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Optimization of hydrolysis conditions for antioxidant activity of whey protein hydrolysate obtained from Ash Gourd (*Benincasa hispida*) protease

Dambar Bahadur Khadka[1,2]* Laxmi Adhikari[1], Pragya Khadka[3] and Dhan Bahadur Karki[1]

[1]Tribhuvan University-Central Department of Food Technology, Dharan, Nepal; [2]Central Campus of Technology, Tribhuvan University, Dharan, Nepal; [3]National Academy of Applied Science and Technology (NAAST), Dharan, Nepal

***Corresponding Author:** Dambar Bahadur Khadka, PhD student, Tribhuvan University-Central Department of Food Technology, R7CQ+HMM, Bijayapur Sadak, Dharan 56700, Nepal.

Submission Date: October 23rd, 2024; **Acceptance Date:** December 5th, 2024; **Publication Date:** December 9th, 2024

Please cite this article as: Khadka D. B., Adhikari L., Khadka P., Karki D. B. Optimization of hydrolysis conditions for antioxidant activity of whey protein hydrolysate obtained from Ash Gourd (Benincasa hispida) protease. *Functional Foods in Health and Disease* 2024; 14(12): 921-933. DOI: https://doi.org/10.31989/ffhd.v14i12.1494

ABSTRACT

Background: Plant serine proteases are emerging as viable alternatives to animal and microbial proteases, particularly for whey protein hydrolysate preparation to enhance their nutritional and bio-functional properties.

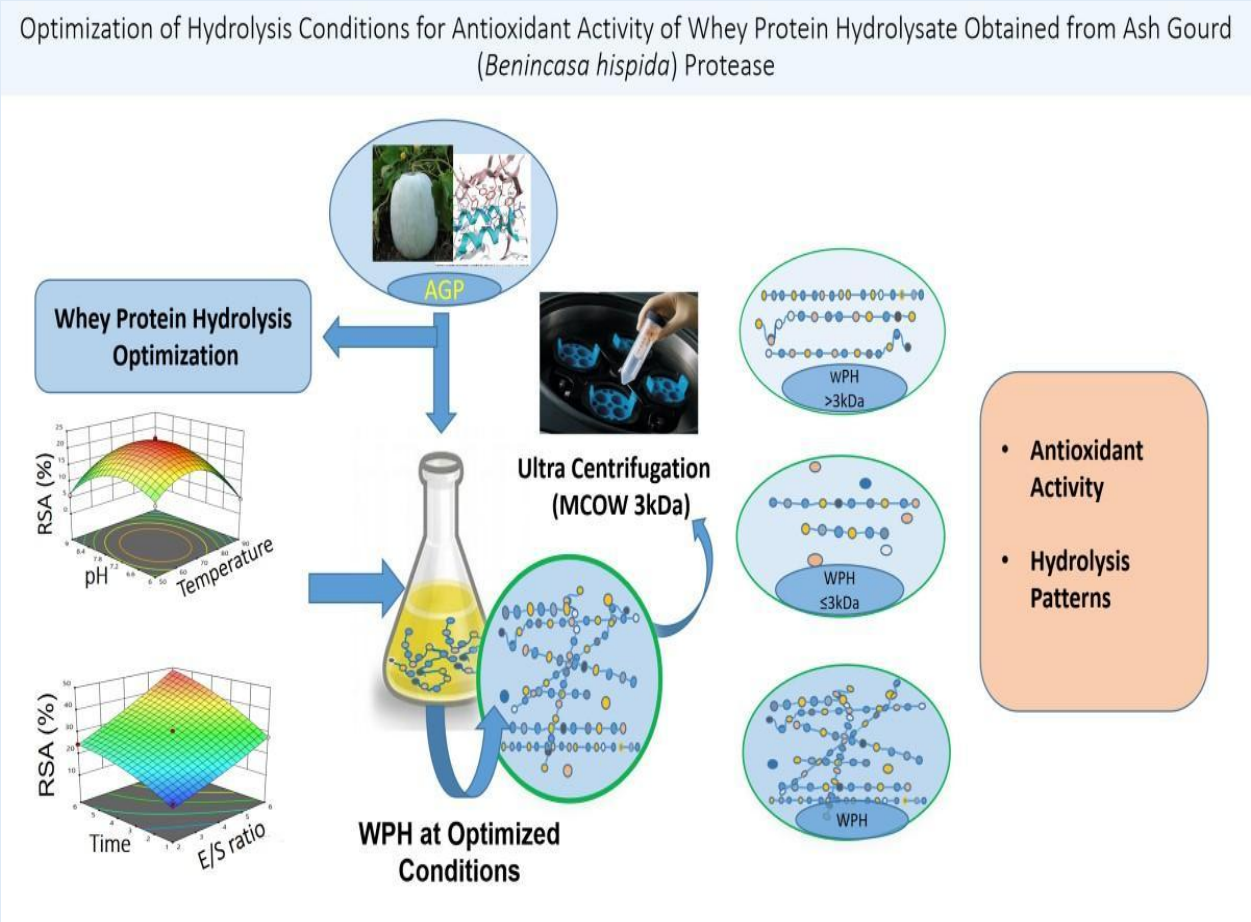
Objectives: The present study thus aims to optimize the conditions for whey protein hydrolysis employing ash gourd protease (AGP) to achieve maximum 2, 2 diphenyl -1-picrylhydrazyl (DPPH) radical scavenging (antioxidant) activity and degree of hydrolysis of the generated hydrolysate.

