PROJECT COMPLETION REPORT

Project Title: Characterisation of technofunctional and biofunctional properties of napin protein hydrolysates of rapeseed meal

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Director
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Submitted by

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1. **PROJECT TITLE: CHARACTERISATION OF TECHNOFUNCTIONAL AND BIOFUNCTIONAL PROPERTIES OF NAPIN PROTEIN HYDROLYSATES OF RAPESEED MEAL**

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5. Duration of the project: 17.01.2014 to 13.07.2016

6. Objectives of the project:
   i) To enzymatically hydrolyse proteins from rapeseed meal and analyse the hydrolysates for technofunctional and biofunctional properties.
   ii) To identify hydrolysates with better technofunctional properties of foam and emulsion stability, viscosity and water absorption.
   iii) To identify hydrolysates with ability to inhibit angiotensin I converting enzyme and bind bile acid by in-vitro methods.

I. OPTIMISATION OF PROTEIN EXTRACTION FROM RAPESEED MEAL

1. Introduction

Rapeseeds belong to the *Brassica* species. They contain about 35 to 40 % oil and 20 to 25 % protein. The dehulled and solvent-extracted seed meal contains 35 to 45 % protein [1,2]. There are two major families of storage proteins termed cruciferins and napins [3,4]. Cruciferins belong to the 12S globulin class of proteins. These neutral proteins, with a high molecular weight, are composed of several subunits and constitute 26-65% of the total rape seed protein content, depending on the variety [4]. Napins belong to the 2S albumin class of proteins (and are hence water soluble) and represent 15-45% of the total rapeseed protein content depending on the variety [5]. Rapeseed also contains some minor proteins of interest, such as thionins, trypsin inhibitors and a lipid transfer protein (LTP). Mature napins are highly basic proteins with pI around 11 [6]. Napins (2S albumins) are therefore, low molecular weight basic proteins

Napins are rich in glutamine, lysine, and cysteine [10]. Rapeseed seed protein has a well-balanced amino acid profile, compared to grains and legumes, which are deficient in sulfur-containing amino acids, and a favorable protein efficiency ratio of 2.6 in comparison with 2.2 for soybean [11].

Rapeseed proteins compare favourably with soy proteins and the FAO pattern of essential amino acid requirement of human adults [7]. The proteins are rich in lysine and methionine,

which are limiting essential amino acids in most of the cereals and vegetables proteins. Rapeseed protein isolates, obtained from defatted rapeseed meal and free of antinutritional components such as glucosinolates, phenolics, phytic acid or fiber, can be used as food ingredients [8]. But the use of these proteins is often limited by their low solubility and poor functional properties. This is a particular problem in oilseeds, such as rapeseed, because the proteins suffer denaturation during industrial oil extraction that further reduces their solubility. Conventional rapeseed processing for oil production generates low-valued meal as a co-product which is mainly used in animal feeds and fertilizers.

Proteins, besides being a source of nutrients, have other uses in the food industry. Proteins can be hydrolyzed to improve water solubility. Proteins can be adsorbed at oil-water and air water interfaces to decrease surface tension values, and, hence facilitate the formation of emulsions and foams. In addition, proteins form a continuous viscoelastic film around the oil droplets or air bubbles that stabilize emulsions and foams [9]. The production of enzymatic protein hydrolysates has undergone considerable development in recent years. Protein hydrolysates with low DH (<10%), with better functional properties than the original proteins and with enhanced functional properties such as water and oil absorption, foaming capacity or emulsifying activity are used as food ingredients [12].

Rapeseed (*Brassica juncea* L.) seed meal was studied for protein recovery by different methods and enzymatically hydrolysed peptides were studied for functional properties [13,14].

2. Materials and methods

Collection of raw material

Oil pressed rapeseed defatted meal was collected from a local factory. The meal was further defatted with hexane so as to prepare an oil free meal to the maximum extent possible (**Fig. 1**). All the other chemicals were of analytical grade. pH meter (pH 700, Eutech) was used for adjustment of pH.

Extraction of protein

Protein was extracted by combination of method of Vioque et. al. [15] and Sadeghi and Bhagya [13]. Defatted rapeseed flour (10 g) was suspended in solvent in the required ratio. The solvent contained 0.05 M Tris, 0.25% Na_2SO_3 and NaCl of given concentrations. Na_2SO_3 was used to prevent oxidation of polyphenols and to avoid the darkening of the final product while NaCl was used to prevent the binding of phenolic acids to the protein [16]. The suspension was extracted by stirring continuously at room temperature for different time intervals. After centrifugation at 8,000 × *g*, two more extractions were carried out with half of the volume of alkaline solution. The supernatants were pooled, and the pH of the soluble

25 g of sample + 50 ml of n-hexane

↓

Stirred for 2.5 h in magnetic stirrer (RT)

↓

Hexane decanted

↓

Process repeated thrice

↓

Filtered

↓

Meal was dried in a vacuum oven overnight at 35°C (to remove the residual hexane)

↓

Defatted meal amount (19.28 g)

Fig. 1. Flow chart for defatting of the meal

proteins was adjusted to the isoelectric point (pH 3.8) and the precipitate formed was recovered by centrifugation as above. The precipitate was washed with distilled water that was adjusted to pH 3.8 and freeze-dried.

Protein isolate yield

The weight of the protein isolate was taken as protein isolate yield (Digital balance, Denver instruments).

Protein content

The protein content of the rapeseed meal was determined by micro-kjehdahl method (1998). 250 mg of the sample was digested in 10 ml of sulfuric acid at 420°C using copper sulphate and sodium sulphate catalyst mixture (4 g) until the solution becomes greenish in colour so that nitrogenous compounds convert into ammonium sulphate. The ammonium sulphate formed was decomposed with an alkali (NaOH). Ammonium liberated is absorbed in excess of neutral boric acid (20 ml) solution. It is titrated against standard 0.1 N HCl. The nitrogen content was calculated as

$$\text{Nitrogen (\%)} = \frac{(\text{Sample titre} - \text{Blank titre}) \times \text{Normality of HCl} \times 14 \times 100}{\text{Weight of sample taken} \times 1000}$$

Protein (%) =Nitrogen content (%) × Conversion factor (6.25)

Colour measurement

The *L,a,b* parameters of the extracts were taken with Color Measurement Spectrophotometer (Hunter Color-Lab Ultrascan Vis). *'L'* denotes lightness of the sample, *'a'* denotes the

redness or greenness and *'b'* denotes the blueness or yellowness. The whiteness index was calculated as WI= L-3b [18].

Experimental design

To study the response pattern and to determine optimum combination of the variables, a central composite experimental design with four variables was used. Experimental range and levels of the independent variables, viz. X_1 (pH, P), X_2 (solvent:meal ratio, S), X_3 (NaCl concentration, C), and X_4 (time, T) at five levels used to design the process are given in **Table 1**. The effect of the independent variables (**Table 1**) in the extraction process is shown in **Table 2**. Six centre points of the design were used to allow for estimation of a pure error sum of squares. All the experiments were carried out in random order so as to maximize the effects of unexplained variability in the observed responses due to extraneous factors. Low and high level of factors were coded as -1 and +1, and midpoint was coded as 0. The factor level of trials that ran along axes drawn from the middle of the cube through the centers of each face of the cube were coded as $-\alpha$ and $+\alpha$.

Table1. Experimental range and levels of the independent variables used to design the process

Independent variables	Symbol		Levels				
	Uncodified	Codified	-α	-1	0	+1	+α
pH	X_1	x_1	8	9	10	11	12
Solvent:Meal	X_2	x_2	5:1	10:1	15:1	20:1	25:1
NaCl (M)	X_3	x_3	0	0.5	1	1.5	2
Time (min)	X_4	x_4	40	50	60	70	80

The variables were coded according to the following equation:

$x_i = (Xi - \overline{Xi}) / \Delta Xi$

where, x_i is the dimensionless value of an independent variable, Xi is the real value of an independent variable, Xi is the real value of an independent variable at the center point, ΔXi is the step change.

The specific codes are:

x_1 (pH) = (P- 10)/1,

x_2 (solvent: meal) = (S-15)/5,

x_3 (concentration) = (C-0.25)/0.13, and

x_4 (time) = (T-60)/10

Central composite design (CCD) for the preparation of protein isolate and its responses are given in **Table 2**. A total of 30 runs were conducted and analysed.

Table 2. Central composite design (CCD) for the preparation of protein isolate and its responses

Run	Factors				Responses		
	pH	Solvent:Meal (v/w)	NaCl (M)	Time (min)	Protein isolate yield (g)	Protein content (%)	Whiteness index
1	9	10:1	0.5	50	1.17	67.25	61.03
2	11	10:1	0.5	50	2.1	71.15	53.03
3	9	20:1	0.5	50	0.72	69.97	47.29
4	11	20:1	0.5	50	1.52	73.2	45.44
5	9	10:1	1.5	50	1.68	68.15	65.3
6	11	10:1	1.5	50	2.14	72.5	55.91
7	9	20:1	1.5	50	1.08	72.45	56.8
8	11	20:1	1.5	50	2.1	74.21	53.56
9	9	10:1	0.5	70	1.27	76.55	55.67
10	11	10:1	0.5	70	2.28	80.35	49.34
11	9	20:1	0.5	70	0.88	79.35	54.7
12	11	20:1	0.5	70	1.89	82.85	54.53
13	9	10:1	1.5	70	1.58	77.15	60.79
14	11	10:1	1.5	70	2.88	80.15	53.08
15	9	20:1	1.5	70	1.11	79.75	65.07
16	11	20:1	1.5	70	1.8	84.45	63.51
17	8	15:1	1	60	0.86	69.95	58.79
18	12	15:1	1	60	2.45	76.85	49.22
19	10	5:1	1	60	1.35	70.21	63.39
20	10	25:1	1	60	1.48	76.37	60.08
21	10	15:1	0	60	1.68	73.55	53.22
22	10	15:1	2	60	1.54	74.31	66.47
23	10	15:1	1	40	1.77	65.24	55.87
24	10	15:1	1	80	1.86	83	60.46
25	10	15:1	1	60	1.68	75.15	56.72
26	10	15:1	1	60	1.54	77.25	56.73
27	10	15:1	1	60	1.37	74.85	56.72
28	10	15:1	1	60	1.87	76	56.72
29	10	15:1	1	60	1.63	74.15	59.22
30	10	15:1	1	60	1.58	73.15	59.21

Statistical analysis

Design-Expert Version 6.0.11 (Stat-Ease Inc., Minneapolis, USA) was used to conduct the experimental design. The meal yield, percentage of protein content and whiteness index obtained were taken as dependent variables or responses. The model proposed for the response is given by the following equation.

$$Y_i = b_0 + \sum_{n=1}^{4} b_n x_n + \sum_{n=1}^{4} b_{nn} x_n^2 + \sum_{n \neq m=1}^{4} b_{nm} x_n x_m$$

where, Y_i (i =1, 2, 3) is predicted response for protein isolate yield, protein content and whiteness index; b_0 is the value of the fitted response at the center point of the design that is point (0,0,0,0). b_n, b_{nn} and b_{nm} are the linear, quadratic and interaction regression terms, respectively. The quadratic model was used for the analysis.

