inc., Minneapolis, USA) was employed to predict the optimal hydrolysis conditions of *L acidissima* using pepsin with 17 experiments (Box and Draper 1987) (Table 2). The three levels of the variables: pH (2.0, 2.2 and 2.4), E/S (1.5, 2.0 and 2.5%) and time (30, 60 and 90 min) were selected for enzymatic hydrolysis using pepsin. The DH was used as response to measure hydrolysis (Table 2).

The experimental data obtained were fitted by the following regression equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2, \quad (1)$$

where Y is DH (predicted response), $\beta_0, \beta_1, \beta_2, \beta_3, \dots, \beta_{33}$ were the regression coefficients, X_1 -H, X_2 -E/S ratio and X_3 -time.

Table 1 Evaluation of parameters used for protein extraction optimization from defatted *L acidissima* seed flour

Variable parameter (range)	Constant parameters			
	Alkali concentration	Temperature	Time	Solid to liquid ratio
Alkali concentration (0.2–1.2%)	–	40	60	1:40
Temperature (20–50 °C)	O.C	–	60	1:40
Time (0–150 min)	O.C	O.C	–	1:40
Solid to liquid ratio	O.C	O.C	O.C	–

O.C optimized parameter

Table 2 Enzymatic hydrolysis experimental design for *L. acidissima* seed protein

Run	pH	ES	Time	DH
1	2	1.5	60	36.55 ± 1.17
2	2.4	1.5	60	28.97 ± 3.99
3	2	2.5	60	40.31 ± 6.34
4	2.4	2.5	60	32.94 ± 5.45
5	2	2	30	25.95 ± 0.84
6	2.4	2	30	10.55 ± 0.38
7	2	2	90	21.11 ± 0.46
8	2.4	2	90	17.60 ± 0.32
9	2.2	1.5	30	12.11 ± 0.98
10	2.2	2.5	30	16.94 ± 1.71
11	2.2	1.5	90	17.35 ± 1.43
12	2.2	2.5	90	19.77 ± 1.60
13	2.2	2	60	32.20 ± 2.40
14	2.2	2	60	32.16 ± 4.23
15	2.2	2	60	32.03 ± 1.66
16	2.2	2	60	33.45 ± 0.58
17	2.2	2	60	33.10 ± 0.87

Assessment of molecular weight (MW) of peptides

The MW of peptides formed during hydrolysis was determined by SDS-PAGE. For electrophoresis, 30% acrylamide-bis-acrylamide solution mixture was used for the preparation of 4% stacking gel and 15% resolving gel. 10–20 µl of sample mixed with the sample buffer was loaded into the well. Gel was run at 60 V for ~90–120 min for the visualization of bands, silver staining was done.

Antioxidant studies of *L. acidissima* seed protein hydrolysate

The optimal conditions provided by the BBD were used to produce protein hydrolysate from *L. acidissima*. The supernatant was collected by centrifuging (10,000 rpm for 20 min at 4 °C). Furthermore, supernatant (neutralized) was dried in vacuum oven at 40 °C until protein hydrolysates were in dried form.

ABTS assay

Antioxidant activity of protein hydrolysate was verified using ABTS method (Re et al. 1999). The 7 mM ABTS and 2.45 mM potassium persulfate was mixed and incubated at 37 °C for 16–17 h. The OD was set at 0.7 (±0.02) by diluting with 0.01 M phosphate buffer saline at 734 nm. The ABTS cations 260 µl (absorbance of 0.700 ± 0.02) mixed to the 40 µl of the hydrolyzed sample and absorbance was measured at 734 nm immediately after 6 min.

DPPH activity

DPPH free radicals were used to assess activity of protein hydrolysate using Sahreen et al. (2010) method with slight modification. The sample (40 µl) mixed with 120 µl of methanol which further mixed with 40 µl of DPPH (0.15 mM) prepared in methanol. After 15 min incubation in dark room OD was taken at 517 nm.

Determination of ferric reducing antioxidant power (FRAP)

FRAP assay was developed by Benzie and Strain (1996). The mixture for FRAP reagent preparation were tenfold acetate buffers: onefold TPTZ: onefold FeCl₃. The reaction mixture 200 µl of FRAP reagent was added to 20 µl of the sample and absorbance was recorded at 593 nm after incubation of 15 min at 37 °C.