Methods: Optimization was accomplished using response surface methodology and treatment combinations applying generated through a central composite design. This was conducted in two steps: first, temperature (50-90°C) and pH (6-9) was optimized adding same concentration of AGP (0.3% v/v) at reconstituted WPC (0.6% protein), and second, E/S ratio (2-6 %v/v) and hydrolysis time (1-6h) was optimized at reconstituted WPC (4% protein). Furthermore, antioxidant potency of hydrolysate and its ultra-centrifuged fractions ≤3kDa and >3kDa obtained from the optimized condition was also evaluated. The AGP (Adjusted to activity 2U/mL) was partially purification using the three phase partitioning method for hydrolysis experiments.

Results: The optimum conditions for enzymatic hydrolysis of whey protein to achieve maximum degree of hydrolysis (13.99%) and DPPH radical scavenging activity (23.05%) were observed at pH 7.48 and 66.40°C. Similarly, the enzyme performed optimally at enzyme by substrate ratio of 5.85% (v/v) for 6 hours of hydrolysis, providing 19.31 % of degree of hydrolysis and 47.71 % of DPPH radical scavenging activity. The lower molecular weight peptide fraction ≤3kDa was found to be more effective (1.45 times) than the peptide fractions >3kDa. However, it was less effective (1.19 times) than the whole hydrolysate regarding antioxidant activity.

Conclusion: Overall, the study showed that the AGP employed whey protein hydrolysate has the potential for use as a natural antioxidant.

Keywords: Ash gourd protease, whey protein, hydrolysis, antioxidant activity



Graphical Abstract: Optimization of Hydrolysis Conditions for Antioxidant Activity of Whey Protein Hydrolysate Obtained from Ash Gourd (Benincasa hispida) Protease

INTRODUNCTION

Whey, a high-volume byproduct of dairy industries mainly resulted from cheese production, accounts for aproximately 190 million tons of annual production [1]. However, an appreciable amount (Approx. 47%) of cheese whey is discarded as waste [2], leading to environmental pollution issues, high economic loss [1], and the unnecessary disposal of essential whey proteins useful in various products like sports drinks, supplements, infant formulas, bakery, and meat [3]. Due to its nutritional and functional importance, efforts to recover and modify whey proteins remain ongoing [1-2]. Enzymatic hydrolysis is one approach to add value to whey protein converting it to hydrolysate [4]. Identification and preparation of food bioactive compounds is an initial step in the development of functional food [5]. Food bioactive compounds, derived from plants, animals, or fundi, exhibit positive impacts on human health beyond its nutritive values [5]. The production of bioactive peptides (Antioxidant, antihypertensive, anticarcinogenic and immune modulatory, etc.) from whey protein hydrolysis is a growing are of research aimed at value addition and addressing the global rise of non-communicable diseases [6-7]. Proteases mainly from animal or microbial sources were used for protein hydrolysate preparation to reduce allergenicity and to improve bio-functionality [4]. However, due to insufficient animal production and wide application of proteases in various sectors, their availability is reported to be insufficient in the future. In addition, changes in consumer perception because of ethical, cultural beliefs, and safety concerns, have shifted consumer preference towards the use of plant-based protease for food and food ingredient production [8].

As an alternative, plants protease are now viewed as a cost-effective substitute to microbial and animal protease, playing a significant role in the area of whey protein hydrolysate (WPH) production [6, 8]. In an attempt to enhance bio-functionality and the generation of bioactive peptides, aspartic proteases such as cardosin from the Cyanara species and arctiumisin from Arctium minus have been used for whey protein hydrolysis [6, 9]. Similar potential was also illustrated with plant cysteine proteases such as papain, bromelain [10], and ficin [10-11]. However, research regarding the use of plant protease in protein hydrolysate preparation are still few in number [6]. Plant serine proteases as compared to cysteine and aspartic types, are more flexible and stable, making them cost-efficient alternative for protein hydrolysis [12]. Protease from the Cucuribita family, including cucumisin-like enzymes, have shown promise in enhancing the functional and nutritional properties of hydrolyzed protein [6]. For example, plant serine proteases such as pomiferin from Maclura pomifera, proteinase from Cucurbita ficifolia, and proteinase extract from trompillo and melon have been successfully used in whey protein hydrolysis to enhance bio-functionality [6, 9, 13]. Moreover, melon protease, in particular, has been shown to outperform alcalase in the hydrolysis of fish protein for enhancing antioxidant activity [14].

