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Utilization of Partially Purified Milk Clotting Protease from Ginger Rhizome in the Manufacturing of Fresh Cheese

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Abstract

This study was carried out to utilize the partially purified ginger rhizome protease in fresh cheesemaking. The crude ginger rhizome extract was partially purified by employing 30-80% (NH4)2SO4 saturations (w/v). The maximum milk clotting activity was achieved at 50% saturation, exhibiting the purification fold of 1.64 and activity recovery of 85.76%. The milk pH and temperature for optimum time of coagulation and milk clotting activity were optimized by using response surface methodology (RSM). A numerical optimization study revealed that the optimum milk pH and temperature for producing cheese were 6.5 and 50 C respectively. The cheese produced by ginger protease had significantly ($p<0.05$) higher moisture, ash and yield, but lower fat content than the rennet cheese. No significant difference ($p>0.05$) was found in the calcium and protein content of both cheeses. The prepared cheese using ginger protease had significantly ($p<0.05$) higher spreadability, but lower aftertaste than the rennet cheese. However, non-significant difference ($p>0.05$) was observed in the flavor and overall acceptance between the two cheeses. Hence, this study demonstrated that the ginger rhizome protease has the potential to be utilized as an effective milk coagulating enzyme in the manufacturing of fresh cheese.

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1. Introduction

Cheese is the generic name for a group of fermented milk-based food products, produced throughout the world in a great diversity of flavors, textures and forms (Fox & McSweeney, 2017). It is a concentrated protein gel that comprises fat and moisture. Its manufacture generally involves gelation of cheese milk, dehydration of the gel to form a curd and treatment of the curd (cheddaring, texturization, salting, moulding, and pressing). The moulded curd may be consumed fresh (Johnson & Law, 2010). The method used to clot milk for cheesemaking influences the overall structure, characteristics and firmness of the cheese (Farkye, 2004). The calf rennet (mainly chymosin) is principal enzyme used in the cheesemaking since it cleaves the Phe_{105}–Met_{106} bond of κ-casein, resulting in casein micelle disintegration and milk clotting (Fox et al., 2017). Due to rising global cheese demand and a limited supply of calf rennet, the selection of appropriate rennet substitute is important (Ben Amira et al., 2017). Vegetarianism, religious convictions, rising rennet prices, ethical concerns about enzyme manufacturing, and restrictions on genetically modified foodstuffs are the additional issues to search for rennet alternatives (Roseiro et al., 2003).

Many extracellular proteases of microbial origin act similar as chymosin and are partially suitable for

cheese production. Such coagulants can be easily produced by fermentation and are, therefore, widely accessible. However, these coagulants possess higher proteolytic activity, which lead to a loss of protein degradation products into whey and thus adversely affect the yield of cheese (Jacob et al., 2011). Besides microbial proteases, plant proteases have the ability to coagulate milk in a broad range of pH and temperature (Mazorra-manzano et al., 2018). Based on catalytic mechanism used during the hydrolytic process, main three classes of plant proteases (aspartic, cysteine and serine) are milk clotting proteases (Shah et al., 2014). Several researches on milk coagulating ability of plant proteases have been conducted. The examples of such plants include kiwifruit (*Actinidia deliciosa*) (Nicosia et al., 2022; Maskey & Karki, 2022), papaya (*Carica papaya*) (Rana et al., 2017; Maskey & Shrestha, 2020), pineapple (*Ananas comosus*) (Bahmid, 2013; Kartawiria et al., 2019), sodom apple (*Calotropis procera*) (Abebe & Emire, 2020; Silva et al., 2020), cardoon (*Cynara cardunculus*) (Gomes et al., 2019; Folgado et al., 2020), trompillo (*Solanum elaeagnifolium*) (Gutiérrez-Méndez et al., 2019; Nájera-Domínguez et al., 2022), doda paneer (*Withania coagulans*) (Khan & Masuq, 2013; Nazish et al., 2022) and anjeer (*Ficus carica*) (Fguiri et al., 2020; Hachana et al., 2021). In addition to milk coagulation abilities, plant proteases also contain antioxidant properties, and a high concentration of bioactive substances (Gupta et al., 2015).