Metal chelation activity

Metal chelating activity described by Decker and Welch (1990) was used to determine the activity with small modifications. In chelation test, protein hydrolysate (50 µl) sample was added into 10 µl of FeCl₂ (2 mM) and then it was mixed with 250 µl of double distilled water. Furthermore, 20 µl of ferrozine solution (5 mM) was mixed to the reaction by shaking for 2 min. The color reduction was recorded by measuring the absorbance at 562 nm after 10 min of incubation. The chelating activity was calculated as a percentage using

$$[1 - (A_{\text{sample}})/(A_{\text{control}})] \times 100.$$

Statistical analysis

All experiments were performed in triplicate and were expressed as a mean ± standard deviation. The SPSS (Statistical Package for Social Sciences) for Windows version (16.0) was used to analyze the data (SPSS Inc., Chicago, IL). Statistical significance was considered at $p < 0.05$.

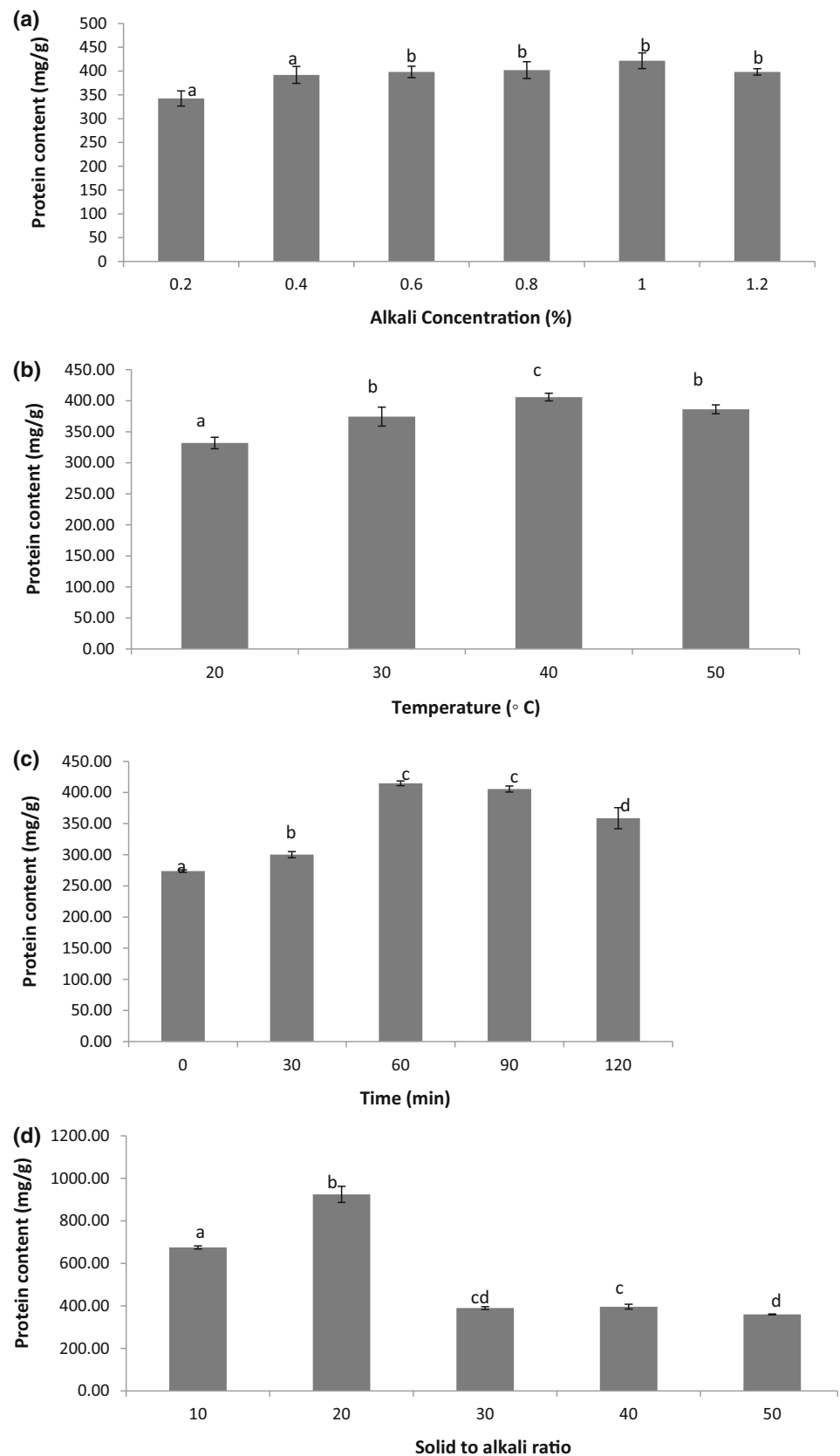
Results and discussion

Extraction and optimization of process parameter for protein from defatted *L. acidissima* seed

Extraction of protein depends upon the alkali concentration, a buffer to sample ratio, pH, temperature, and time (Wani et al. 2006).