Ash gourd (Benincasa hispida), a native vegetable of South and Southeast Asia, belonging to the Cucurbitaceae family [15], has long been consumed for both nutritional and therapeutic purposes (anti-diarrheal, anti-obesity, anti-ulcer, antioxidant, and diuretic) [15]. A serine protease (Mw; 67 kDa) that acted on different synthetic peptides was previously isolated from B. hispida [16]. However, its application in whey protein hydrolysis as concerned to bio-functionality has not been discovered to date. Additionally, hydrolysis conditions can also alter specific compositions of generated hydrolysate causing significant changes in the bio-functionality of hydrolysate [17-18]. Response surface methodology (RSM) was used successfully by various researchers for optimization of process variables

in whey protein hydrolysis to achieve maximum bio-functionality [19-21]. Hence, this study aimed to optimize the hydrolysis conditions (pH and temperature, enzyme to substrate ratio, and time of hydrolysis) using ash gourd protease (AGP) to produce WPH having a maximum degree of hydrolysis (DH) and antioxidant activity. Optimization was accomplished using RSM in two steps: first temperature and pH were optimized, followed by enzyme to substrate (E/S) ratio, and hydrolysis time. Furthermore, the antioxidant potency of generated hydrolysate at optimized conditions and their ultra-centrifuged fractions; ≤3kDa and > 3kDa were also evaluated.

MATERIALS AND METHODS

Materials: Ash gourd was collected from the local grower of Dharan, Nepal. Whey protein concentrate (WPC-80%) was obtained from Medizen Labs Pvt. Ltd, India. 2, 2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich, TEMED (N, N, N', N'-Tetramethylethylenediamine), N-N´-methylene-bis-acrylamide, 2- mercatoethanol, Ammonium persulfate, Bovine serum albumin, O-pthaldehyde (OPA), Sodium dodecyl sulfate was procured from HiMedia laboratories Pvt. Ltd, India. Acrylamide, pre-stained protein ladder 26619, HPLC water were purchased from Thermo Fisher Scientific Pvt. Ltd, India.

Isolation and Purification of Ash Gourd Protease (AGP): The crude protease extract of ash gourd was prepared employing the method as reported by Gagaoua et al. [22] with appropriate adjustments. Ash gourd pulp was homogenized with a 0.1M sodium phosphate buffer, (pH 6.8) at a 1:1 w/v ratio using mortar and pestle. The buffer extract was stirred using a magnetic stirrer at 250 ± 50 rpm for 15 min under refrigerated condition. After centrifugation at 4 °C for 10 min at 5000 rpm, the supernatant was collected and precipitated, with ammonium sulfate (60%). The resulting solution was centrifuged (13000 rpm for 10 min at 4 °C) to obtain the pellet which was resuspended in a minimal volume of the same buffer and dialyzed to obtain the crude protease extract (AGPCE). The AGPCE was subjected to further purification and concentration by using three phase partitioning process at pre-optimized conditions (50% ammonium sulfate, AGPCE: tert-butanol ratio of 1:1, 8 pH and 25°C temperature). The purified ash gourd protease (AGP), concentrated in intermediate phase, was carefully collected and dialyzed. The resulting AGP demonstrated a 5.17-fold purified with 122.67% activity recovery of, a protease activity of 8.4 U/mL, and protein content of 0.37 mg/mL (specific activity 22.55U/mL) (See Supplementary information_1: AGP isolation and purification). The obtained AGP was subsequently diluted to adjust its protease activity to 2U/mL for use in this study.

Experimental Design and Preparation of WPH for Optimization of pH and Temperature: RSM was used to optimize pH and temperature conditions for whey protein hydrolysis by AGP to achieve maximum DH and antioxidant activity. A central composite design (CCD) using Design of Expert (DOE Version 13, Stat-Ease Inc., Minneapolis) was applied to generate experimental treatment combinations. Two factors, pH (6-9) and temperature (50ºC- 90ºC), were considered as variables, while DH and antioxidant activity were evaluated as response variables. The temperature and pH ranges were selected according to the preliminary results of the thermal and pH stability of the AGP. A total of 11 independent treatment combinations (Table 1) were conducted including: four factorial levels to check linear and interaction effects, three center point to estimate experimental error, and four axial point to estimate quadratic terms were run in a random manner. The

quadratic equation as mentioned below was built-in to obtain experimental data for responses.

$$Y = \beta0 + \beta1\ A + \beta2\ B + \beta11\ A2 + \beta22\ B2 + \beta12\ AB + \varepsilon$$

Where: Y is the response value; A and B are coded values of the process variables; temperature and pH respectively. β0 is the intercept; β1 and β2 are the linear coefficients; β11, β22 are the quadratic coefficients; β12 is the interaction coefficient and ε denotes the experimental error.

Whey protein concentrate (WPC-80%) was reconstituted to 0.6% protein concentration in 50 mM buffer of the respective pH conditions (Table 1). Enzymatic hydrolysis was intiated by adding AGP (0.3 % v/v) to the WPC solution at specfic combinations of temperature and pH (Table 1) for 2 hours. The enzyme activity was terminated by applying a heat treatment (95°C for 15 min). The hydrolyzed solution was centrifuged at 12,000 rpm for 10 minutes at 4°C to separate the soluble peptide fraction from unhydrolyzed protein. The supernatant was collected into two vials: one vial was used to examine DH and protein determination, and the other set of vials were syringe filtered (0.2 µm nylon filter membrane), lyophilized, and frozen at -20°C until analysis. The lyophilized sample was reconstituted in HPLC water to get 20 mg/mL protein concentration for evaluation of antioxidant activity.