The 'p' value of the regression co-efficient explains the pattern of mutual interaction between variables, the smaller the value of 'p', the corresponding co-efficient is more significant. The optimum level of pH, NaCl concentration, solvent to meal ratio and time was obtained by maximizing the protein isolate yield, protein content and whiteness index through numerical optimization. The quality of fit of second order equation was determined by co-efficient of determination R^2 and its statistical significance was determined by F test. The individual and interactive effects of the independent variables were evident from the model graphs.

Purification of Napin

Purification of the napin protein was done with a combination of chromatography steps. First the extraction of the sample was done under the optimized conditions. The supernatant does obtained was subjected to chromatography.

Desalting of the sample

Desalting of the sample was done remove the compounds such as polyphenols, phytic acid etc. and also to remove the salt. Desalting was done using Sephadex G50. A 60 ml column was prepared manually. The column was then calibrated for 2 days with buffer (0.05M Tris-HCl, pH 8). A 0.5 ml of supernatant was loaded on to the column. Fractions of 1ml of desalted sample were collected manually in Eppendorf tubes. The fractions containing protein was detected by Lowry's method [19].

Cation exchange chromatography

The fractions of desalted samples containing protein was further subjected to cation exchange chromatography. A 2 ml column of SP-Sepharose was taken. SP Sepharose Fast Flow is a strong cation exchanger. Napin at pH 8 will have positive charge and binds to the SP cation exchanger. A 10 ml of the sample was loaded onto the column. Napin will bind to the SP- (sulphopropyl) group of the beads. Napin was then eluted with an increasing

concentration of (0.1 – 1M) NaCl. One ml fractions were collected. Fractions containing napin were pooled and then desalted as above.

SDS-PAGE profiling

The purity of the sample was determined by running the purified sample in a SDS-PAGE in a vertical slab. A polyacrylamide gel consisting of 12 % resolving gel and 5 % stacking was prepared. The protein sample (30µg) was precipitated with TCA. The sample was then mixed with loading buffer in reducing and non reducing conditions. A 20µl of loading buffer containing reduced and the non reduced samples were loaded on to the wells of the gel. A broad range marker (10 – 170 kB, Fermentas) was used for calibration. Electrophoresis was performed at a voltage of 120 V and 25 mA current. The gel was run until the tracking dye reached the bottom of the gel. After that the protein bands were silver stained according to Sammons et. al. [20]

3. Results and discussion

ANOVA for protein isolate yield

Proposed model (2nd order polynomial regression) equation for response is

Meal yield = +1.61 + 0.21*A + 0.090*B + 0.14*C + 0.45*D + 0.093*A^2 - 0.026*B^2 + 0.19* C^2 + 0.20* D^2 + 0.032*A* B - 0.012*A*C + 0.022*A*D - 0.003*B*C + 0.013*B*D - 0.086*C*D

Here, A is pH, B is Solvent to meal ratio, C is NaCl concentration and D is time. The equation in terms of coded factors can be used to make predictions about the response for the level of each factor. The coded equation is useful for identifying the impact of the factors by comparing the factor coefficient. The ANOVA for response surface quadratic model is given in **Table 3**. The model F value of 14.49 implies that the model is significant. There is only 0.01 % that an F-value this large could be due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case, A, C, D, A^2, C^2, D^2 are significant model terms. The "Lack of fit" is not significant relative to pure error which further indicates the validity of the model. The "Lack of fit" value of 1.89 indicates that there is 25.03 % chance that the value this large could be due to noise. The prediction equation showed a good fit with the experimental design since the R^2 value of 0.9312 indicated that 93.12% of the variability within the range values studied could be explained by the model (**Fig. 2**). The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of observed response expressed as a percentage. It is a measure of reproducibility of the models. The CV of the model was 10.55%. It means that the model was quite reproducible. "Adeq precision" measures the signal to noise ratio. A value greater than 4 is desirable. The ratio of 14.082 indicates that the model can be used to navigate the design space. 3D contour plots were drawn to demonstrate the main and interactive effects of the independent variables on

the dependent variables. These graphs (**Fig. 3**) were obtained by fixing two variables at coded zero level while the other two variables varied to predict the response variable (protein isolate yield).

Table 3. Co-efficient of the fitted model for the determined response

Co-efficient	**Determined response (Protein isolate yield)**
Model F-value	14.49
Prob>F	<0.0001
Lack of fit	1.89
R^2 value	0.9312
CV value	10.55 %
Adeq precision value	14.082

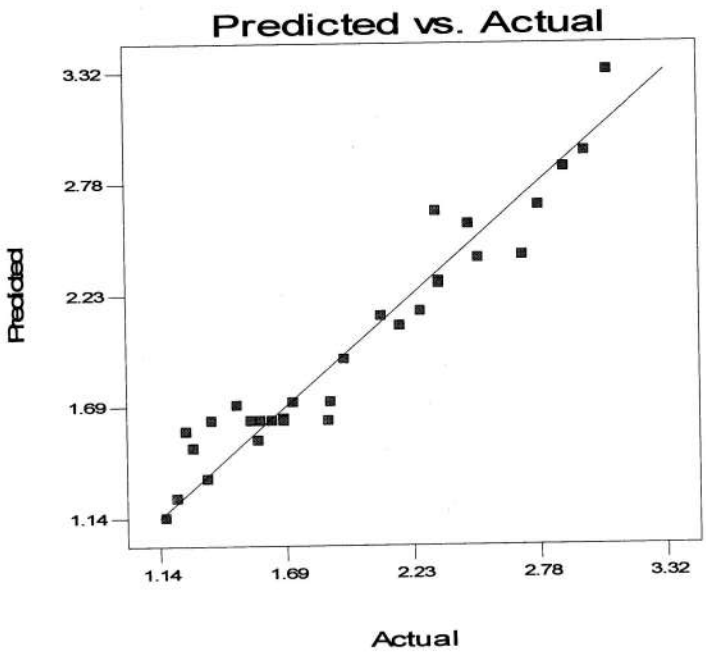
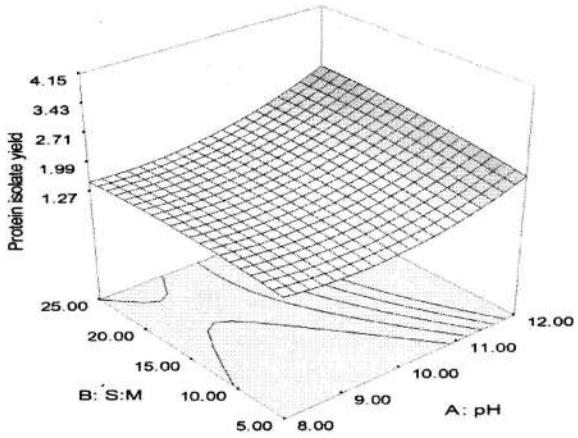
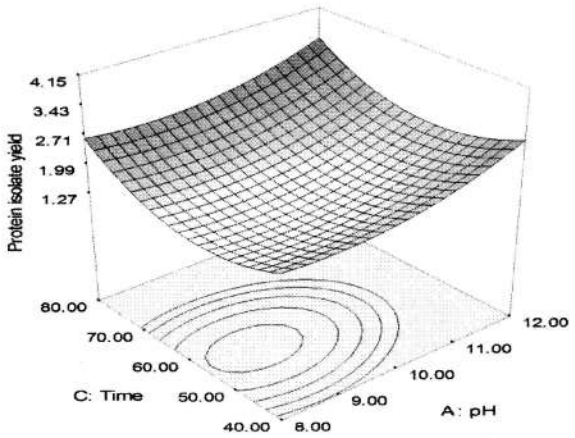


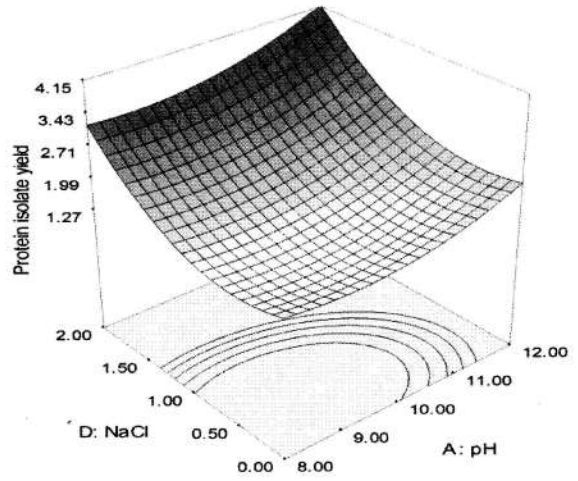
Fig 2: Comparative predicted and actual values for protein isolate yield at R^2= 0.93


Protein isolate yield
4.15
3.43
2.71
1.99
1.27
25.00
20.00
15.00
10.00
5.00
B: S:M
8.00
9.00
10.00
11.00
12.00
A: pH

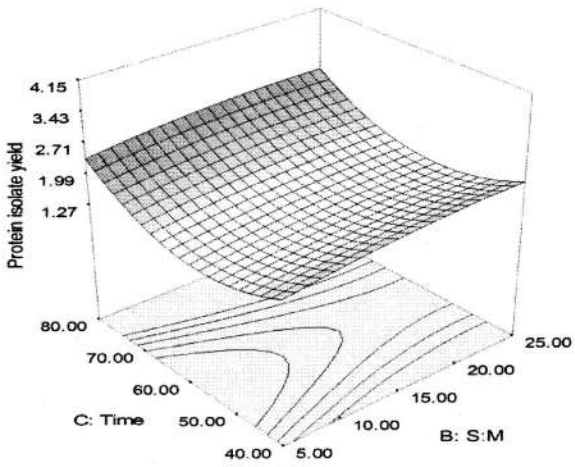
(a)


Protein isolate yield
4.15
3.43
2.71
1.99
1.27
80.00
70.00
60.00
50.00
40.00
C: Time
8.00
9.00
10.00
11.00
12.00
A: pH