Effect of various alkali concentrations on protein content is signified in Fig. 1a). From figure it can be seen that

Fig. 1 Effect of **a** alkali concentration; **b** Temperature; **c** Time; **d** solid to alkali ratio on protein content of defatted *L. acidissima* seed flours (Duncan test)



as concentration increased up to 0.4%; insignificant difference in protein content of *L. acidissima* seed was observed. Further, as the concentration of alkali increased; an increase in protein content up to 1% was observed followed by a significant decrease in the concentration. Thus, an optimal concentration for extraction was considered as 1% alkali. This could be due to fact that acidic proteins are soluble in alkali solution thus causing an increase in protein content with respect to increased alkali concentration (Zhang et al. 2009). However, further decrease in protein concentration was observed and this could be due to the denaturation of proteins.

Temperature is a key factor which affects protein content during extraction (Fig. 1b). As the temperature increased from 20 to 40 °C; significant improvement in protein concentration (331.94–405.88 mg/g) was observed; however, significant decrease was seen at 50 °C. Hence, 40 °C was considered as an optimum temperature in the experiment. A similar kind of study was performed for grape seed protein (Lv et al. 2011). Deng et al. (2014) also observed a decrease in protein content of kiwifruit seeds with increased temperature (above 35 °C) due to the denaturation of proteins.

The influence of time on protein extraction of *L. acidissima* is shown in Fig. 1c). As time increased from 10 to 60 min; significant increase in protein content, i.e. from 273.80 to 414.77 mg/g was observed followed by a significant decrease. The increase in the protein concentration due to the longer time protein dissolves. Thus, an optimum time of 60 min was finalised for protein extraction. The plausible explanation for such observation is given by Lv et al. (2011). They observed 30 min as the total time of extraction of protein later it was stable due to the protein solubility equilibrium.

The effect of solid to alkali ratio is represented in Fig. 1d. Initially at 1:20 ratio; significantly high protein content, i.e., 924.31 mg/g was recorded and as further ratio increased from 30 to 50 ml/g, it decreased 390.17–360.19 mg/g. Hence, 1:20 (solid to alkali ratio) was selected as optimal to increase protein content in extraction. A similar solid/solvent ratio was obtained by Lv et al. (2011), during grape seeds protein extraction.

From the single factor test, optimum extraction parameters were obtained as following alkali concentration 1%, temperature 40 °C, time 60 min, and solid to alkali ratio 1:20. Under this condition total protein content was 924.31 mg/g achieved from *L. acidissima*. This may be due to the high alkali, which is responsible to inhibit the common activity of protease and causes ionization of phenolic compounds and also prevent the forming hydrogen bonding with protein (Loomis and Battaile 1966; Hochstrasser et al. 1988).

Optimization of process parameter for hydrolysis of protein from defatted *L. acidissima* seed protein

The DH is a useful tool to understand the effect of pH, an enzyme to substrate ratio, and time on hydrolysis of *L. acidissima* protein. In protein hydrolysates, PCL (peptide chain length) is produced with desired functional properties which explain that higher the DH value corresponds to low formation of PCL or inverse (Adler-Nissen 1986).

Effect of pH on hydrolysis of protein was observed and depicted in Fig. 2a. As the pH increased from 2 to 3, the degree of hydrolysis was decreased. There was no significant difference observed after pH 2.2 ($p < 0.05$). Hence, pH range of 2–2.4 was selected for further optimization study.

As enzyme concentration increased from 0.5 to 3% (w/w, enzyme/substrate); DH was increased (Fig. 2b). However, 2–3% was selected for further optimization.