Experimental Design and Preparation of WPH for Optimization of E/S ratio and Time of Hydrolysis:

In the second experiment, RSM with a quadratic model was used to optimize the E/S ratio and time of hydrolysis for whey protein using AGP, focusing on DH and DPPH-RSA as response variables. Using a CCD, combinations of E/S (2- 6 % v/v) and time of hydrolysis (1-6 hours) were used to generate treatment combinations. A total of 11 obtained combinations (Table 1) were run in a random manner for obtaining the values for DH and antioxidant activity for the optimization process.

For the WPH preparation, WPC-80 was reconstituted with in 50 mM Tris-HCl buffer buffer (pH 7.48) to make the final concentration of 4% protein. AGP was then added to adjust the E/S ratio and hydrolysis was conducted for the designated durations in Table 1. The enzyme activity was terminated, and WPH samples were prepared as mentioned previously for analysis of DH, protein concentration, and antioxidant activity.

Analytical Procedures

Degree of Hydrolysis (DH): The DH, representing the percentage of peptide bonds cleaved, was estimated based on free amino groups present in the sample determined by OPA method with some modification [23]. A total of 40 µL of hydrolysate samples (after appropriate dilution) was added with 3 ml freshly prepared OPA reagent, kept exactly for 2 min at room temperature, and the absorbance was read at 340 nm. L-serine 0.9516 meqv/L (0.1mg/ml) as a standard was applied for the quantification. DH was calculated as:

$$DH\ (\%) = h*100 / h_{tot}$$

Where, h_{tot} is the total number of peptide bonds per protein equivalent and h is the number of hydrolyzed bonds and is calculated as,

$$h\ (meqv\ serine/g\ protein] = Serine\text{-}NH2 - \beta/\alpha$$

$$Serine\text{-}NH2 = [Asample\text{-}Ablank) /(Astandard\text{-}\ Ablank)] \times (0.9516\ meqv/L) \times\ 0.1 \times (100/X) \times P$$

Where, Serine-NH2= meqv serine NH2/g protein; X= g sample; P = % protein in sample and 0.1 = sample volume in liter (L). The value of htot α, β were obtained from Nielsen et al. [23].

Protein Determination: The protein concentration of WPH and its fraction were estimated by Lowry method using the BSA (0-1mg/mL) standard curve [24].

SDS-Polyacrylamide Gel Electrophoresis: Whey protein hydrolysis patterns were analyzed using Tricine sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) applying stacking gel (6%) and separating gel (15%) as described described by Mazarro-Manzano et al. [13].

Antioxidant Activity: The antioxidant activity of hydrolysate was measured based on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging methods as described by Hussein et al. [20] with appropriate adjustment. 100µL (20 mg/ml) of hydrolysate was mixed with 3ml of DPPH solution (0.1mM prepared in 80% methanol) and incubated in the dark for 30 minutes, with the absorbance measured spectrophotometrically at 517nm (Carry UV-VIS, Agilent, USA). Sample was replaced with the same volume of distilled water keeping all other reagents same to perform control. For sample blank and solvent blank, 100 µL of sample and 100 µL of distilled water respectively were added to the 80% methanol. The antioxidant activity was stated as percentage DPPH radical scavenging activity (DPPH-RSA) utilizing the formula:

$$\text{DPPH–RSA (\%)} = [1 – (As\text{-}Asb\text{-}Ab) / (Ac\text{-}Ab)] \times 100$$

Where As, ASb, Ac and Ab are the absorbance of sample, sample blank, control, and solvent blank respectively.

The IC50 values of the hydrolysate from optimization condition and their fractions (≤3kDa and > 3kDa) were separated through Amicon® ultra-centrifugal filter (MWCO: 3kDa) were estimated by determining DPPH-RSA at different concentrations (0 - 80 mg/mL).

Data Analysis: DOE version 13 (Stat-Ease Inc., Minneapolis) was used for regression analysis of the models, goodness of fit and ANOVA of the model terms and coefficients, and RSM analysis.

RESULTS AND DISCUSSIONS

Optimization of Temperature and pH by RSM: RSM was applied to evaluate the pH and temperature influences on whey protein hydrolysis by AGP. Table 1 shows the experimental combinations of factors and the values obtained for the response variables. The DH and DPPH-RSA for WPH were found to be varied from 1.22-14.78% and 1.23-23.03% respectively (Table 1).