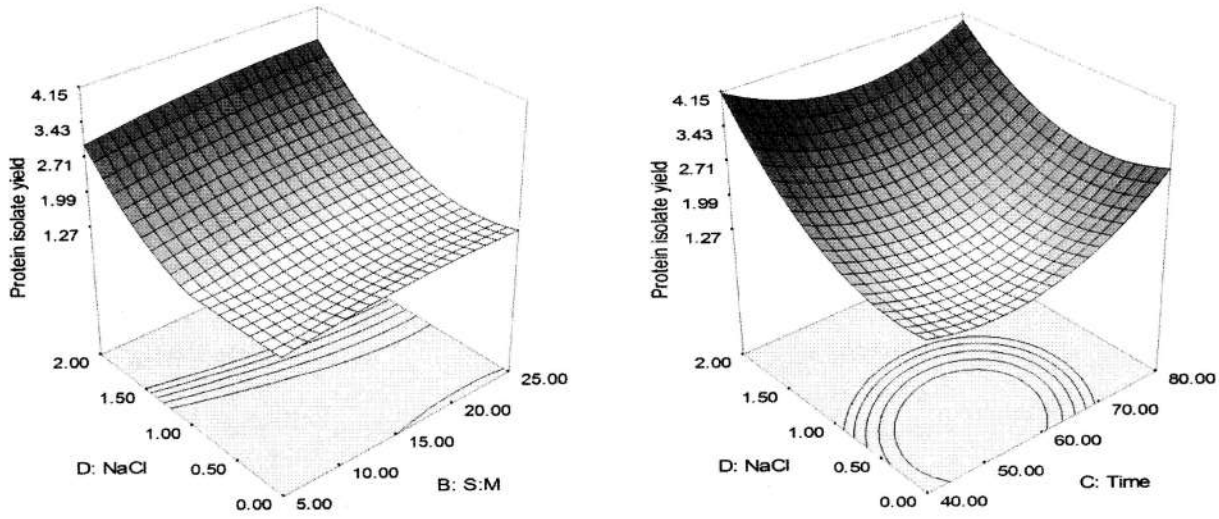
(b)


Protein isolate yield
4.15
3.43
2.71
1.99
1.27
2.00
1.50
1.00
0.50
0.00
D: NaCl
8.00
9.00
10.00
11.00
12.00
A: pH

(c)


Protein isolate yield
4.15
3.43
2.71
1.99
1.27
80.00
70.00
60.00
50.00
40.00
C: Time
5.00
10.00
15.00
20.00
25.00
B: S:M

(d)



(e) (f)

Fig. 3. Effect of interaction of the factors on protein isolate yield

ANOVA for protein content

Proposed second order polynomial regression equation for response is

Protein content = + 75.09 + 1.75*A + 1.47*B +0.40*C + 4.47*D - 0.11*A^2 - 0.13*B^2 + 0.027*C^2 + 0.075*D^2 - 0.12*A* B - 0.039*A*C + 0.11*A*D + 0.18*B*C + 0.089*B*D - 0.21*C*D

The factors are coded as stated earlier. The equation in terms of coded factors can be used to make predictions about the response for the level of each factor. The coded equation is useful for identifying the impact of the factors by comparing the factor coefficient. The ANOVA for response surface quadratic model is given in **Table 4**. The model F value of 20.05 implies that the model is significant. There is only 0.01 % that an F-value this large could be due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case, A, B and D are significant model terms. The "Lack of fit" is not significant relative to pure error which is good as we want the model to fit. The "Lack of fit" value of 1.10 indicates that there is 48.71 % chance that the value this large could be due to noise. The prediction equation showed a good fit with the experimental design since the R^2 value of 0.9493 indicated that 94.93% of the variability within the range values studied could be explained by the model (**Fig. 4**). The ANOVA for response surface quadratic model is given in **Table 4**. The CV of the model was 1.97%. As a general rule, a model can be considered reasonably reproducible if its CV value is not greater than 10% [21]. "Adeq precision" measures the signal to noise ratio. A value greater than 4 is desirable. The ratio of 17.125 indicates that the model can be used to navigate the design space. 3D contour plots were drawn to demonstrate the main and interactive effects of the independent variables on the dependent variables. These graphs

(**Fig. 5**) were obtained by fixing two variables at coded zero level while the other two variables varied to predict the response variable (protein content).

Table 4. Co-efficient of the fitted model for the determined response

Co-efficient	**Determined response (Protein content)**
Model F-value	20.05
Prob>F	<0.0001
Lack of fit	1.10
R^2 value	0.9493
CV value	1.97 %
Adeq precision value	17.125

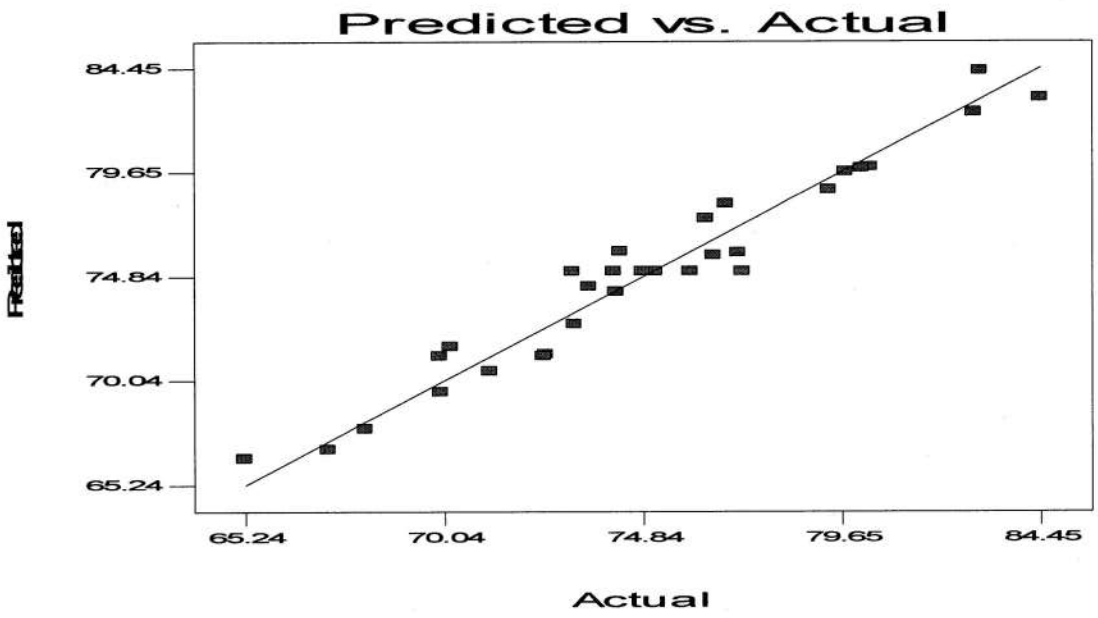


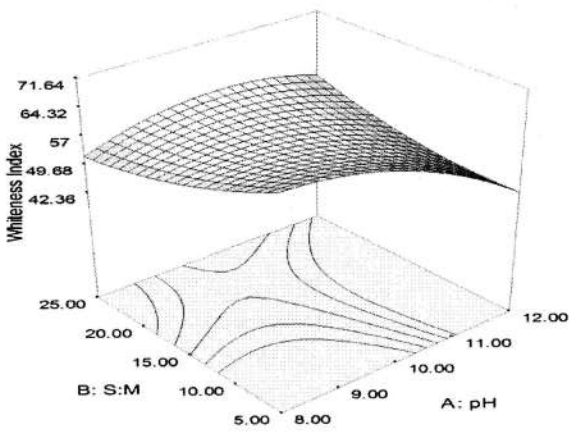
Fig. 4. Comparative predicted and actual values for protein content at R^2= 0.94

Effect of the different factors and their interaction on the responses

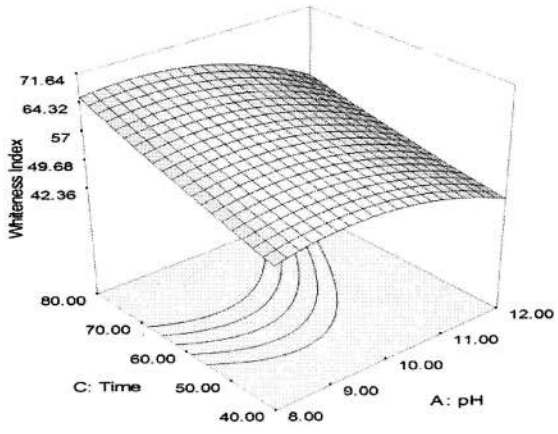
From review of several published literature on protein extraction the four parameters viz, pH, time, solvent to meal ratio and NaCl concentration [21, 22, 23, 24] were selected to study their effects on the responses. Therefore, the central composite design was done using the four factors. The minimum and maximum levels were chosen from related published literature.

Nitrogen extractability increases at high pH. It was found to be maximum at pH 12 [25]. Extraction at higher pH was not done as it had undesirable effect on protein isolate. Wang et.al. [26] reported that high pH caused protein hydrolysis and denaturation resulting in an unacceptable odour and flavour. Increased pH also increased Maillard reaction that darkened the product, decreased nutritive value of protein, especially essential amino acid such as lysine, while increased the extraction of non-protein component, which coprecipitated with protein leading to lower protein purity. The effects of the four parameters seem to be similar for both protein isolate yield and protein content. With increase in the pH both protein isolate yield and protein content increased. The extraction was more beyond pH 10 but it was quite linear from pH 11 to 12 [27]. A further increase in pH could not be attained as the meal turned dirty green in colour which is an undesirable characteristic and the solvent to meal ratio could not be maintained.

The meal to solvent ratio had a positive effect on both protein isolate yield and protein content i.e. with increase in meal to solvent ratio the extraction of protein increased. The extraction of protein increased with increase in solvent to meal ratio in the beginning but the rate of increase slowed down later. The increase of extraction in the beginning was because of availability of more solvent that allowed more extraction. But the slow increase in extraction with further



(a)



(b)