Effect of time on DH is represented in Fig. 2c. Initially high DH was observed, i.e., up to 60 min, later, it was in stationary phase up to 150 min (which showed no significant difference between 60 and 150 min) and further it significantly increased due to the longer extraction time. Hence, 30–90 min ranges were selected for optimization study. Thus, these results reconfirmed that hydrolysis is a function of time and enzyme concentration.

Response surface methodology

The interaction of hydrolysis parameters: pH (X_1), ES (X_2) and time (X_3) having impact on DH of *L. acidissima* seed protein, were studied by employing a BBD design. The single factor helps in the designing experiment on the basis of DH as response. Table 2 shows that the experimental outcomes obtained by design. The various conditions of software DH was varied in between 10.55 and 40.31%.

Model fitting

In our experiment, pepsin was most suitable enzyme which produces peptides with a high DH.

Furthermore, efforts were made to investigate interaction of pH, E/S ratio and time on the DH using BBD. The results are shown in Table 2. The best explanatory model equation fitted into Eq. (1) is given in Eq. (2) which corresponds to DH (%),

$$\text{DH} = 32.59 - 4.23 X_1 + 1.87 X_2 + 1.29 X_3 + 2.97 X_1 X_3 + 2.18 X_1^2 - 15.97 X_3^2. \quad (2)$$

The significance of the coefficient evaluated using analysis of variance (ANOVA) and presented in Table 3. Quadratic model was significant ($p < 0.05$) and in coded

