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Optimization of antioxidant activity of buffalo casein hydrolysates using response surface methodology

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Present study mainly explores the antioxidative function derivable from casein hydrolysates. Flavourzyme and alcalase were used for casein hydrolysis under varying time at their optimum pH and temperature. The effects of enzyme concentration and reaction time of sequancial treatment of alcalase and flavourzyme on degree of hydrolysis and antioxidant activity of casein hydrolysates were investigated. Response surface methodology (RSM) was employed to optimise the reaction conditions. Enzymatic hydrolysis with sequencial alcalase and flavourzyme could eanhance the antioxidant activity of casein hydrolysates effectively and the increase of antioxidant activity could be controlled by regulation of the reaction conditions. The quadratic model was used for selecting optimal reaction conditions of casein hydrolysates with higher antioxidant activity. Under the range of conditions studied, maximum activity found with alcalase concentration 3.0 nkat/mg protein, flavourzyme concentration 2.5 nkat/mg protein, alcalase digestion time 4.5 h, and flavourzyme digestion time 6.0 h with desirability 0.94.The correlation was noticed between enzyme activity and degree of hydrolysis.

Keywords: ***Alcalase, Flavourzyme, Casein hydrolysates, Antioxidative activity*.**

Introduction:

Biological antioxidants are defined as substances present at relatively low concentrations that can delay or inhibit oxidation of a substrate (Frankel and Meyer 2000). Commonly, bioactive peptides composed of 2 to 9 amino acids (Kitts and Weiler, 2003). However, some of the peptides containing more than 20 amino acids can still have biological activity (Korhonen and Pihlanto, 2003). Enzymatic hydrolysis is an effective means of liberating bioactive peptides from protein sequence (Korhonen and Pihlanto 2006). Enzymatic hydrolysis cause three distinct effects: Decrease in molecular weight, increase in the number of ionizable groups, exposure of hydrophobic groups (Dinakar Panyam and Arun Kilara., 1996). Milk protein derived bioactive peptides are claimed to be health enhancing components that can be used to reduce the risk of disease or to enhance a certain physiological function. Clausen *et al.* (2009) demonstrated that caseins were quantitatively the major radical scavenging species in milk. Kumar *et al.* (2010) worked on

whole casein and its fractions (α_{s1}-, β-, κ-) both from buffalo and cow milk and their radical scavenging activity was evaluated and found that , buffalo whole casein had greater DPPH IC_{50} compared to cow whole casein. Based on DPPH radical scavenging activity significant ($p<0.05$) difference between casein and its fractions both from cow and buffalo was observed. The radical scavenging activity of different casein fractions both from buffalo and cow milk followed the order of α_{s1}- >β- >κ-casein. For this reason, this study was conducted to check the effect of sequancial treatment of alcalase and flavourzyme to generate buffalo casein hydrolysates for maximum degree of hydrolysis, antioxidant activity and to reduce the overall bitterness profile of hydrolysates so that they can be incorporated in functional foods.

MATERIALS AND METHODS:

Material

The enzymes used were Flavourzyme 1000 L, Alcalase 2.4 L (Novozymes) were gift samples from M/s. ABTS [2, 2'-azinobis (3 ethyl beizothiazoline)-6-sulfonic acid], Albumin (Bovine), caffeine and Ciocalteau's phenol reagent was procured from Sigma Aldrich. Boric acid, Copper sulphate, Ferric chloride, Sodium hydroxide and Sodium potassium tartarate, Sodium thiosulphate, Hydrochloric acid, Glacial acetic acid was procured from Qualigens Fine Chemicals, Glaxo India Ltd. Potassium dichromate was procured from s.d. Fine- chem Ltd., Mumbai, India).

Methods:

Casein was separated by isoelectric precipitation of buffalo casein, the precipitates were filtered through muslin cloth, wash the same thrice with distilled water and the wet casein was collected, and dispersed in distilled water and dissolved by adding 1N NaOH solution with thorough stirring till it reached pH 7.0. Casein solution was lyophilized and stored at−20 °C for further use.

Enzymatic hydrolysis of casein:

Buffalo casein was hydrolyzed using substrate solutions (2.5g/l on protein basis) in distilled water ,followed by stirring for 15 min at room temperature .The pH was adjusted to 7.5 using 0.5 M HCl. Based on preliminary laboratory trials, the minimum and maximum levels of different variables (Enzyme concentration ,and time of hydrolysis) were selected. The central model values were 2 nkat/mg protein alcalase, 1.65 nkat/mg protein flavourzyme, 3 h alcalase digestion time and 4 h sequential flavourzyme time.