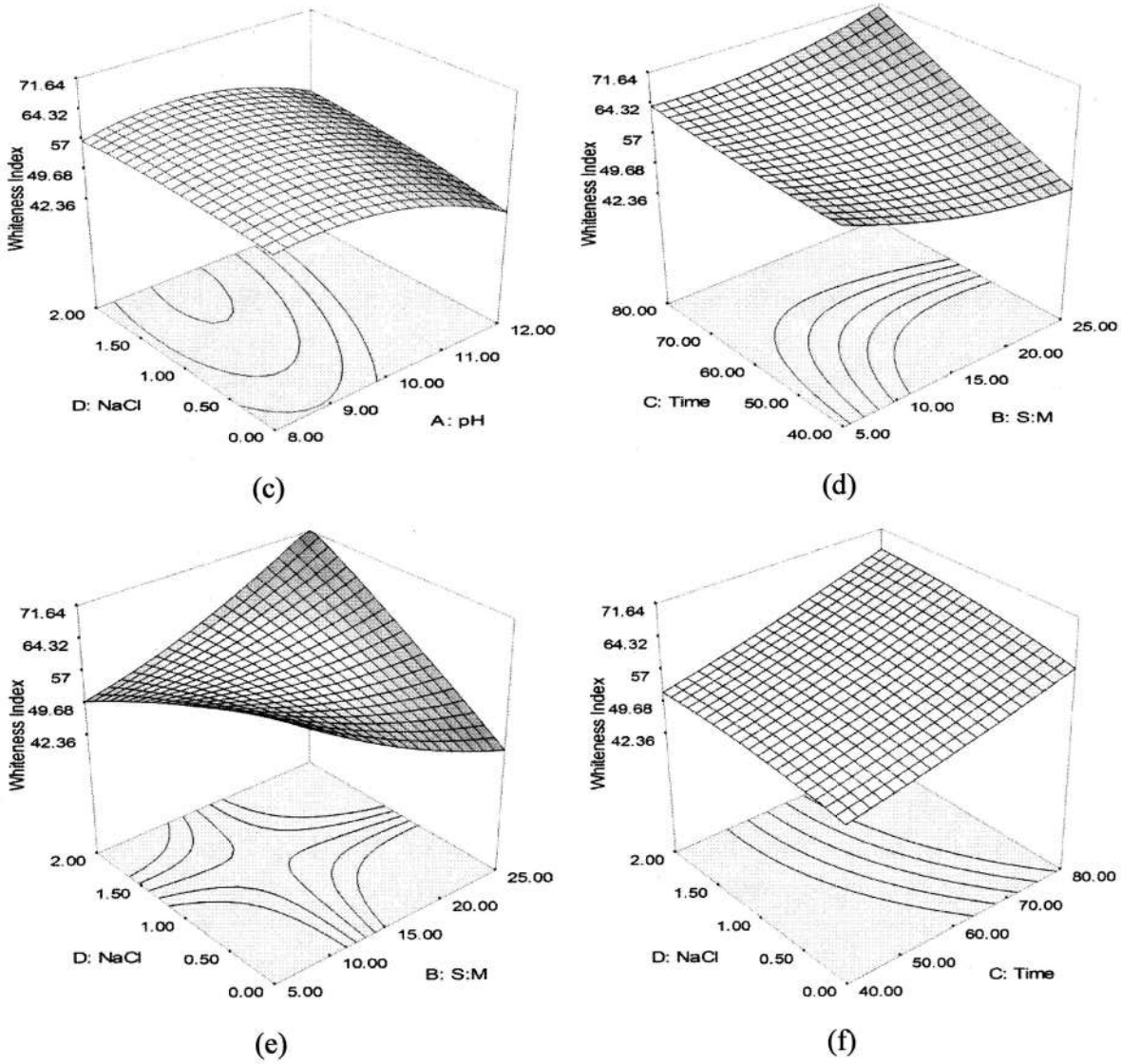


Fig. 5. Effect of interaction of the factors on whiteness index

increase in ratio may be due to solvent which was not enough to disperse and extract protein [28].

NaCl concentration also had a positive effect on extraction of protein. With the increase of NaCl the effect on extraction increases. The phenolic compounds present in rapeseed are bound to the protein by various mechanisms in aqueous medium such as hydrogen bonding [29], covalent bonding [28], hydrophobic interaction [30] and ionic bonding [31]. Extraction of protein in aqueous medium in presence of NaCl prevents the formation of the complexes thus aids in the process of extraction of protein. But excess of the salt interferes with the functional properties such as foaming capacity, foaming stability, emulsion capacity and

emulsion stability of the protein. NaCl has a negative effect on both the emulsion capacity and stability of protein concentrates [32, 33]. NaCl have a positive effect on the foaming capacity at lower concentrations only [34].

Extraction of protein increased with time i.e. the factor also had a positive effect. Increase in time increases the interaction between the solvent and meal which increases the extraction of protein. However, increasing the time beyond 1 h causes frothing of the solution, which is due to denaturation of the protein and coagulation of protein matrix. [35].

Whiteness index (WI) of the extracted protein was also affected by the four factors. The interaction of the factors and their effect on WI index is shown in **Fig. 5**. pH and solvent meal ratio had negative effects while NaCl concentration and time had positive effects. The values of whiteness index ranged between 45 and 66. The results were comparable to Purkayastha et. al. [36]. Decrease in phenolic compounds increases the WI [36]. Negative effect on WI because of increase in pH may be due to the fact that high pH accelerates the protein and phenolic reaction [38, 39]. Positive effect of NaCl is explained by the fact that NaCl prevents the binding of phenols to protein as explained earlier. The longer interaction time helped in breaking protein phenol bonds. Xu and Diosady [17] found that a 72.5% decrease in phenolic compound would cause an increase of WI by 28.4%.

Verification of the results

The capability of the mathematical model obtained to predict the optimization of the response values using the recommended levels of the factors was tested. The values of the responses obtained experimentally were in agreement with the predicted ones (**Table 5**). Thus, it can be said that the model can be successfully used for prediction of optimized responses.

Table 5. Optimum of condition (based on graphical optimisation), predicted and experimental value of the response at that condition

Factors	**Optimum conditions**	
pH	10.47	
Solvent:Meal	14.15:1	
NaCl (M)	0.9	
Time	53.06	
Responses	**Predicted**	**Experimental**
Meal Yield (g)	1.61	1.67±0.12[a]
Protein Content %	75.09	74.21±0.03[a]
Whiteness Index	57.56	57.42±0.03[a]

[a]Mean value of five determinations

Purification of Napin

The presence of napin protein in the eluent was verified electrophoretically from a SDS-PAGE gel. The appearance of a single band confirms the purity of the protein (**Fig. 6**). Comparing with the marker band the molecular size of the protein band is around 14 kDa. Thus, this confirms the

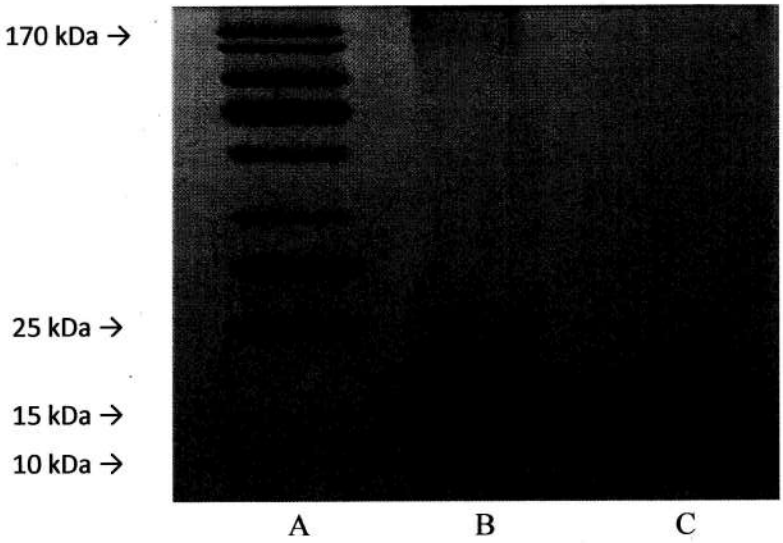


Fig. 6 SDS-PAGE pattern of the eluent. (A) Marker lane (B) Non Reducing conditions (C) Reducing conditions

presence of napin protein [40]. Lane A is the pattern of the protein in non reducing condition while lane B is the pattern of the protein in reducing condition. Under reducing condition the napin molecule breaks down into two bands 9 kDa and 4 kDa. The 4 kDa is not visible as it may have run out of the gel because of its small size.

II. HYDROLYSIS OF THE PURIFIED PROTEIN AND DETERMINATION OF ACE INHIBITORY PROPERTY AND BILE ACID BINDING CAPACITY

1. Introduction

Proteins have biofunctional properties in their native structure. But modification in their structure enhances their functional properties. Enzymatic modification changes the structure of protein to linear thus exposing many hidden amino acid group to the surface. This change in structure helps in enhancing the properties of the proteins. Extensively hydrolysed rapeseed protein obtained by the sequential use of Alcalase and Flavourzyme have found application as food supplements [41]. Partially hydrolyzed rapeseed protein isolates showed improved functional properties such as oil holding capacity, water absorption capacity, whipability, foaming and emulsion stability. The emulsifying properties of rapeseed protein

hydrolysates make them a potential ingredient in food formulations. Bandyopadhyay et al. [42] reported that protein hydrolysates produced from defatted Indian rice bran meal showed better functional properties such as foam capacity, foam stability, emulsion stability than the protein isolate.

Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase, which participates in regulation of blood pressure by virtue of two different reactions, i.e. conversion of inactive peptide Angiotensin I into powerful vasoconstrictor Angiotensin II and inactivation of the vasodilator peptide Bradykinin [45]. Many synthetic ACE inhibitors including Captopril, Enalapril, Lisinopril and others are available for clinical use [46]. ACE inhibitors are accepted well by most patients, but some undesirable side effects may occur such as cough, lost of taste, renal impairment and angioneurotic oedema [47]. However, peptides generated by enzymatic hydrolysis are shown to have antihypertensive properties. Though the peptide inhibitors are much less potent than synthetic ones, however, they have an advantage of having no known side effects.

2. Materials and methods

The purified napin protein was taken as the substrate. The two hydrolyzing enzymes Alcalase 2.4L and Flavourzyme 500L were used. Both the enzymes were obtained from Sigma.

Enzymatic hydrolysis of napin protein

Enzymatic hydrolysis was performed in a reaction vessel equipped with a stirrer, thermometer and pH electrode. A protein slurry at 5% was prepared. The pH and temperature of the slurry was adjusted at 8 and 50°C prior to hydrolysis. Hydrolysis was done separately for the two enzymes. Enzymes were added at a concentration of 4%. The pH of the reaction was maintained at a constant value by addition of either 0.5 M NaOH or 1 M HCl. The hydrolysis was stopped at regular intervals by heating the samples at 95°C for 15 min. The pH of the sample was brought down to 4 to precipitate the unhydrolysed proteins and large peptides. The sample was then centrifuged at 12,000g to remove the precipitate. The clear supernatant was used for further studies.

Determination of degree of hydrolysis

The degree of hydrolysis was determined according to the method by Tsumura et al. [43]. An aliquot of 0.5ml of the supernatant after hydrolysis was taken. To it an equal volume of 0.44 mol/l trichloroacetic acid (TCA) was added. The mixture was incubated for 30 min. at room temperature. The mixture was then centrifuged at 12,000g (Sigma Zentrifugen) for 15 min. The protein content of the obtained 0.22 mol/l TCA-soluble fraction and the supernatant of reaction mixture were each determined by Lowry [19], using bovine serum albumin as the standard protein. The DH value was calculated as the ratio of 0.22 mol/l TCA-soluble protein to total protein in the supernatant of reaction mixture, expressed as a percentage.