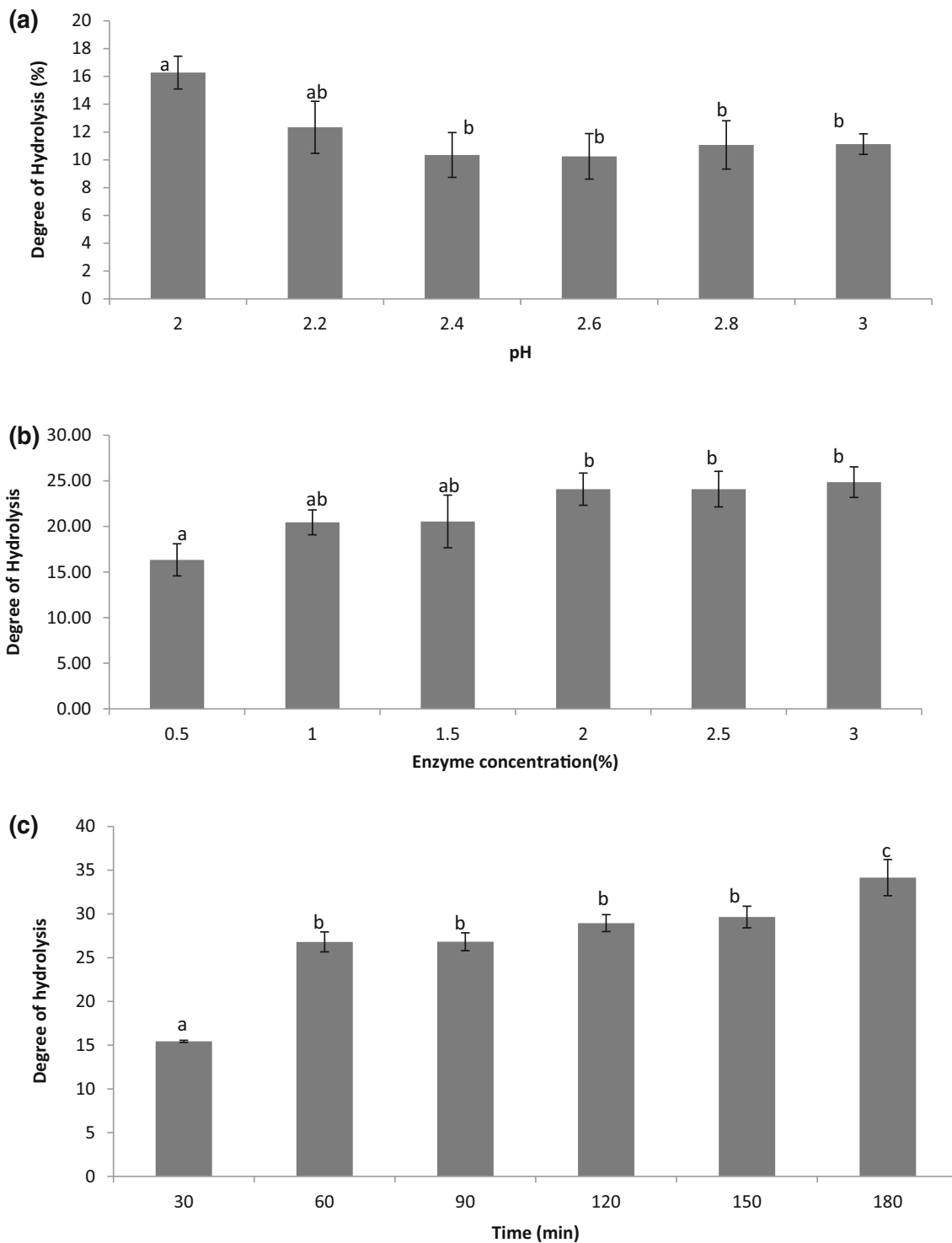


Fig. 2 Effect of **a** pH; **b** Enzyme to substrate (%); **c** Time (min); on hydrolysis of protein from defatted *L. acidissima* seed protein (Tukey test)

variables is given in Eq. (2). F value and p value was used in the determination of each coefficient. If F value is greater than p value, then corresponding variables would be more significant (Atkinson and Donev 1992).

The DH was a linear term of pH (X_1), an enzyme to substrate ratio (X_2) and time (X_3) followed by a quadratic term of pH(X_1^2), and time (X_3^2) and interaction terms X_1X_3 . On the other hand, quadratic terms of the enzyme to

Table 3 ANOVA for Response Surface Quadratic Model

Source	Sum of squares	df	Mean square	F value	p value prob >F	
Model	1305.686	9	145.0762	127.866	<0.0001	Significant
X ₁ -pH	143.3144	1	143.3144	126.3132	<0.0001	
X ₂ -Es	28.01546	1	28.01546	24.69202	0.0016	
X ₃ -time	13.29038	1	13.29,038	11.71376	0.0111	
X ₁ X ₂	0.011,495	1	0.011,495	0.010131	0.9226	
X ₁ X ₃	35.35,149	1	35.35,149	31.15778	0.0008	
X ₂ X ₃	1.484,312	1	1.484,312	1.30823	0.2903	
X ₁ ²	20.01717	1	20.01717	17.64256	0.0040	
X ₂ ²	0.024382	1	0.024382	0.021489	0.8876	
X ₃ ²	1073.634	1	1073.634	946.2703	<0.0001	
Residual	7.94217	7	1.134596			
Lack of fit	6.294256	3	2.098085	5.092704	0.0749	Not significant
Pure error	1.647914	4	0.411979			
Cor total	1313.628	16				
R-squared	0.993954					
Adj R-squared	0.986181					
Pred R-squared	0.921376					
C.V. %	4.086604					

substrate ratio (X_2^2), and other interaction terms of X_1X_2 and X_2X_3 were not significant ($p > 0.05$).

The lack of fit test deals the failure of the model (insignificant p-value of 0.0749) which indicates data in the experiment fitted well and acceptable to describe observed data. The results are presented in Table 3. The $R^2 = 0.9940$ indicated that the fitted model can explain 99.40% of the variation in the data. Also high degree of correlation was observed from Adj R^2 (0.9862) and Pred R^2 (0.9214). This model had a CV (coefficient of variation) of 4.09% suggesting a good precision and high reliability of the experiment achieved.

Interpretation of response surface model

The optimum values of the variables within the given ranges are shown by graphical representation of the regression equation. This can be presented by three-dimensional response surfaces and contour plots of the models. The interaction between E/S and pH on the DH of the hydrolysates of *L acidissima* seed protein at any constant hydrolysis time was depicted in Fig. 3a. As the concentration of enzyme was increased; DH was also increased. Whereas at increased pH; decreased DH was observed. At pH 2; as concentration of enzyme increased; increased DH was observed. Similar trends were observed at high pH 2.4. However, at low (1.5%) and high (2.5%) concentration of enzyme, if pH was varied; decreased order in DH was noted.

The interaction between time and pH on the DH of the hydrolysates of *L acidissima* seed protein at any constant Es is presented in Fig. 3b. Initially, as hydrolysis time increases DH increase and start decrease after a certain time (75 min), decrease in the DH observed as increase in the pH. Whereas in Fig. 3c noted that DH increased up to certain time and further it slightly decreased. But as enzyme concentration increased DH increased.