Experimental design:

In the present investigation, response surface methodology (RSM) was adopted for optimization of the conditions of hydrolysis. Central Composite Rotatable Design (CCRD) with Four independent variables was used for the experiments. The process parameters used in the experiment were Alcalase enzyme unit (X1), flavourzyme enzyme unit (X2), Alcalase Digestion time (X3), Sequential Flavourzyme Digestion time (X4). Based on preliminary laboratory trials, the minimum and maximum levels of different variables were selected. The levels of the four independent variables were coded as $-\alpha$, -1, 0, +1, $+\alpha$ (Table 1). In all, a total of 29 runs as per D-optimal design of RSM were prepared using different combination of the independent variables enlisted in table 2

Evaluation of hydrolysates:

After every hydrolysis condition the hydrolysates were micro filtered using 0.45µm cutoff membrane and the filtered hydrolysates solution was lyophilized, 3kDa fractions also collected from optimized conditions. Hydrolysate solution (10 mg/ml) was prepared by mixing lyophilized hydrolysates in distilled water, followed by stirring for 2-3 min at room temperature.

Degree of hydrolysis was evaluated by OPA method given by Parmar *et al.* (2015). Antioxidant activity was evaluated by in vitro non cellular assay as ABTS by Parmar *et al*. (2015).

Sensory (Bitterness) evaluation of hydrolysates:

Hydrolysates obtained at optimized conditions using different enzymes were evaluated for their bitterness compared to corresponding caffeine concentration from 1mM-10mM in double distilled water.

The sensory evaluations of hydrolysates were performed by five panelists. Initially they were trained for evaluation of bitterness using caffeine solution of concentration (1-10 mM), with the mouth rinse using distilled water after every sample. Then the samples of hydrolysates were assessed for their bitterness isotonic to corresponding caffeine concentration.

Separation of fractions of antioxidant peptides by Analytical RP-HPLC

Hydrolysates obtained at optimized conditions using different enzymes concentration were precipitated with solvent A. The acidic mixture was centrifuged for 20 min at 5,000 rpm. The supernatant, containing TFA soluble peptides were filtered (0.2 µm).The separation was performed using Resource RPC (3 ml) using AKTA purifier with conditions as solvent A 100% water + 0.1% TFA, Solvent B 70% Acetonitrile + 0.01% TFA, injection volume 1ml and detection wavelength 214 nm. Several runs were performed to collect adequate material and were dried by speed vac lyophilize (Henil). The dried fractions were dissolved in distilled water and assessed for its antioxidant activity.

Result and discussion:

Assessment of fitting of models for design variables:

The experimental design and the results of central composite design for optimization of the process conditions to obtain antioxidative hydrolysates from buffalo sodium caseinate with sequential treatment of alcalase and flavourzyme as presented in table 1. The response results were analyzed using design expert 7.0.0, As can be seen in table 2 the relationship between four independent variables (alcalase concentration, flavourzyme concentration, alcalase digestion time, sequencial flavourzyme digestion time) and important process responses (degree of hydrolysis and antioxidant activity) were analyzed using central composite design. Significance model terms are desired to obtain a good fit in a particular model. The probability (p) values were used to identify the effect of independent variables on antioxidant activities and degree of hydrolysis as listed in Table 2. The ANOVA and its R^2 values for degree of hydrolysis and ABTS activity of buffalo sodium caseinate hydrolysates were determined to be 0.89 and 0.86 respectively, which showed that the regression models well defined the true behavior of the system.

Table: 1 The central composite rotatable design (CCRD) for casein hydrolysate production using commercial enzymes

Run	Independent variables				Dependent variables	
	X1	X2	X3	X4	DH (%)	ABTS (µM)
1	1	2.5	4.5	6	32.14	1760.77
2	3	0.8	4.5	6	36.15	1618.30
3	3	2.5	4.5	6	36.42	1915.02
4	3	0.8	4.5	2	29.65	1495.09
5	2	1.6	3	0	20.00	1280.00
6	2	1.6	6	4	25.60	1398.40
7	1	2.5	4.5	2	23.92	1368.22
8	0	1.6	3	4	21.85	1280.00
9	3	0.8	1.5	6	33.95	1485.00
10	1	0.8	4.5	6	24.34	1292.00
11	3	2.5	1.5	6	31.06	1783.23
12	1	2.5	1.5	6	32.43	1443.78