Table 1. Experimental treatment combinations with values of response variables for optimization of Temperature and pH

Run	Experiment 1: Optimization of Temperature and pH				Experiment 2: Optimization of E/S ratio and hydrolysis time			
	Factors		Responses		Factors		Response 1	
	A:Temperature (°C)	B:pH	DH (%)	DPPH-RSA (%)	E/S ratio (%v/v)	Time (h)	DH (%)	DPPH-RSA (%)
1	70.00	7.50	13.62	22.74	2.00	1.00	8.53	13.18
2	70.00	9.60	9.54	7.27	4.00	7.00	20.36	37.33
3	90.00	9.00	1.75	5.80	6.80	3.50	14.11	47.71
4	41.70	7.50	7.14	8.75	4.00	3.50	19.17	30.56
5	70.00	7.50	14.78	21.18	4.00	3.50	19.48	30.71
6	50.00	9.00	11.03	5.82	6.00	6.00	17.76	47.06
7	50.00	6.00	6.47	11.78	4.00	0.00	6.60	11.25
8	98.30	7.50	1.22	1.23	2.00	6.00	11.85	24.44
9	90.00	6.00	5.52	4.59	6.00	1.00	10.05	27.71
10	70.00	7.50	13.03	23.03	1.20	3.50	8.99	16.31
11	70.00	5.40	7.00	12.48	4.00	3.50	17.07	28.51

Based on the regression analysis, a quadratic model was selected for the prediction of DH (Eq1) and DPPH-RSA (Eq 2) of the WPH with respect to change in temperature and pH within the experimental range.

DH (%) = -145.726 +2.092A + 23.739B -0.069AB – $1.234A^2$ - $2.78B^2$ (Eq. 1)

DPPH-RSA (%) = -196.633- 2.511A +37.003B + 0.059AB – $0.0219A^2$ -$2.813B^2$ (Eq. 2)

Where, A and B represent the temperature (°C) and pH respectively.

The model and terms of coefficients; linear (A, B), quadratic (A2, B2) and the interaction (AB) of the effect of variables for the responses were evaluated for adequacy, fitness, and significance by ANOVA (Table 2). The significant model's p-value ($P < 0.0001$), non-significant lack of fit ($p > 0.05$), and high coefficient of determination (R2, adjusted and predicted) supported that the model was fit to determine the optimum hydrolysis condition of WPH for both the responses. The ANOVA results further demonstrated that the temperature (A) had significant linear effect on DH, whereas pH did not exhibit a significant linear effect. However, their quadratic terms and interaction had significant effect on DH (Table 2). Similarly in the case of DPPH-RSA, the linear and quadratic terms of both temperature and pH, as well as their interaction, exhibited significant effect (Table 2).

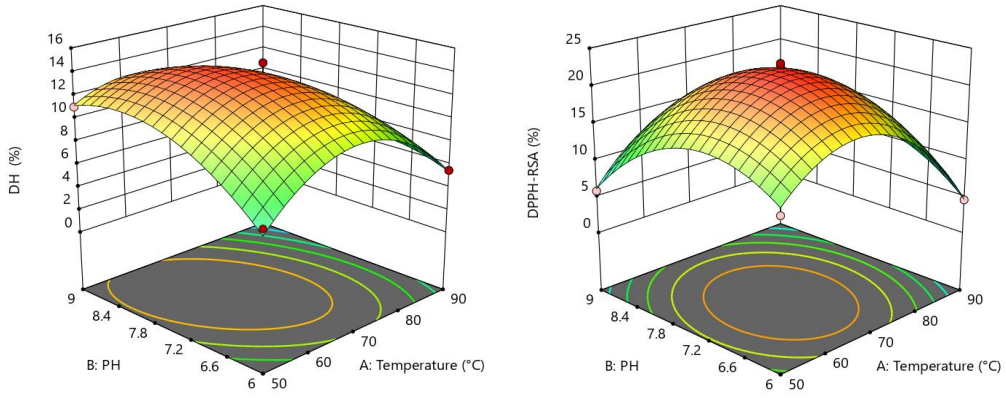
Table 2. ANOVA results of temperature and pH optimization quadratic model and regression coefficients for DH and DPPH-RSA

Source	df	Response 1: DH			Response 2: DPPH-RSA		
		**Coefficient estimate	F-value	*p-value	**Coefficient estimate	F-value	*p-value
Model	5	-	68.76	0.0001	-	118.52	< 0.0001
Intercept	1	13.81	-	-	22.32	-	-
A-Temperature	1	-2.33	72.30	0.0004	-2.23	39.88	0.0013
B-pH	1	0.5460	3.99	0.1024	-1.52	18.37	0.0068
AB	1	-2.08	28.93	0.0030	1.79	12.84	0.0140
A2	1	-4.82	219.47	<0.0001	-8.77	434.65	< 0.0001
B2	1	-2.78	72.82	0.0004	-6.33	226.22	< 0.0001
Lack of Fit	3	-	0.6030	0.6727	-	0.8992	0.0013
Fit Statistics		R^2: 0.9857, Adjusted R^2: 0.9713, Predicted R^2: 0.9346, CV: 9.34			R^2: 0.9922, Adjusted R^2 : 0.9843, Predicted R^2 : 0.9604, CV: 8.54		

*p value <0.05 indicates significantly different ** Regression coefficient estimated in term of coded factors