Validation of experiments

Confirmation of experiments was accomplished to suitability in the experiment. The optimal conditions for the predicted target degree of hydrolysis 36.59% corresponded to pH 2, an enzyme to substrate ratio 2.5% (w/w), and hydrolysis time 42.41 min. The experimental value of 39.82 ± 4.30 (%) was found close to predicted value. Hence, the model is valid for optimizing the process of hydrolysate from *L acidissima* seed protein.

Distribution of molecular weight in *L acidissima* seed protein hydrolysates

Polypeptide band pattern was observed in *L acidissima* seed protein hydrolysates (Fig. 4). *L acidissima* seed protein hydrolysates composed of 15 protein bands with a molecular weight between 95 and 14 and below 14 kDa, low molecular weight peptide band pattern was also observed below 14 kDa. The degree of hydrolysis of *L*

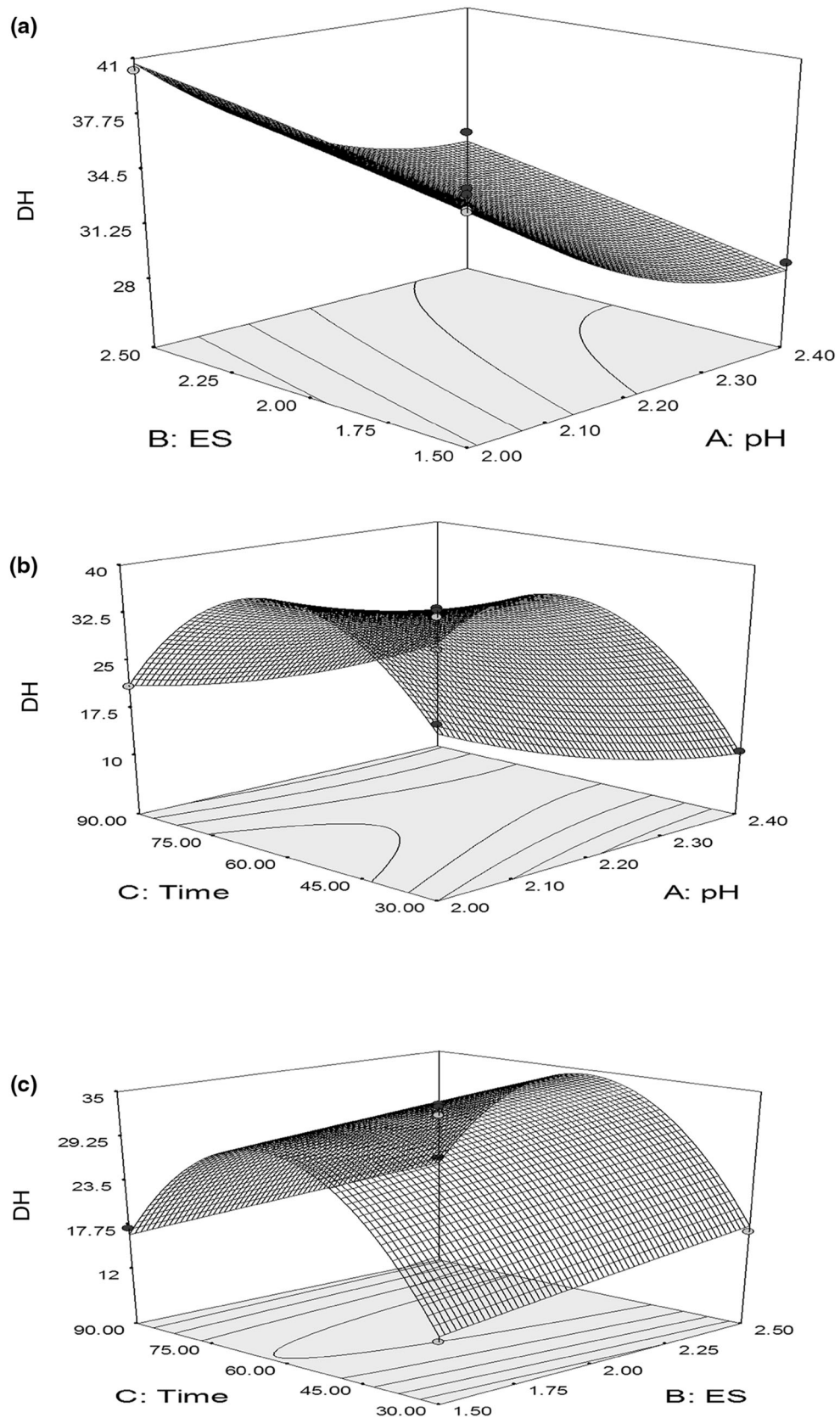


Fig. 3 Response surface plot: effect of **a** Es and pH **b** time and pH **c** ES and time on degree of hydrolysis of protein from defatted *L. acidissima* seed