Belonging to the Zingiberaceae family, ginger is an underground rhizome plant which is one of the most important and widely used spices in the world (Srinivasan, 2017). Ginger is also used in pharmacy due to the presence of the phenolic substances gingerol and shagaol in the rhizome which are reported to have anti-cancer and antioxidant activities (Semwal et al., 2015). Ginger (*Zingiber officinale* Roscoe) rhizome contains zingibain (EC 3.4.22.67), which was first identified in 1973 as a protease with high proteolytic activity (Thompson et al., 1973). The ginger protease has a high affinity for collagen as well as other connective tissue proteins, making it an excellent meat tenderizer (Kim et al., 2007). It also exhibits good milk clotting activity. Hence, it is utilized in the south of China to make a snack called ginger milk curd (Su et al., 2009). It demonstrates the optimum activity at a pH range of 6 to 8, and a temperature range of 50-60°C. Such characteristics of ginger protease are appropriate for cheesemaking in dairy industry (Hashim et al., 2011). Hence, the aim of this present work was to partially purify ginger protease and utilize it in the manufacturing of fresh cheese as well as compare its quality with chymosin cheese.

2. Materials and Methods

2.1 Materials

Fresh mature ginger rhizomes (Nashe variety) and raw cow milk of local breed were purchased from the local market of Dharan, Nepal. Chymosin (CHY-MAX® Powder Extra NB, Chr Hansen) was acquired from Trishul Trade Links, Kathmandu, Nepal. Casein (purified), ammonium sulfate, L-cysteine, bovine serum albumin (BSA) and dialysis membrane were procured from HiMedia Laboratories Pvt. Ltd. (India). Trichloroacetic acid (TCA) and Ethylenediamine tetra acetic acid (EDTA) were acquired from Thermo Fisher Scientific Pvt. Ltd. (India).

2.2 Methods

2.2.1 Ginger rhizome crude extract preparation

The ginger rhizomes were washed, peeled and cut into fine pieces. The pieces were then homogenized in a blender (Model Havel's max grind 14000) after mixing with cold sodium phosphate buffer (50 mM, pH 7.0) in the ratio of 1:1 (w/v). Under cold condition, the homogenate was stirred for 45 min, followed by filtration through cheese cloth. The filtrate thus obtained was centrifuged (4°C) at 5000 rpm (Model DLAB D3024R) for 10 min. The resulting supernatant was marked as crude extract (CE), and stored at 4°C (Gagaoua et al., 2015).

2.2.2 *Crude extract partial purification*

The partial purification of ginger rhizome crude extract was performed by using ammonium sulfate precipitation technique as mentioned by Andevari et al. (2019). The $(NH_4)_2SO_4$ saturations to a concentration of 30, 40, 50, 60, 70, and 80% (w/v) were carried out simultaneously. All the resulting saturations were centrifuged (4ºC) at 15000 rpm for 10 min. The pellets obtained were mixed in a little amount of phosphate buffer containing L-cysteine (10 mM) and EDTA (2.5 mM), followed by overnight dialysis (molecular weight cut off: 12 KDa) at 4°C with three changes of buffer. The obtained partially

purified ginger protease was analyzed for milk clotting activity, caseinolytic activity as well as protein content.

2.2.3 Milk clotting activity determination

The milk clotting activity (MCA) of partially purified ginger rhizome protease was identified using the method given by the International Dairy Federation (IDF, 2007). The reconstituted milk (pH 6.5) in 2 ml vial was incubated at 37°C for 5 min, and 200 µl protease was then added. At regular 10 s interval, the vial was rotated and checked for any signs of clot. One MCA unit is defined as the amount of enzyme that clots reconstituted skim milk (10 ml) in 40 min at 37°C (Berridge, 1952).