Fig.1a shows the interaction effect of temperature and pH on the DH of WPH. The DH was found to be increased with rising pH and temperature till it reached an optimal region, beyond which it began to decline. The maximum DH was observed within a neutral to alkaline pH range (7.2-8.4) and temperature range of 62-68°C. This indicates that AGP is stable and highly active in these pH and temperature ranges, and hence is favored for maximum activity against whey protein cleavage. The pH beyond the isoelectric point of whey protein (pi 5.2) can promote higher DH, likely caused by the disruption of compact protein structure, which converts β-lactoglobulin from dimeric to monomeric form, exposing additional cleaving sites [18]. The influence of pH and temperature in DH found in the study are similar to the patterns reported for whey protein hydrolysis with flavourzyme [19] and alcalase [20]. The drop in DH at higher temperature and higher pH could be attributed to whey protein aggregations and structural changes in the active site of enzyme that reduce enzyme access to the primary sites of proteins [19, 25].

Fig. 1b demonstrates that DPPH-RSA increased as the temperature and pH until it reached the maximal region, after which the antioxidant activity declined. The maximum DPPH-RSA was observed at a temperature between 60-70°C. This increase is consistent with findings by Naik et al. [21], who attributed the enhanced activity at higher te,pertures to exposure of reactive amino groups from proteins. Similar temperature dependent change in DPPH-RSA was also demonstrated in WPC hydrolysis with alcalase [20]. The DPPH–RSA was noted highest at the neutral to alkaline pH range in this study, which may be attributed to increased solubility of the peptides formed during hydrolysis. This is because solubility of WPH has been reported to approach maximum at neutral pH [25]. The neutral to slightly alkaline pH value is responsible for the protein dissociation, driven by repulsion generated between anionic groups and favors bioactive peptide release [19]. The decrease in antioxidant activity observed at the boundary of pH value could be due to the prooxidative natures of certain antioxidant peptides or antioxidative potency modification by isomerization of pH-sensitive peptides [19]. Instead of individual effects, the interaction of temperature and pH plays a crucial role in both DH and released peptide bio-functionality; therefore, optimization is essential in hydrolysis reactions [17, 25].



(A.) (B.)

Fig.1 Response surface 3-D plot. A) Effect of temperature and pH on degree of hydrolysis of WPH. B) Effect of temperature and pH on DPPH-RSA of WPH

Numerical optimization approach setting process variables in ranges, was used to predict the combination of optimum pH and temperature for the maximum DH and DPPH-RSA. The optimum combination was obtained to be 66.40°C temperature and 7.48 pH which provide 13.99% DH and 23.05% DPPH-RSA with desirability 0.96.

Optimization of E/S Ratio and Time of Hydrolysis by RSM: In the second phase, RSM was further applied to study the effect of the E/S ratio (% v/v) and hydrolysis time (hours) on whey protein hydrolysis by AGP. Table 1 shows experimental levels of the independent variables and the outcome of response variables. The DH and DPPH-RSA of the WPHs obtained from experiment were varied from 6.60% – 20.36% and 11.25% - 47.06% respectively. The regression analysis confirmed the quadratic model was best fit for prediction of responses DH (Eq. 3) and DPPH-RSA (Eq. 4) with respect to change in process factors (E/S ratio and hydrolysis time).

DH (%) = -7.353 + 7.500A+ 3.663B+ 0.220AB – 0.918A^2 -0.430B^2 …… (Eq. 3)

DPPH-RSA (%) = 1.832 + 1.587A+ 4.997 B + 0.405AB + 0.265A^2 - 0.461 B^2… (Eq.4)

Where A = E/S ratio (% v/v) and B= Time of hydrolysis (h)

The regression model for both responses were statistically significant ($p < 0.05$) (Table 3). A non-significant lack of fit ($p > 0.05$) and reasonable R2 (predicted and adjusted) for both responses demonstrated that the model is adequate to estimate the optimum hydrolysis conditions (E/S ratio and time of hydrolysis) of WPH within the experimental range (Table 3).

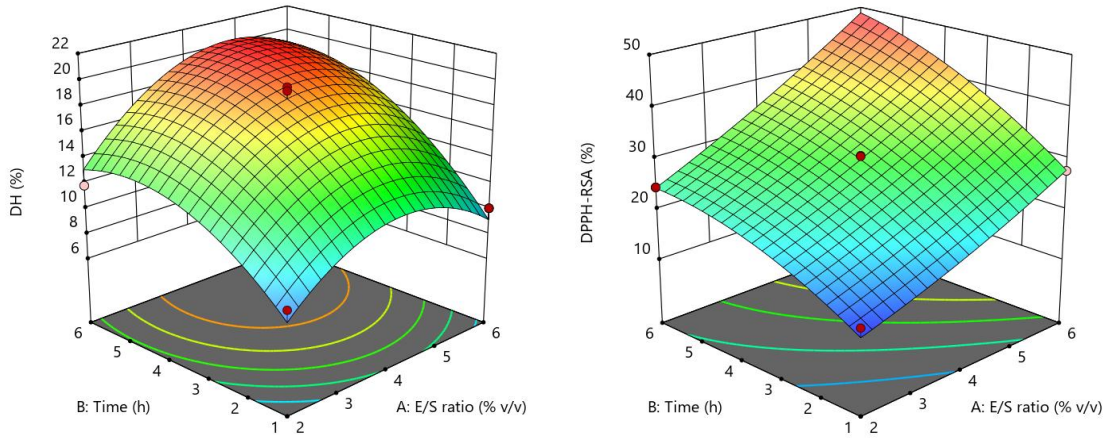
Table 3. ANOVA results of E/S and hydrolysis time optimization quadratic model and regression coefficients for DH and DPPH-RSA