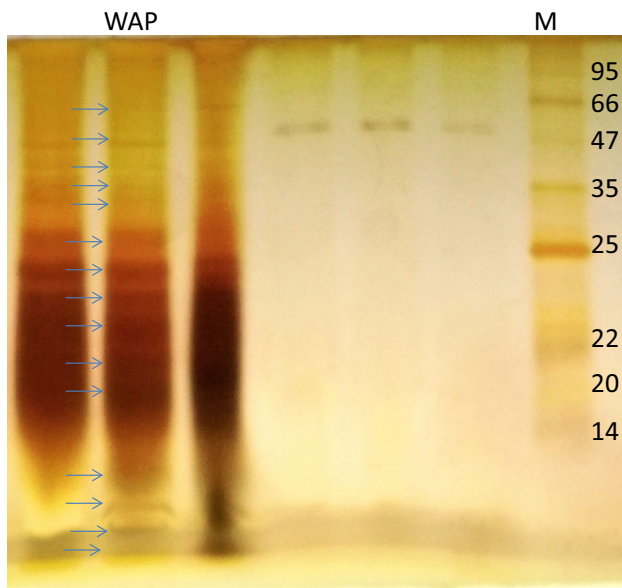


Fig. 4 SDS page of *L acidissima* seed protein hydrolysates

acidissima seed protein (39.82%) produces a range of different peptide bands. Suetsuna and Nakano (2000) documented that pepsin is help in hydrolysis of proteins.

Antioxidant activity of *L acidissima* seed protein hydrolysates

It is a widely suggested to perform more than two assays for estimation of antioxidant activity due to the possible irregularity of different radicals which are measured by DPPH, FRAP and ABTS assays. The DPPH and ABTS assay evaluate the scavenging capacity, and FRAP assay was used to reflect the ferric-reducing activity. The secondary antioxidant potential was measured by MCA (metal chelating assay) and demonstrates inhibitory effect on generation of radicals.

Scavenging activity by DPPH

Radical scavenging activity is measured by reduction in absorbance. Table 4 represents the scavenging activity of the *L acidissima* seed protein hydrolysate at various dosages. The *L acidissima* seed protein hydrolysates have DPPH radical scavenging activity at a dosage of 20–100 $\mu\text{g/ml}$, whereas Trolox at 2–10 $\mu\text{g/ml}$ (Table 4). The protein hydrolysate 100 $\mu\text{g/ml}$ showed 32.94% inhibition which was closed to 6 $\mu\text{g/ml}$ of Trolox. This is a disadvantage of DPPH scavenging activity assay in the case of protein, DPPH is only soluble in methanol or ethanol solution and when the concentration of protein was increased, precipitation occurred, which is a big hurdle to measure antioxidant activity of protein by DPPH. This may

be due to the hydrophilic nature of hydrolysate in which possibility of accumulation of shorter peptides and amino acids. The increased polarity of the low-MW protein hydrolysate makes them more difficult to react with hydrophobic (Zhu et al. 2008).

Scavenging activity by ABTS

Antioxidant activity lipophilic and hydrophilic molecules analysed using ABTS decolourization assay that determined by measuring absorbance at 734 nm in which reaction of hydrogen donating antioxidants with the ABTS radical. ABTS scavenging activity by protein hydrolysate was increased as the concentration of peptides increased (Table 4). 0.2 mg/ml of protein hydrolysate showed 57.09% of scavenging activity which was close to 25 $\mu\text{g/ml}$ of Trolox that is 51.6%. The difference in the inhibition of DPPH and ABTS radical by *L acidissima* seed protein hydrolysates was observed. This may be due to the DPPH assay performed in organic media, while the ABTS assay in aqueous media. The contradictory phenomenon between the activities is due to the differences of solubility and diffusivity of radicals and peptides in the reaction system (Arnao 2000).