Run	X1	X2	X3	X4	DH%	ABTS
13	3	2.5	1.5	2	26.70	1020.00
14	3	0.8	1.5	2	24.83	1343.26
15	2	1.6	3	4	28.69	1619.8
16	4	1.6	3	4	28.42	1612.31
17	2	1.6	3	4	28.69	1619.80
18	2	3.3	3	4	28.93	1542.45
19	2	1.6	0	4	28.95	1353.71
20	1	0.8	1.5	2	16.32	1198.72
21	2	1.6	3	4	28.69	1619.80
22	3	2.5	4.5	2	27.86	1720.92
23	2	1.6	3	4	28.69	1619.80
24	2	1.6	3	4	28.69	1619.80
25	2	0	3	4	17.15	1110.00
26	1	2.5	1.5	2	27.93	1222.83
27	1	0.8	4.5	2	18.41	1317.78
28	1	0.8	1.5	6	22.49	1625.54
29	2	1.6	3	8	31.80	1680.83

X1:Alcalase enzyme unit
X2:Flavourzyme enzyme unit
X3: Alcalase digestion time
X4: Flavourzyme digestion time

Study utilized RSM to develop a prediction model for optimizing the antioxidant activity of hydrolysates and the corresponding response value for dependent variables were analysed to obtain a regression equation that could predict the response within given range. The regression equations for dependent variables is as follow:

DH% = $-2.94099 + 72.35284x_1 + 131.18285x_2 + 0.51544x_3 + 1.86810x_4 - 236.59404\, x_1x_2 + 2.18336x_1x_3 + 1.15611x_1x_4 - 2.98738x_2x_3 + 1.40055x_2x_4 - 0.049423x_3x_4 - 47.07310x_1^2 - 147.78161x_2^2 + 0.025635x_3^2 - 0.072658x_4^2$

ABTS = $+678.71267 + 1336.85309x_1 + 1454.62879x_2 + 143.91642x_3 + 53.66435x_4 + 1017.32136x_1x_2 + 75.17325\, x_1x_3 + 70.34352\, x_1x_4 + 291.68232\, x_2x_3 + 335.10038\, x_2x_4 - 4.19994\, x_3x_4 - 3121.87675\, x_1^2 - 9107.19773\, x_2^2 - 22.01294\, x_3^2 - 5.85978\, x_4^2$

Effects of independent variales on response :

Regression coefficients and response surface graphs were used to study the effect of various independent variables on degree of hydrolysis and antioxidant activity. From table 2 it was shown that , for linear variables alcalase and flavourzyme concentration had varying effects ($p<0.01$) on DH% and antioxidant activity (ABTS). Alcalase digestion time had non significant effect on DH%, but it showed significant effect ($p<0.05$) on antioxidant activity. Flavourzyme digestion time had highly significant effect ($p<0.01$) for DH% and antioxidant activity (ABTS).

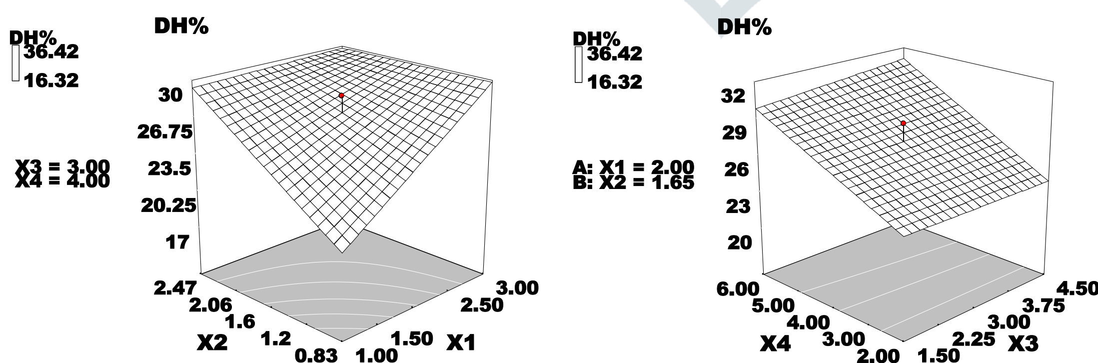
From quadratic variables flavourzyme concentration had negatively significant effect ($p<0.05$) on degree of hydrolysis and antioxidant activity on the other hand Alcalase concentration and digestion time both had non significant effect ($p>0.05$) on degree of hydrolysis and antioxidant activity.