Source	df	Response 1: DH (%)			Response 2: DPPH – RSA (%)		
		**Coefficient estimate	F-value	*p-value	**Coefficient estimate	F-value	*p-value
Model	5	-	18.47	0.0031	-	93.60	< 0.0001
Intercept	1	18.58	-	-	29.93	-	-
A-E/S ratio	1	1.84	10.44	0.0232	10.24	262.21	< 0.0001
B-Time	1	3.83	44.98	0.0011	8.47	179.57	< 0.0001
AB	1	1.10	1.87	0.2297	2.02	5.16	0.0722
A2	1	-3.67	28.79	0.0030	1.06	1.94	0.2220
B2	1	-2.69	15.42	0.0111	-2.88	14.39	0.0127
Lack of Fit	3	-	1.83	0.3723	-	2.83	0.2720
Fit Statistics		R^2: 0.9486, Adjusted R^2: 0.8973, Predicted R^2: 0.7013, CV: 11.47			R^2: 0.9894; Adjusted R^2: 0.9789; Predicted R^2: 0.9346, CV: 6.22		

*P value <0.05 indicate significantly different ** Regression coefficient in term of coded factors

Regarding DH, the linear and quadratic terms for E/S ratio and hydrolysis time had significant effect ($p< 0.05$) but their interaction effect was not significant (Table 3). With respect to DPPH-RSA; the linear term for E/S ratio and hydrolysis time had significant influence ($p< 0.05$) but their interaction was not significant. As concerned to quadratic terms, hydrolysis time had significant effect but not with the E/S ratio ($p<0.05$) (Table 3).

The maximum DH seems to lie between the E/S ratio of 4-6 % (v/v) and hydrolysis time of 5-6 hours (Fig 2a). As seen in Fig. 2a, DH is increased as the E/S ratio and hydrolysis time rises until it reaches maximum, and then remain almost constant at extremities of both the factors. Similarly, DPPH-RSA was also found to increase with rising E/S ratio and hydrolysis time, exhibiting a maximum antioxidant activity level towards the extremities of E/S ratio (4-6 %; v/v) and hydrolysis time (4-6 h) in a quadratic surface (Fig. 2b). The increase in DH and DPPH-RSA with increase in E/S ratio and hydrolysis time was also reported with alcalase [20] and trypsin [26] hydrolysed WPC, supporting the results obtained in this study. When numerical optimization approach was used, the ideal E/S ratio of 5.85% and 6 hours of hydrolysis time with desirability of 0.96 were predicted for maximum DH (19.31%) and DPPH-RSA (47.71%) of hydrolysates.



(A) (B)

Fig 2. Response surface 3-D plot; A) Effect of E/S ratio and hydrolysis time on DH. B) Effect of E/S ratio and hydrolysis time on DPPH-RSA

Hydrolysis Patterns of Whey Proteins

SDS-PAGE results of WPHs (from both the optimization conditions) showed that AGP had effective tendency to cleave β-lactoglobulin (β-Lg) and α-lactoalbumin (α-La) as indicated by degradation of both the bands and appearance of shorter peptides below 10kDa. (Fig. 3). Similar effective cleavage against β-Lg and α-La were reported for the plant serine protease from trompillo and melon fruits [13].

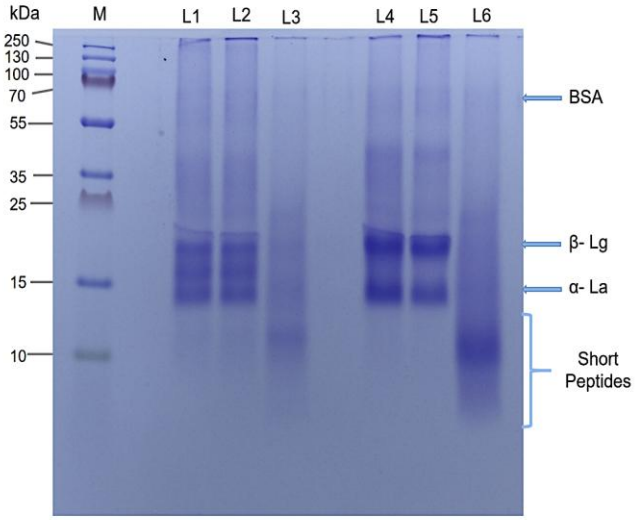


Fig 3. SDS-PAGE analysis of whey protein hydrolysis patterns by AGP. M: Standard protein marker; L1: WPC, L2: WPH (0h) and L3: WPH (2h) at optimum pH and temperature; L4: WPC, L5: WPH (oh) and L3: WPH (6h) at optimum E/S and hydrolysis time.