Determination of ACE activity

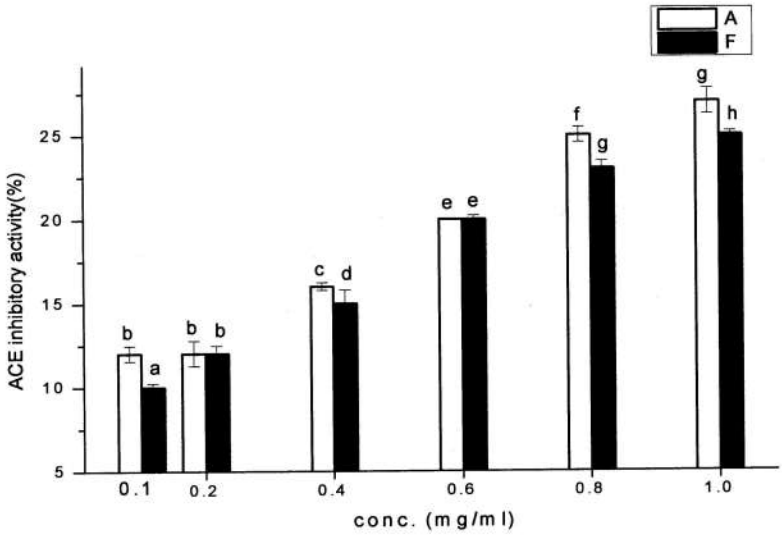
ACE activity was determined according to the method of Cushman and Cheung, 1971 [48]. The substrate (HHL), protein sample, and ACE solutions were prepared using 50 m*M* sodium borate buffer, pH 8.3 containing 0.5 *M* sodium chloride. An aliquot (50 µL) of the protein hydrolysate (sample) or 50 µL of borate buffer (control) was mixed with 50 µL of ACE solution (25 mU/mL) and incubated at 37 °C for 5 min. An aliquot (150 µL) of the substrate solution was then added, and incubation continued for 1 h. The reaction was stopped by addition of 250 µL of 1 *M* HCl solution followed by addition of 1.5 mL of ethyl acetate to extract the hippuric acid. After mixing for 1 min, the mixture was centrifuged at 1000 × *g* for 5 min and 1 mL of the upper layer transferred into a glass test tube. The ethyl acetate was evaporated to dryness using a heating block and the residue dissolved in 3 mL of distilled water; concentration of hippuric acid was determined at 228 nm. The concentration of protein sample that inhibited 50% of ACE activity was defined as the IC_{50} value.

3. Results and discussion

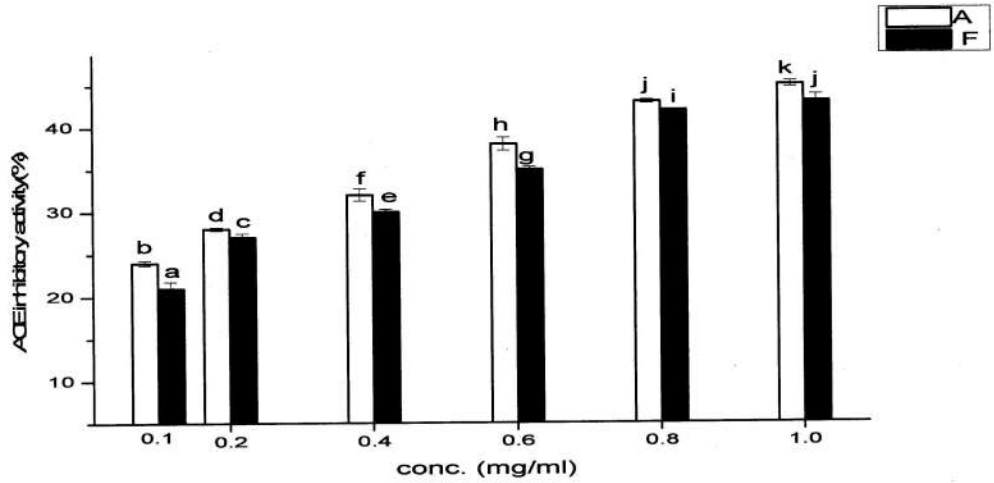
Alcalase is an alkaline non-specific serine protease from *Bacillus subtilis* that initiates the nucleophilic attack on the peptide bond through a serine residue at the active site; it catalyzes the hydrolysis of proteins and peptide amides. Flavourzyme is a protease from *Aspergillus oryzae* that catabolizes proteins by hydrolysis of peptide bonds. It contains both endoprotease and exopeptidase activities. The degree of hydrolysis with relation to time is seen in **Table 6**. The degree of hydrolysis of the individual enzyme with time is comparable to Villanueva et.al. [44].

Table 6. Enzymatic hydrolysis of napin protein with alcalase and flavourzyme

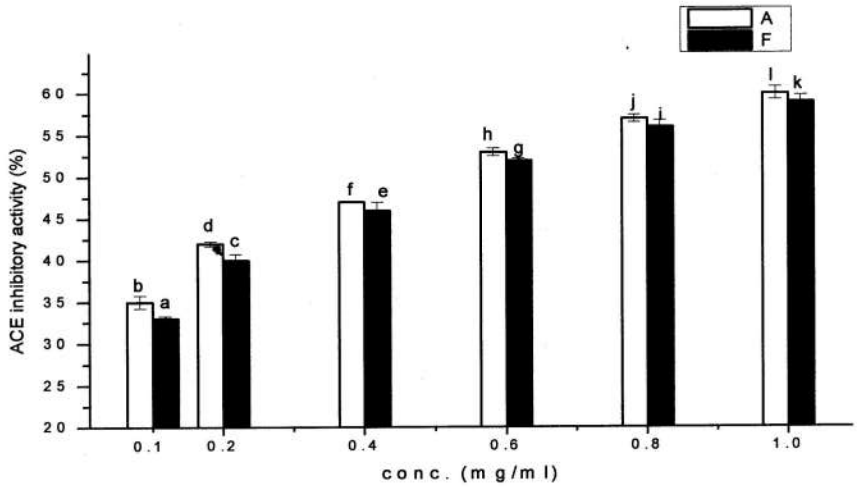
Time (min)	Degree of hydrolysis (%)	
	Alcalase	Flavourzyme
10	3.76±0.07	4.08±0.02
20	17.12±0.01	16.72±0.11
30	20.23±0.05	20.11±0.01


A
F
ACE inhibitory activity(%)
5
10
15
20
25
b
a
b
b
c
d
e
e
f
g
g
h
0.1
0.2
0.4
0.6
0.8
1.0
conc. (mg/ml)

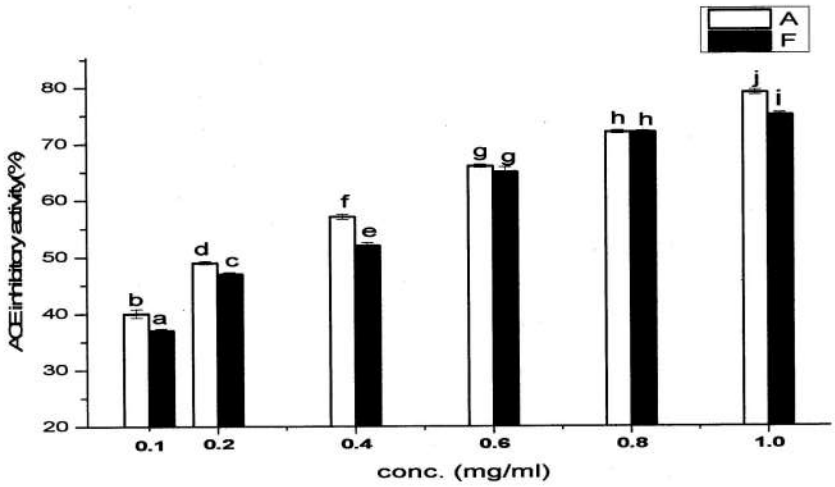
(i)


A
F
10
20
30
40
b
a
d
c
f
e
h
g
j
i
k
j
0.1
0.2
0.4
0.6
0.8
1.0
conc. (mg/ml)

(ii)



(iii)



(iv)

Fig. 7. ACE inhibitory property shown by napin hydrolysates at (i) at 30 min hydrolysis, (ii) at 60 min hydrolysis, (iii) at 90 min hydrolysis and (iv) at 120 min hydrolysis

The ability of the hydrolysates increases with increase in concentration of the hydrolysates whereas the potency of the hydrolysates increases with decrease in the molecular length as shown in **Fig 7**. The ACE activity results were found similar to Jeon et. al., [49] and Aluko

and Monu [50]. The ACE inhibitory activities of the peptides are determined by the composition of the peptides. Increase aromatic groups increase the potency of the peptides. Thus, more hydrolysis exposed the aromatic group with increase in inhibitory activity of the peptides [51].

III. DETERMINATION OF BILE ACID BINDING CAPACITY

1. Introduction

Bile acids are steroid acids found predominantly in the bile of mammals and other vertebrates. Different molecular forms of bile acids can be synthesized in the liver by different species. Bile acids are conjugated with taurine or glycine in the liver, forming bile salts [52].

Primary bile acids are those synthesized by the liver. Secondary bile acids result from bacterial actions in the colon. In humans, taurocholic acid and glycocholic acid (derivatives of cholic acid) and taurochenodeoxycholic acid and glycochenodeoxycholic acid (derivatives of chenodeoxycholic acid) are the major bile salts in bile and are roughly equal in concentration. The conjugated salts of their 7-alpha-dehydroxylated derivatives, deoxycholic acid and lithocholic acid, are also found, with derivatives of cholic, chenodeoxycholic and deoxycholic acids accounting for over 90% of human biliary bile acids [53].

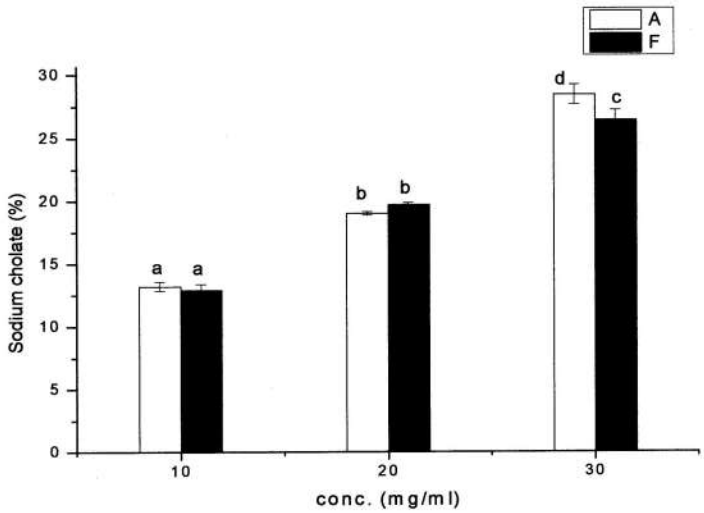
Bile acids, synthesized in the liver from cholesterol, help the emulsification and absorption of fats by micelle formation. Increased dietary fat and cholesterol are known to raise the level of total cholesterol. Cholesterol first solubilizes in bile salt micelles and then are absorbed in the body [54]. Many studies have reported that peptides produced from soy protein, casein protein, lupin protein have high bile acid-binding capacity could inhibit the reabsorption of bile acid in the ileum or decrease the micellar solubility of cholesterol in small intestinal epithelial cells and decrease the blood cholesterol level [55].