For interaction effect sequential flavourzyme concentration and alcalase digestion time had highly significant effect ($p<0.01$) on ABTS assay and for degree of hydrolysis it showed non significant effect. Alcalase concentration and flavourzyme concentration had negatively significant($p<0.01$) effect on DH % and for antioxidant activity it showed non significant effect.

Table 2: Regression coefficients and ANOVA response for independent variables

Term	Regression Coefficient		p-values	
	DH%	ABTS	DH%	ABTS
Intercepts	28.70	1619.8	-	-
X1	2.57**	75.65**	0.00	0.00
X2	2.57**	71.83**	0.00	0.00
X3	0.63	60.62*	0.00	0.02
X4	3.21**	126.60**	0.20	< 0.00
X1*X2	-1.98**	8.49	0.00	0.77
X1*X3	0.33	54.37	0.00	0.08
X1*X4	0.23	12.98	0.58	0.66
X2*X3	-0.37	76.52**	0.69	0.01
X2*X4	-0.23	56.55	0.53	0.07
X3*X4	-0.15	-54.29	0.69	0.08
$X1^2$	-0.48	-32.00	0.80	0.18
$X2^2$	-1.01*	-61.98**	0.31	0.01
$X3^2$	0.05	-49.52	0.04	0.04
$X4^2$	-0.29	-23.43	0.53	0.31
MODEL			0.00	0.00
R-Squared			0.89	0.86
Adeq Precision			10.63	9.73
Std.Dev.			2.35	115.59

The response surface graph for DH% & antioxidant activity of hydrolysates from buffalo casein as a function of digestion time and enzyme concentration as shown in figure-1 inferred that degree of hydrolysis increased from 16.32 to 36.42%, and antioxidant activity increased upto 1020-1915µM tolox equivalant/mg protein for ABTS. The effect of alcalase and flavourzyme concentration on DH%, ABTS assays was illustrated in response surface plots (Fig.1) where as alcalase and flavourzyme digestion time effect on DH%, ABTS assays was illustrated in response surface plots (Fig.1). Higher alcalase and flavourzyme concentration resulted in significant ($p<0.01$) increase in DH%. Increase in digestion time of flavourzyme resulted in significantly ($p<0.05$) increase in DH% but alcalase digestion time shows constant effect on DH%. Lower flavourzyme concentration and increased alcalase concentration resulted in significant increase ($p<0.01$) in ABTS activity. Increase in digestion time of flavourzyme resulted in significantly ($p<0.05$) larger increase in ABTS activity but alcalase digestion time shows constant effect on activity.



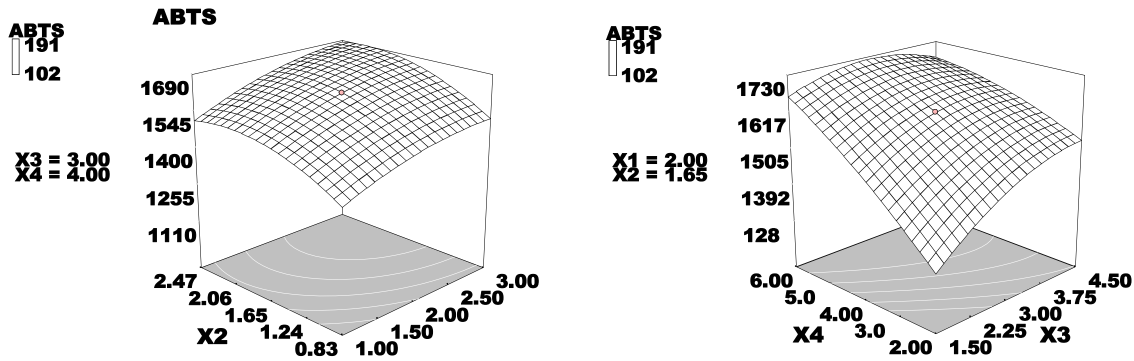


Figure 1: Response surface graphs for degree of hydrolysis (DH %) and antioxidant activity

Optimum conditions for the generation of casein hydrolysates:

The influence of enzyme concentration and digestion time on degree of hydrolysis and antioxidant activity was determined using central composite design as mention previously. From the quadratic model, under optimum conditions, the maximum degree of hydrolysis (34.26%) and antioxidant activity (1928.21µM trolox equivalent) of buffalo casein hydrolysates were obtained using 3.0 nkat/mg protein alcalase, 2.5 nkat/mg protein flavourzyme, 4.5 h alcalase digestion time and 6.0 h sequential flavourzyme digestion time with desirability 0.94.