2.2.4 Caseinolytic activity

The determination of caseinolytic activity (CA) of partially purified protease was conducted following the procedure mentioned by Ladd & Butler (1972) using 1% casein as substrate (w/v) in phosphate buffer (pH 7). After incubation of equal volume (1 ml) of diluted enzyme and casein at 37°C for 30 min, 3 ml of 10% TCA was mixed for the reaction termination, followed by incubation on ice for 1 h, and centrifugation at 5000 rpm for 10 min. The absorbance readings of supernatant were noted using a double beam spectrophotometer (Agilent Cary 60 UV-Vis, US) at 280 nm. One CA unit is referred to as the quantity of enzyme which releases 1 µg tyrosine at standard assay condition.

2.2.5 Protein content

The Bradford protein-dye binding method was used to estimate quantitative protein determination of partially purified protease (Bradford, 1976). The absorbance readings were noted by a double beam spectrophotometer at 595 nm. The protein content was determined by using BSA (bovine serum albumin) standard curve.

2.2.6 Experimental design for cheesemaking

The influence of two independent variables (pH and temperature of milk) on response variables, namely TOC (time of coagulation) and MCA of partially purified protease, was analyzed using response surface methodology (RSM). The selection of independent variables and their limits was performed on the basis of preliminary researches (Hashim et al., 2011; Nafi et al., 2014). Two-factor central composite design (CCD) comprising 13 experimental runs was utilized for the optimization of milk pH and temperature in cheesemaking. The experiments were carried out in triplicate and the results were expressed as an average of three independent trials. The three coded levels utilized in the optimization study were -1, 0 and +1 as shown in Table 1. The fixed factor was enzyme concentration i.e., 200 µl was added in every 2 ml of milk. The experimental design, analysis of data, and building of model were performed using the Design Expert software (Version 13, Stat-Ease Inc., Minneapolis, MN).

Table 1: Range of factors for RSM/CCD

Factors	**-1**	**0**	**+1**
Milk pH	6	6.5	7
Milk temperature (°C)	35	45	55

The responses TOC and MCA for different experimental combinations were related to coded controlling variables (X_i, i = 1 and 2) by a second-degree polynomial equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1.X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon$$

Where Y was the predicted response and β terms were the coefficients computed by least squares method. The polynomial coefficients were expressed by β_0 (constant); β_1, β_2 (linear effects); β_{12} (interaction effect); β_{11}, β_{22} (quadratic effects); and ε (random error). The multiple regression analysis was carried out for data modelling, whereas analysis of variance (ANOVA) was performed for statistical significance of terms.

2.2.7 Cheesemaking

Two fresh cheeses were manufactured by following the procedure mentioned by Walstra et al. (2006) with some modifications. The first cheese produced by using chymosin was labeled as cheese A, whereas the second cheese produced by using ginger rhizome protease as cheese B. Fresh cow milk (2.93% fat and 7.98% SNF) was first heated until it reached a temperature of 80-85°C. For cheese A, the milk pH

was adjusted to 6.0 using 2% food grade GDL (glucono-delta-lactone) at 37°C, followed by the addition of chymosin at the rate of 2.5g/100L of milk. Similarly, the milk pH was first adjusted to 6.5 at 50°C, and then ginger protease (0.5%) was added for cheese B. The milk was stirred gently and left to coagulate for 40 min. The resulting curd was cut vertically and horizontally into 1 cm3 by the help of cheese wire, and it was then cooked at 45-50°C for 45 min with constant stirring. The whey was drained and the resulting curd grains were pressed for 6 h by placing it in cheese mould (195 × 125 mm) lined with cheese cloth. After pressing, the cheese obtained was immersed in 15% brine solution for 24 h, and the wiped dry cheese was vacuum packed in low-density polyethylene (LDPE) pouch to store at 4°C.

2.2.8 Physicochemical analysis of cheese

Fat: Fat content of cheese was determined by the method as per AOAC (2005).