2. Materials and methods

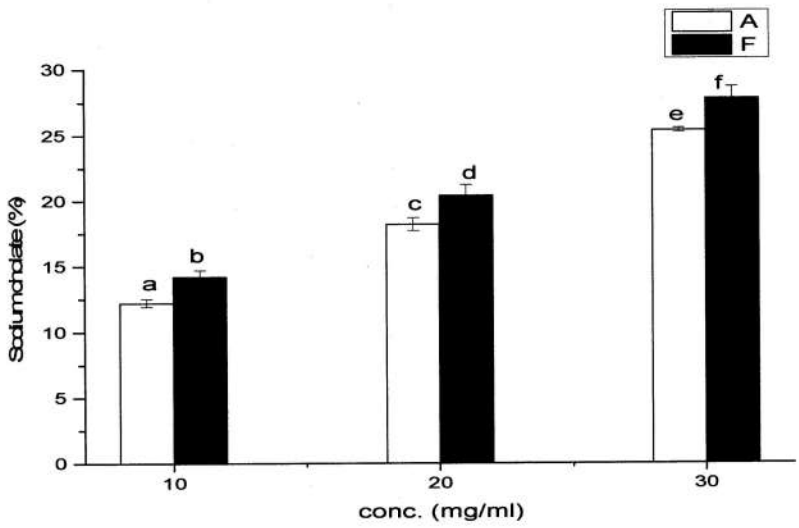
The in vitro bile acid-binding assay was carried out according to the method of Yoshie-Stark and Wäsche [56]. Napin protein hydrolysates at the said concentration were mixed with 1.5 mM sodium cholate/sodium deoxycholate solution in 100 mM sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 2 h. Samples were centrifuged at 3000xg and the supernatants were collected and analyzed for bile acid using a spectrophotometer at 530 nm. Cholestyramine resin, a bile acid-binding and cholesterol lowering drug, was also evaluated for its bile acid-binding ability.

3. Results and discussion

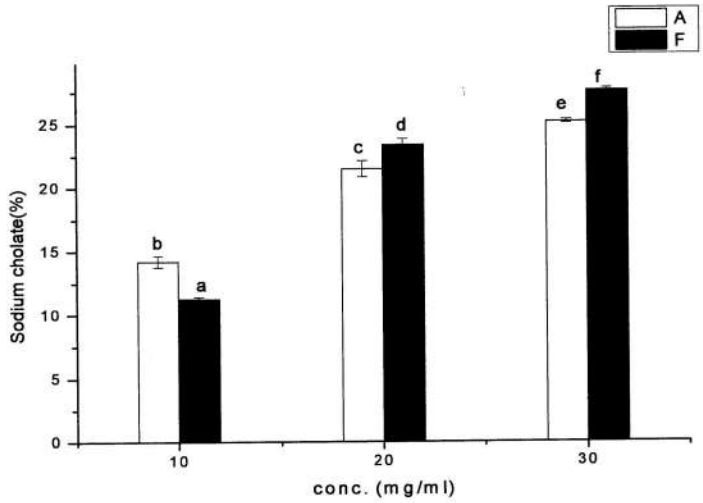
Bile acid binding capacity of napin protein hydrolysates was determined with sodium cholate and sodium deoxycholate. Napin protein after purification was hydrolysed with Alcalase and Flavourzyme for 30, 60, 90 and 120 min. Since the hydrolyzing property of both the enzymes are different so different types of peptides are produced after hydrolysis. The degree of hydrolysis did not seem to affect the binding capacity; however the binding capacity was dose dependent.



(i)



(ii)



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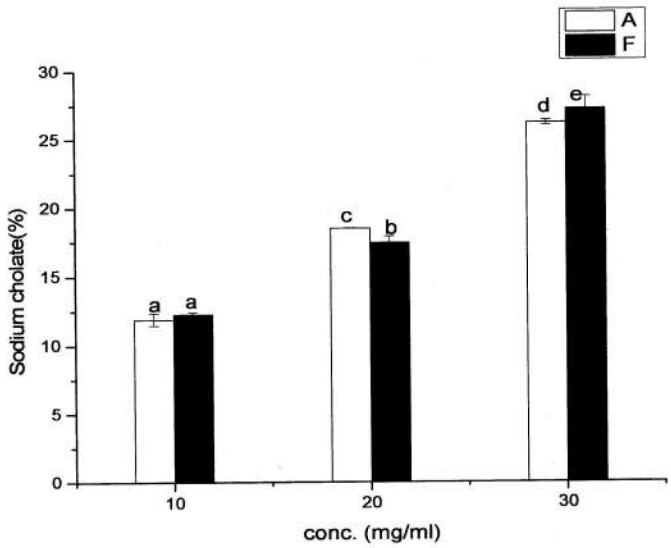
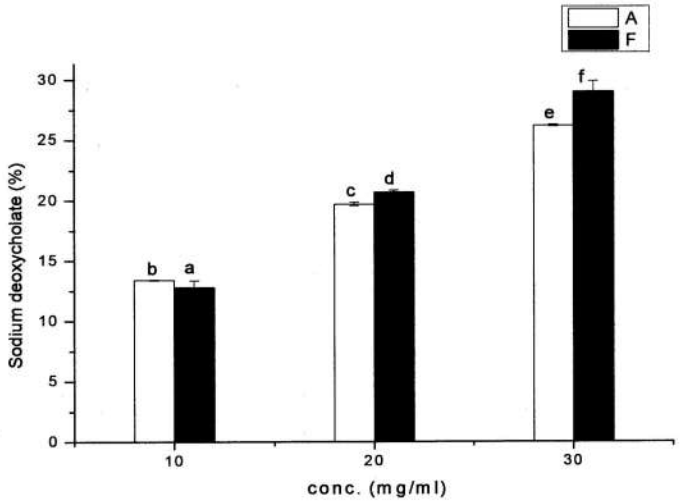
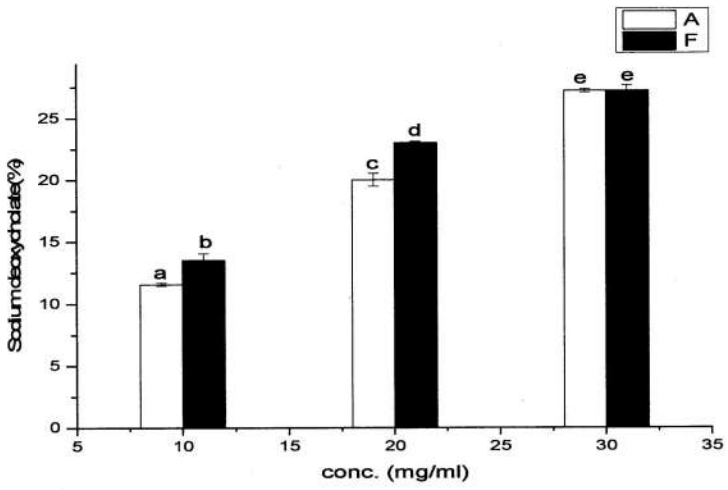


(iv)

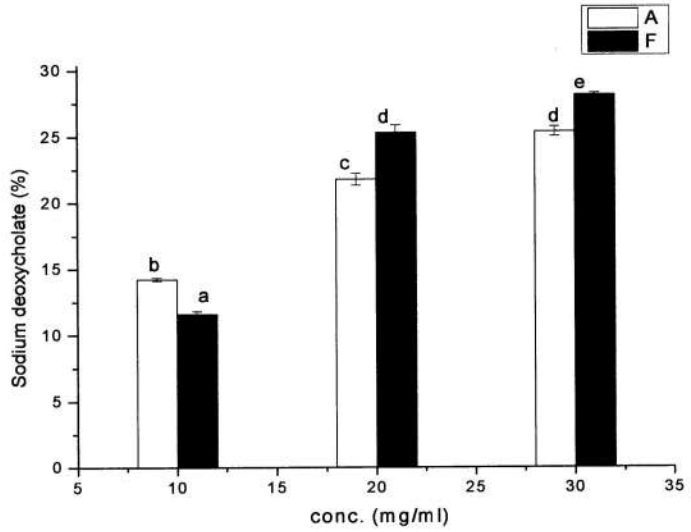
Fig 8. Sodium cholate binding capacity shown by napin hydrolysates after hydrolysis for (i) 30 min, (ii) 60 min, (iii) 90 min and (iv) 120 min.


A
F
Sodium deoxycholate (%)
conc. (mg/ml)
a
b
c
d
e
f

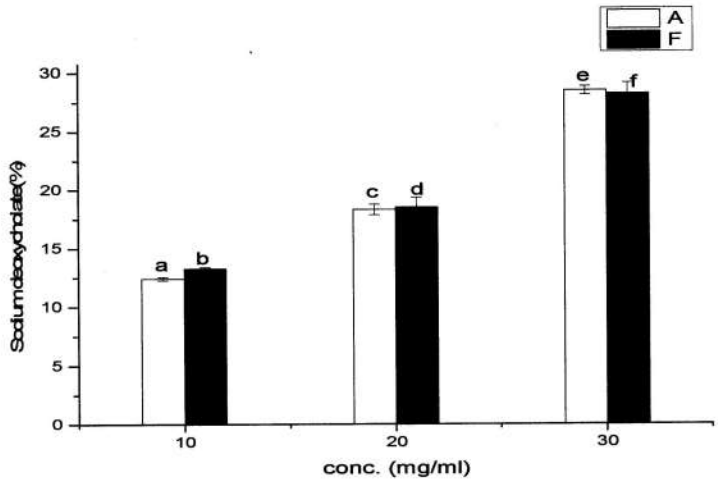
(i)


A
F
conc. (mg/ml)
a
b
c
d
e
e

(ii)



(iii)



(iv)

Fig 9. Sodium deoxycholate binding capacity shown by napin hydrolysates after hydrolysis for (i) 30 min, (ii) 60 min, (iii) 90 min and (iv) 120 min.

The binding capacity ranged from 11.89 % to 28.4 % for sodium cholate and 11.56% to 29.0 % (**Fig.8, Fig.9**). Flavourzyme hydrolysed samples showed better binding capacity than those produced with Alcalase hydrolysed samples.

IV. DETERMINATION OF EMULSIFYING ACTIVITY INDEX (EAI) AND STABILITY INDEX (ESI) AND FOAMING CAPACITY (FC) AND STABILITY (FS)

1. Introduction

Emulsifying activity index (EAI) and emulsion stability index (ESI) are two important functional characteristics of proteins that affect the behavior of various industrial products, including adhesives, cosmetics and packing material. EC and ES are critical parameters that affect the choice of a protein for use in an industrial process. Proteins can reduce tension at the water–oil interface and help prevent coalescence [57]. A protein's stabilizing effect in an emulsion comes from the membrane matrix that surrounds the oil drop and prevents its coalescence [58].

Another practical application of proteins in industrial production comes from their ability to generate foam. Foaming capacity (FC) and foam stability (FS) are important parameters in the characterization of the functional properties of proteins. Proteins must be highly soluble in water, flexible and form part of a cohesive film at the water–air interface to ensure good foam formation [59]. The film should possess sufficient viscosity to maintain stability and prevent rupture and subsequent coalescence.