The chromatogram of 3 kDa permeates obtained from casein hydrolysates by sequancial alcalase and flavourzyme treatment at optimized condition shows 15 major peaks. The antioxidant activity ranges from 328 µM trolox/mg protein to 2381 uM trolox/mg protein. The fraction from peak at 80.43 min shows highest activity shown in Fig. 2

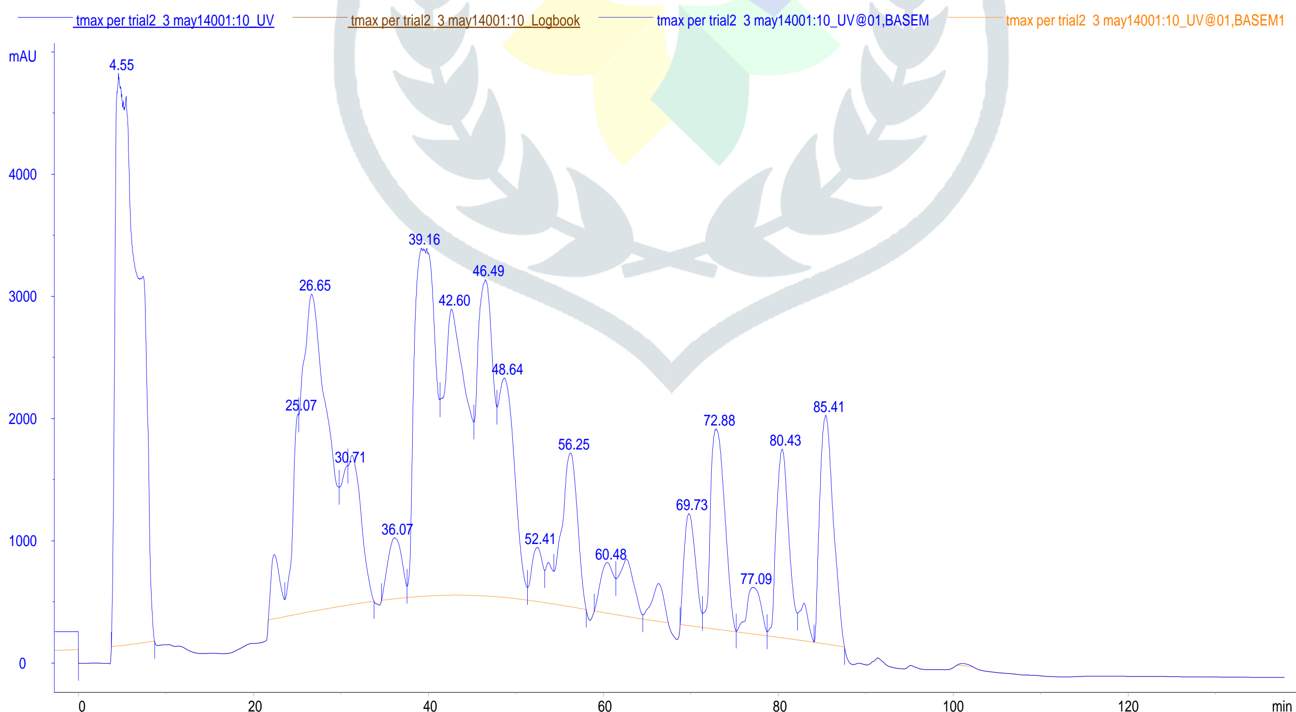


Figure 2: RP-HPLC profile of 3KDa permeate of optimized condition

Sensory evaluation:

Sequential treatment of casein hydrolysates derived from optimized conditions resulted in caffeine isointensity of bitterness < 3mM showing that treatment of endo-exoproteases resulted in decrease in bitterness together with improvement in antioxidant activity.

Conclusion:

Commercial alcalase and flavourzyme are highly effective for the production of casein hydrolysates with higher antioxidative property and lower bitterness can find applicability for the development of functional beverages.

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