Protein: Protein content of cheese was determined by Kjeldahl method as per AOAC (2005).

pH: pH of cheese was estimated by the method as per AOAC (2005).

Moisture: Moisture content of cheese was determined by oven-drying method as per AOAC (2005).

Ash: Ash content of cheese was determined by dry ashing method as per AOAC (2005).

Acidity: Acidity of cheese was determined by titrimetric method as per in AOAC (2005).

Calcium: Calcium content of cheese was estimated by the method as per Ranganna (1986).

Yield: The yield of cheese was determined by using the equation as per Nasr et al. (2016).

$$\text{Yield (\%)} = \frac{\text{Weight of cheese (kg)}}{\text{Weight of milk (kg)}} \times 100$$

2.2.9 Sensory evaluation of fresh cheeses

The prepared fresh cheeses were evaluated organoleptically by 10 semi-trained panelists, following the recommendations of IDF (2009) and Puglisi et al. (2014). The training session was first conducted to define the sensory terminologies and then, the cheese samples were presented in a properly ventilated and well-lit laboratory. The panelists evaluated each sample for sensory attributes (texture, spreadability, flavor, aftertaste and overall acceptance) by using a five-point hedonic scale, with 1 being poor and 5 being excellent quality.

2.2.10 Statistical analysis

The data were analyzed by RStudio (Version 2023.06.1 build 524) using Two Sample t-test at 5% level of significance. The graph was created using Microsoft Excel (2019). The data from RSM/CCD were analyzed in Design Expert software using ANOVA and fit statistics for significant model as well as R^2.

3. Results and Discussion

The crude extract was first isolated from ginger rhizome and then, partially purified by $(NH_4)_2SO_4$ precipitation. The impact of milk pH and temperature on TOC and MCA was analyzed by using RSM. The cheeses, manufactured from chymosin (A) and partially purified ginger protease (B), were examined for the physicochemical and sensory qualities.

3.1 Crude extract partial purification

Ginger rhizome crude extract (CE) was partially purified using various (NH4)2SO4 saturations (30-80%, w/v). The protease recovered at 50% (NH4)2SO4 saturation demonstrated the highest MCA (845.61 U/ml) (Figure 1). Similar finding in the purification of ginger (*Zingiber officinale* Roscoe) rhizome protease was expressed by Nafi' et al. (2013) and Gagaoua et al. (2015). Maskey & Karki (2022) also highlighted the highest activity of kiwifruit (*Actinidia deliciosa*) protease precipitated with 50% $(NH_4)_2SO_4$. At greater $(NH_4)_2SO_4$ concentration, the irreversible denaturation of protease might take place, whereas at lower concentration the SO_4^{2-} ions might not affect the hydrophobic protease surface.

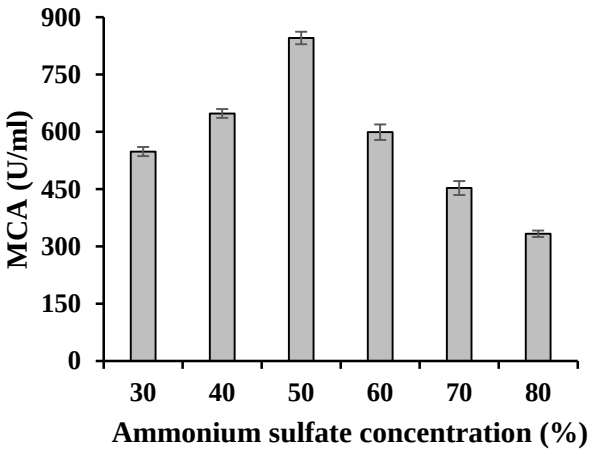

Figure 1. MCA of ginger rhizome protease precipitated at various $(NH_4)_2SO_4$ concentrations. Values are means ± standard deviation (SD) of the triplicates.