2. Materials and methods

Dephenolization

The defatted meal was dephenolized using a solvent of methanol and acetone in ratio of 1:1. The meal to solvent ratio was kept at 1:20. The meal was stirred (magnetic stirrer) in the solvent for one hour and then centrifuged (Zentrifugen, Sigma Aldrich) at 8000g for 10 min. The dephenolized meal was collected and used for extraction of protein.

Extraction of protein isolate from rapeseed meal

Defatted meal was dispersed in solvent containing 0.5M Tris buffer, 0.25% sodium sulfite, 0.9M NaCl. The pH was maintained at 10.5 and the solvent to meal ratio was taken at 14:1 (v/w). The parameters and the levels were taken according to previously optimized experiment using response surface methodology. The mixture was stirred for 53 min (Spinot, Tarsons) and then centrifuged at 8000 g for 20 min. The supernatant was collected and the above process was repeated two more times with half the volume of solvent. All the

supernatant were pooled. Protein was then precipitated by bringing the pH to 3.5. The precipitated protein was then centrifuged again as above. The residue was then collected and freeze dried until further use.

Enzymatic hydrolysis of extracted protein

Enzymatic hydrolysis was done with both Flavourzyme and Alcalase separately. The protein content of the precipitate was determined by Lowry method and a suspension of the rapeseed protein isolate (RPI) at a concentration of 4% was prepared. The enzymes were added at 5% (w/v). Hydrolysis with Alcalase was done at 60°C and pH 8.5 while hydrolysis with Flavourzyme was done at 50°C and pH 7. To stop the hydrolysis, the reaction mixture was immersed in water bath at 95°C for 5 min. The hydrolysed mixture was used for preparation of mayonnaise.

Emulsifying activity index (EAI) and emulsifying stability index (ESI)

EAI and ESI were determined by the turbidimetric method of Pearce and Kinsella [60]. Protein samples (0.1%, w/v) were prepared and adjusted to pH 3 and 7 (0.1M HCl or NaOH) with final volume 10 ml. A 50 µl of emulsion was pipetted from the bottom of the container into 5 ml of 0.1% sodium dodecyl sulfate (SDS) (w/v) solution immediately (0 min) and 10, 30 and 60 min after homogenization. Absorbance of the SDS solution was measured at 500 nm (UV Vis Spectrophotometer, Aquarius 7000, Cecil). Absorbance at 0 time was expressed as EAI of protein, and ESI was calculated by the equation:

$$\text{ESI (min)} = \frac{T_0 \times t}{T'}$$

where, T_0 =the turbidity at 0 min after homogenization, T' = the change in turbidity between 0 and 10 min, t was interval time between 0 and 10 min.

Foaming capacity (FC) and foaming stability (FS)

FC and FS was done as described by Chau et al [61]. These properties were evaluated from suspensions (25 ml) of sample (1.5% w/v) adjusted to pH 3 and 7. Suspensions were mixed and beaten at low speed. They were then transferred to a 50 ml graduated cylinder. Foam capacity (FC) was expressed as the increase in percentage of foam volume measured after 30 seconds. Foam stability (FS) was expressed as residual foam volume at 10, 30 and 60 minutes after blending.

Coding of samples

The sample codes are presented in **Table 7**.

Table 7. Coding of the samples according to different treatment and time

Sample	Code
Untreated sample	U
Untreated sample hydrolysed with Alcalase for 10 min	UA1
Untreated sample hydrolysed with Alcalase for 20 min	UA2
Untreated sample hydrolysed with Alcalase for 30 min	UA3
Untreated sample hydrolysed with Flavourzyme for 10 min	UF1
Untreated sample hydrolysed with Flavourzyme for 20 min	UF2
Untreated sample hydrolysed with Flavourzyme for 30 min	UF3
Dephenolised sample	D
Dephenolised sample hydrolysed with Alcalase for 10 min	DA1
Dephenolised sample hydrolysed with Alcalase for 20 min	DA2
Dephenolised sample hydrolysed with Alcalase for 30 min	DA3
Dephenolised sample hydrolysed with Flavourzyme 10 min	DF1
Dephenolised sample hydrolysed with Flavourzyme 20 min	DF2
Dephenolised sample hydrolysed with Flavourzyme 30 min	DF3

3. Results and discussion

Emulsifying activity index (EAI) and emulsifying stability index (ESI)

EAI and ESI of the hydrolysates were compared (**Fig. 10, 11, 12**). Hydrolysed samples had better EAI and ESI. EAI and ESI of the untreated samples were less than the dephenlised samples. EAI of the untreated hydrolysed samples ranged from 0.42 to 0.84 while dephenolised hydrolyseates had EAI of 1.29 to 1.51. Samples hydrolysed with alcalase had better EAI and ESI than those hydrolysecwith flavourzyme. Alcalase is non-specific serine protease having endoprotease activity while Flavourzyme had both endo and exoprotease activity. Thus difference in properties may be because different kinds of peptides are produced. Similar results were obtained by Kanu et al [62]. Improvement of emulsifying and

foaming properties with partial hydrolysis of protein has been reported. However, excessive hydrolysis causes loss of functional properties [63]. The increase in EAI may be due to exposed hydrophobic groups which enhanced the interaction between proteins and lipids. Emulsifying property also depends on the initial solubility of protein as more dissolved protein would be in interface between oil and water phase [64]. Dephenolised samples had better EAI as phenolic acid bound to peptides hampers functional properties [65].

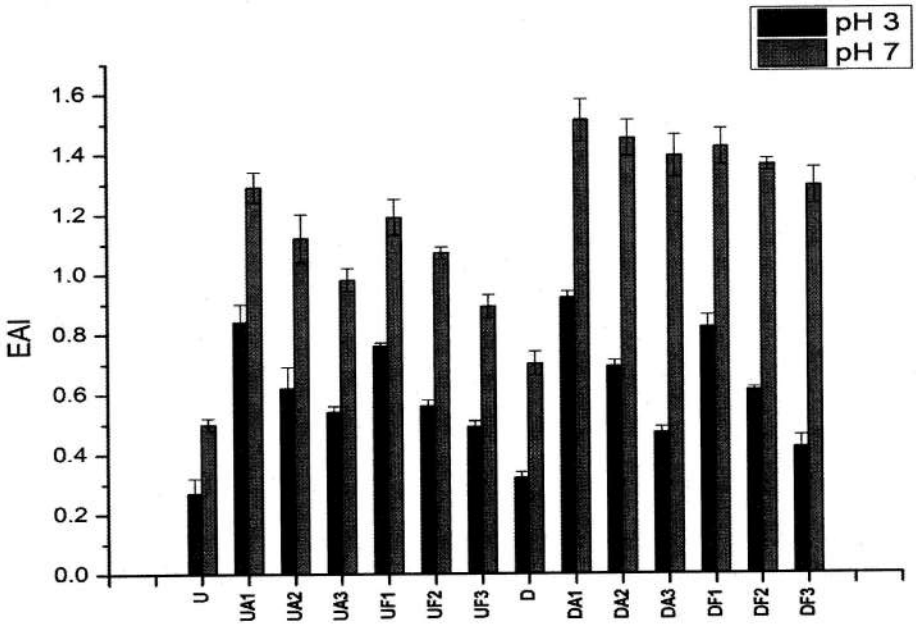


Fig 10. Emulsifying activity index of the samples at pH3 and pH 7

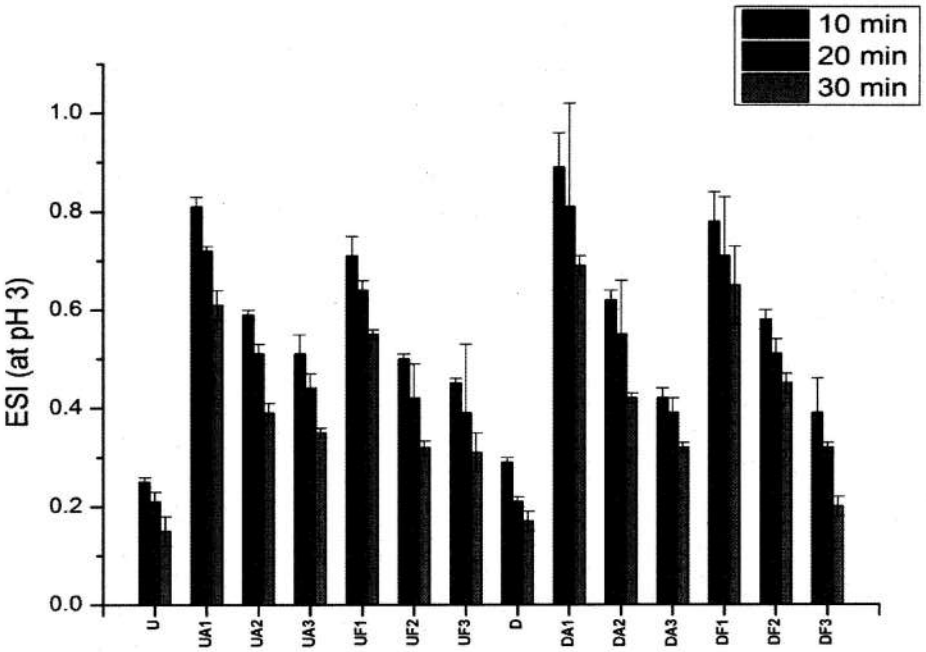


Fig 11. Emulsifying stability index at pH 3 at 10min, 30 min, 60 min.

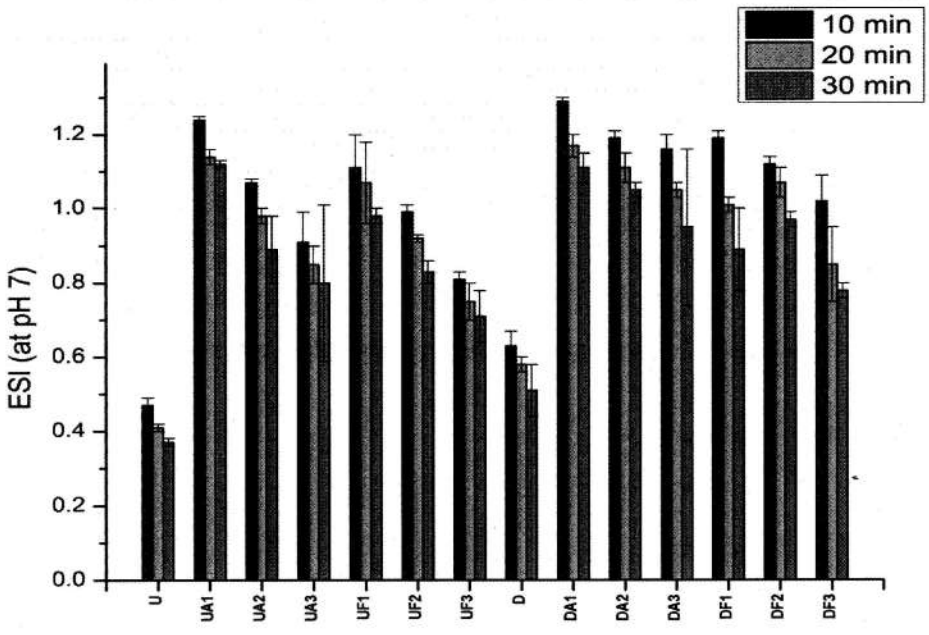


Fig 12. Emulsifying stability index at pH 7 at 10 min, 30 min, 60 min.