FRAP

A compound shows its potential antioxidant activity by reducing capacity that may serve as a marker of antioxidant power. The reducing agents in tested samples results in the development of the complex tripyridyltriazine (TPTZ)–Fe(II), which is monitored at 595 nm. As the concentration of *L acidissima* protein hydrolysate (1–5 mg/ml) increased; the absorbance was increased (0.03–0.16) at 593 which were comparatively low to Trolox (Table 4). At 5 mg/ml of protein hydrolysate concentration; the absorbance value of 0.16 was observed that was corresponding to 0.22 absorbance (at concentration of 10 $\mu\text{M/ml}$ of Trolox). An increased absorbance was noted as the concentration of protein hydrolysates and Trolox increased. This increase could be due to the formation of peptide cleavages which resulted into improved hydrogen ions availability during the course of reaction (Kong and Xiong 2006).

Metal chelating ability

The ferrous ion chelating ability of *L acidissima* protein hydrolysates is shown in Table 4. The high chelating effect was observed at a high concentration of hydrolysates that is 1–5 mg/ml. 87.30% of chelating activity was showed by 5 mg/ml of hydrolysate. It is well-known that the side chains of the acidic and basic amino acids plays an important role in chelating metal ions (Saiga et al. 2003).

Table 4 Radical scavenging activity and reducing capacity of *L. acidissima* seed peptides

Peptides (µg/ml)	Scavenging activity of DPPH (%)	Trolox (µg/ml)	Scavenging activity of DPPH (%)	Peptides (mg/ml)	Scavenging activity of ABTS (%)	Trolox (µg/ml)	Scavenging activity of ABTS (%)
20	10.14 ± 0.32	2	11.9	0.2	57.09 ± 5.12	5	12.0
40	15.02 ± 0.48	4	24.8	0.4	74.06 ± 1.19	10	22.3
60	16.73 ± 0.54	6	35.4	0.6	81.48 ± 0.85	15	38.8
80	22.66 ± 0.73	8	48.0	0.8	85.00 ± 0.58	20	46.2
100	32.94 ± 1.05	10	58.5	1	88.18 ± 0.71	25	51.6

Reducing capacity of wood apple seed peptides							
Peptides (mg/ml)	FRAP Abs _{593 nm}	Trolox (µM/ml)	FRAP Abs _{593 nm}	Peptides (mg/ml)	Metal chelating activity (%)	Trolox (µg/ml)	Metal chelating activity (%)
1	0.03 ± 0.02	10	0.220	1	51.91 ± 1.19	0.1	3.67
2	0.05 ± 0.01	20	0.426	2	67.23 ± 1.55	0.2	14.69
3	0.07 ± 0.01	30	0.635	3	80.65 ± 1.86	0.3	16.33
4	0.09 ± 0.01	40	0.809	4	81.73 ± 1.88	0.4	21.41
5	0.16 ± 0.01	50	0.986	5	87.39 ± 2.01	0.5	30.83

Results were expressed as the mean ± SD of three determinations

Antioxidant activity obtained from different assay showed variation due to reaction mechanism such as single electron transfer (SET) which corresponds to FRAP and hydrogen atom transfer (HAT) mechanism corresponds to ABTS assay (Prior et al. 2005).

Antioxidant capacity of proteins and their hydrolysates comes from their ability to inactivate reactive oxygen species (ROS), quench free radicals, chelate prooxidative transition metals, and donate electron/hydrogen (Elias et al. 2008). The amino acid composition and sequence, the size and the configuration (Chen et al. 1996; Kong et al. 2008) and hydrophobicity (Sarmadi and Ismail 2010) having influence on the antioxidant properties of proteins and their hydrolysates. Xie et al. (2008) reported amino acids, such as Tyr, Lys, Arg, Gly, Leu, and His is playing key role for antioxidant activity.

Conclusions

In summary, the study of single factors test employed in the extraction and hydrolysis of protein from *L. acidissima* seed. The predicted profile showed that the optimum conditions for protein hydrolysis were a degree of hydrolysis 39.82% corresponded to pH 2, an enzyme to substrate ratio 2.5% (w/w), and hydrolysis time was 42.41 min. Through the confirmative test, model can be widely used for hydrolysis of protein. This investigation confirms that *L. acidissima* seed protein hydrolysate could be used as a valuable source of antioxidative peptides. This may forecast their future application into functional food formulations and/or dietary supplements.

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Compliance with ethical standards

Conflicts of interest We do not have any conflicts of interest.

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