Antioxidant Activity and Potency of WPH and Its Fractions: Table 4 presents the IC50 values of the un-hydrolysed whey protein, WPH and its fractions obtained from optimal hydrolysis conditions. WPH exhibited the highest antioxidant activity followed by WPH ≤3kDa and then WPH > 3kDa. Based on IC50 values, the antioxidant potency of WPH and its fractions, ≤3kDa and > 3kDa, were respectively 3.12, 2.62 and 1.81 times higher than un-hydrolyzed whey protein. Similarly, WPH and its fraction ≤3kDa had respectively 1.72- and 1.45-times higher

antioxidant potency than the fractions >3kDa (Table 4). Higher antioxidant potential of WPH compared to WPH≤ 3kDa fractions found in this study was consistent with the findings obtained by Ballatore et al. [26]. In contrast, lower molecular weight fraction has also shown to be higher antioxidant potential than WPH, but the molecular cut-off used during ultrafiltration was 5kDa [21]. This is likely due to the chances of retaining more active short peptides in 5kDa than in ≤3kDa fraction. The shorter antioxidant peptides (<10kDa) had a greater ability to adsorb in the cell and could act effectively against the free radicals [27]. Additionally, antioxidant activity of WPH is largely influenced by the peptide composition, hydrophobic natures, and sequences of amino acids mostly by presence of aromatic amino acid (histidine, tyrosine, and phenyl alanine) and hydrophobic amino acids (leucine, isoleucine, valine, alanine and methionine) [20]

Table 4. IC50 values and potency of WPH and its fractions for antioxidant activity.

Samples	Antioxidant activity	
	IC50 value*	Potency
Un-hydrolysed whey protein	71.13±0.558	1
WPH	22.79±0.461	3.12
WPH ≤ 3kDa	27.13±0.995	2.62
WPH > 3kDa	39.09±3.88	1.81

*Value means of triplicate ± SD.

The higher antioxidant potency WPH and WPH ≤3kDa over un-hydrolysed whey protein confirm that AGP can be used as an alternative to animal and microbial protease for generation the WPH and peptides having improved antioxidant activity. Furthermore, AGP can hydrolyze both major whey proteins. This action of AGP could be advantageous over the limited catalytic ability of many commercial animal proteases like pepsin and trypsin [13]. Additionally, the production of WPH with the use of this natural and simply (TPP) purified plant protease also improves effectiveness and cost-efficient production for antioxidant peptides that could be ethically acceptable to be used by the wider populations, including vegetarians. However, further studies on peptide structure, bio-functional relationships, efficacy in vivo, and clinical trials are essential for the confirmation of antioxidant effects and the suitability of AGP-derived WPH and peptides to use as functional food ingredients [28].

CONCLUSIONS

This study for the first time, illuminated the potential of of AGP on hydrolysis of whey protein regarding antioxidant activity. This study demonstrated the ideal pH of 7.48, temperature 66.40°C, E/S ratio of 5.85% (v/v) , and 6 hours of whey protein hydrolysis by AGP in order to obtain highest antioxidant activity and DH. AGP can effectively cleave the major whey proteins (β-Lg and α-La) and release shorter peptides having antioxidant potential. Overall, the WPH and its peptide fractions produced by AGP displayed its possibility to use as natural antioxidant for food and pharmaceutical applications and further work in these aspects is recommended.

List of Abbreviations: AGP: Ash Gourd Protease ANOVA: Analysis of Variance, BSA: Bovine Serum Albumin, CCD: Central Composite Design, DH: Degree of Hydrolysis, DOE: Design of Expert, DPPH: 2, 2-Diphenyl-1-

Picrylhydrazyl, DPPH-RSA: DPPH Radical Scavenging Activity, Ig: Immunoglobulin, OPA: O-pthaldehyde, RSM: Response Surface Methodology, WPC: Whey Protein Concentrate, WPH: Whey Protein Hydrolysate, α-La: α-Lactoalbumin; β-Lg: β-Lactoglobulin

Competing Interests: The authors declare that they have no competing interests.

Authors' Contributions: Dambar Bahadur Khadka conceived and designed the experiment; performed the experiment; analyzed and interpreted the data; and prepare the manuscript, Laxmi Adhikari and Pragya Khadka performed the experiment; helped in data management and analysis, Dhan Bahadur Karki supervised the work, conceived and designed the experiment; contributed to analysis tools.

Acknowledgements and Funding: The authors are grateful to the Central Department of Food Technology, TU, Nepal; Central Campus of Technology, TU, Dharan and Manmohan Technical University for providing the necessary facilities and support to conduct this study. The authors are very thankful to University Grant Commission, Nepal for providing financial research support (PhD-77/78-S&T- 08) to conduct this research.

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