Foaming capacity (FC) and foaming stability (FS)

FC and FS of the hydrolysates were compared at both pH 3 and pH 7 (**Fig. 13, 14, 15**). Hydrolysates had better FS and FC than the unhydrolysed samples. FS and FC were better at pH 7 than at pH 3. FS at pH 3 ranged from 91% to135% whereas at pH 7 FS ranged from 175% to 210%. Dephenolised samples had better FS as phenolic acid hampers functional properties of the peptides [63]. Foaming properties are determined by many factors such as balance of hydrophilic and hydrophobic gruops and net charge of the peptides [66].

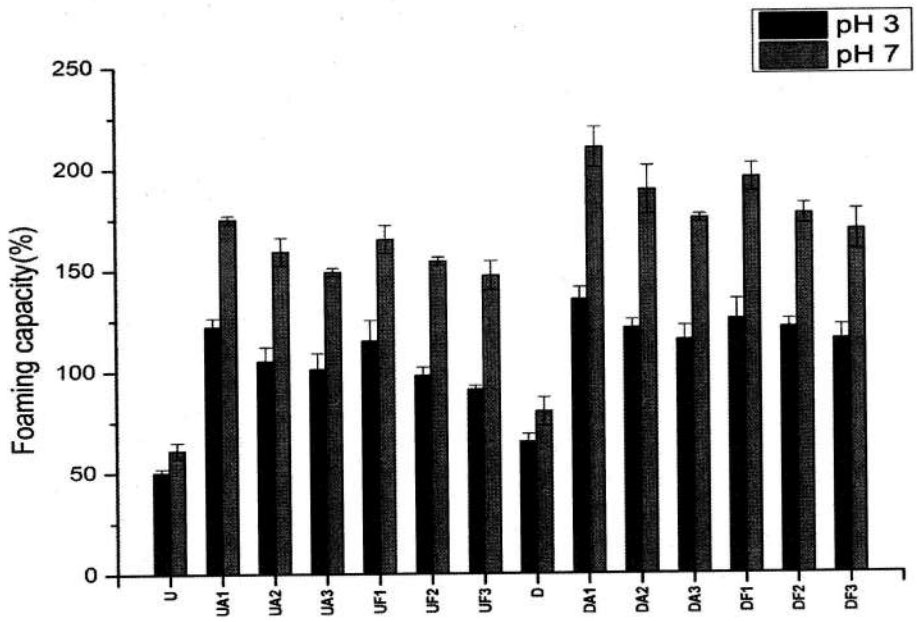


Fig. 13. Foaming capacity of the samples at pH 3 and pH 7

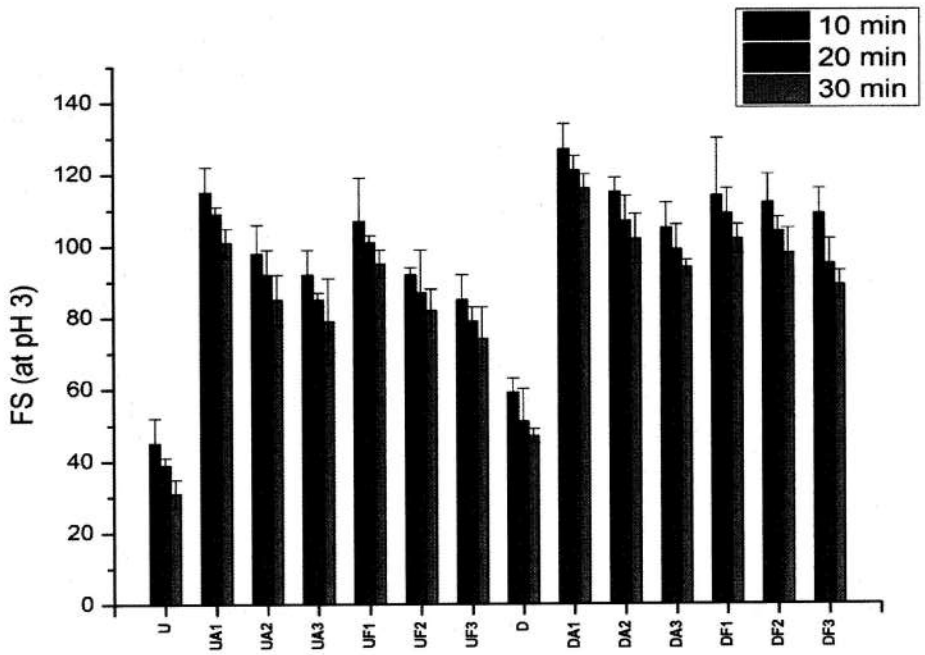


Fig. 14. Foaming stability of the samples at pH 3

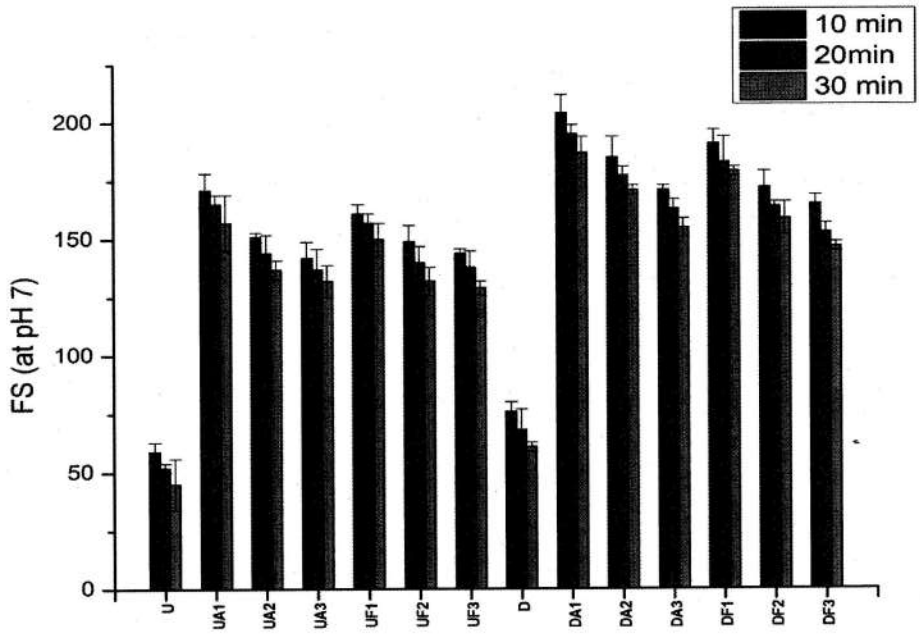


Fig. 15. Foaming Stability of the samples at pH 7

CONCLUSION

Studies on the biofunctional and technofunctional properties of the peptides from purified napin protein have not been reported. Through this study, the central composite design of Response Surface Methodology was successfully modeled to obtain protein isolate with optimized properties. Purification of the extracted protein through various chromatographic steps resulted in purified napin protein. The single band confirmed the purity of the protein. The purified protein was then enzymatically hydrolysed with Alcalase and Flavorzyme. The hydrolysed peptides showed ACE inhibitory activity and bile acid binding capacity. Upon hydrolysis the emulsifying activity index, emulsifying stability index, foaming capacity and foaming stability increased manifold. Dephenolisation further improved the biofunctional and technofunctional properties. Thus, hydrolysis of napin proteins yields peptides that can be explored for utilization for their biofunctional and technofunctional properties.

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26/9/2016

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(P.I.) 26/9/2016

Principal Investigator
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GFR 19 – A

(See Rule 212 (1))

Utilization Certificate

Sl.	Letter No. & Date	Amount
1.	**No. DRL/1205/TC Dated 12/11/2013**	(₹) **2,68,000/-**
2.	**No. DRL/1205/TC Dated 12/11/2013**	(₹) **2,32,000/-**
		(₹) **5,00,000/-**

Certified that out of ₹ **5,00,000/-**of Grants- in-aid sanctioned during the years 2013-2014 and 2016-2017 in favour of Registrar, Tezpur University, Napaam-784028, Assam from Defence Research Laboratory, Tezpur letter No. **No. DRL/1205/TC dated 12/11/2013** given in the margin, a sum of ₹ **5,00,000/-** has been utilized for the purpose of salary, consumables chemicals and miscellaneous expenditure for which it was sanctioned and that **NIL** balance remain unutilized.

1. Certified that I have satisfied myself that the conditions on which the grants- in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of check s exercised

1. Accounts audited by qualified Chartered Accountant appointed by this University as Internal Auditor
2. All the glassware, chemicals, raw materials, etc., purchased from the grant are entered in the stock book.

Charu Lata Mahanta 31/8/2016
Signature of Principal Investigator with date
Principal Investigator
Dept. of Food Engg. & Tech.
Tezpur University
Tezpur -784 028, Assam

31/8/16
Signature of Registrar/ Accounts Officer
Finance Officer
Tezpur University

31/8/16
Signature of Head of the Institute
Registrar
Tezpur University

Statement of Expenditure (2016-2017)

S. No.	Particulars	Grant Sanctioned (₹)	Amount released in installment(₹)		Expenditure (₹)				Total Expenditure (₹)
			1st	2nd	17/1/2014 to 31/3/2014	1/4/2014 to 31/3/2015	1/4/2015 to 31/3/2016	1/4/2016 to 13/7/2016	
1	Staff salary @ ₹14000/- p.m.	3,36,000/-	2,68,000/-	2,32,000/-	19,500	98,000	70,000	1,47,226	3,34,726
2	Consumables, store, chemicals	1,50,000/-			-	72,749	5,367	86,258	1,64,374
3	Miscellaneous	14,000/-			-	-	900	-	900
Grand Total		**5,00,000**			**19,500**	**1,70,749**	**76,267**	**2,33,484**	**5,00,000**

Total grant sanctioned : ₹ 5,00,000/-
Total grant received: ₹ 5,00,000/-
Total grant utilized: ₹ 5,00,000/-

Charu Lata Mahanta
Name and Signature of Principal Investigator

Date: 31/8/2016

Principal Investigator
Dept. of Food Engg. & Tech.
Tezpur University
Tezpur -784 028, Assam

31/8/16
Signature of Competent financial/ audit authority with seal

Date:

Finance Officer
Tezpur University