3.1.1 Recovery profile

The recovery profile of ginger rhizome protease precipitated at 50% (w/v) $(NH_4)_2SO_4$ precipitation is presented in Table 2. The purification fold and recovery % of partially purified ginger protease were found to be 1.64 and 85.76% respectively. Hashim et al. (2011) also mentioned similar recovery, but higher purification fold (2.66) of ginger protease. In partial purification of bromelain from pineapple core, Gul et al. (2021) observed a greater purification fold (2.28) and recovery (215.25%). However, a lower purification fold (1.56) and recovery (78.84%) of kiwifruit protease were reported by Maskey & Karki (2022). The reason behind the variation in both purification fold and recovery might be due to the difference in plant species, plant growth conditions, fruit maturity, temperature and ionic strength of extraction buffer, presence of contaminants and inhibitors, as well as protease stability.

3.2 RSM optimization in cheesemaking

For response surface methodology (RSM) analysis, the central composite design (CCD) was employed. The pH of milk (6, 6.5 and 7) and temperature of milk (35, 45 and 55°C) were utilized to determine the optimum time of coagulation (TOC) and milk clotting activity (MCA) of partially purified ginger protease.

3.2.1 CCD analysis

The measured values of TOC and MCA for partially purified ginger protease varied 18 to 140 s and 171.43 to 1333.33 U/ml respectively. The experimental outcomes are presented in Table 3. The outcomes from the experimental runs were fitted with second order quadratic polynomial regression equations. The equations of both TOC and MCA (in terms of coded factors) are demonstrated in Equations (1) and (2) respectively. Statistical significance of these regression equations was analyzed by ANOVA for the quadratic model of responses (Table 4 and 5).

$$TOC = 44.03+21.00\times A-40.50\times B-17.00\times AB+6.38\times A^2 +13.88\times B^2 \quad (1)$$

$$MCA = 560.32-167.32\times A+437.01\times B-51.67\times AB -24.13\times A^2+151.84\times B^2 \quad (2)$$

Where A and B are coded values of milk pH and milk temperature; AB, A^2 and B^2 are model terms.

From the analysis of TOC regression model (Table 4), the milk pH (A) exhibited positive significant ($p<0.05$) impact on TOC, whereas the milk temperature (B) exhibited negative significant ($p<0.05$) impact at a 95% confidence level. The interaction term of A and B (AB) showed negative significant ($p<0.05$) impact. But the quadratic term of A (A^2) showed non-significant ($p>0.05$) positive impact, while the quadratic term of B (B^2) showed significant ($p<0.05$) positive impact on TOC. This regression model is significant as evidenced by the p-value less than 0.0001, and the lack of fit is insignificant ($p>0.05$) indicating that the regression model can be fitted well. The predicted R^2 value was 0.9390, which is in reasonable agreement with the adjusted R^2 of 0.9824. Therefore, this regression model was used for analysis and prediction of TOC.

From the analysis of MCA regression model (Table 5), A exhibited significant ($p<0.05$) negative impact on MCA, whereas B exhibited significant ($p<0.05$) positive impact at a 95% confidence level. AB showed significant ($p<0.05$) negative impact. A^2 exhibited non-significant ($p>0.05$) negative impact, while B^2 exhibited significant ($p<0.05$) positive impact on MCA. The regression model is significant as revealed by the p-value less than 0.0001, and the

insignificant (p>0.05) lack of fit indicated well fitted model. Since the predicted R^2 of 0.9521 was in reasonable agreement with the adjusted R^2 of 0.9851, the MCA was analyzed and predicted using this regression model.

Figures 2 and 3 demonstrate the interactive effects of milk pH and temperature used to estimate the optimum TOC and MCA levels in the response surface plots. The TOC of ginger protease enhanced with the increase in milk pH, and decreased with the increase in milk temperature. The MCA of ginger protease enhanced with increase in milk temperature, and decreased with increase in milk pH. Similar properties of ginger protease was expressed by Nafi et al. (2014).

3.2.2 Optimization

Numerical optimization technique was utilized to determine the optimal combination of milk pH and temperature for the most effective TOC and MCA. The different parameters for this RSM study were presented in Table 6.

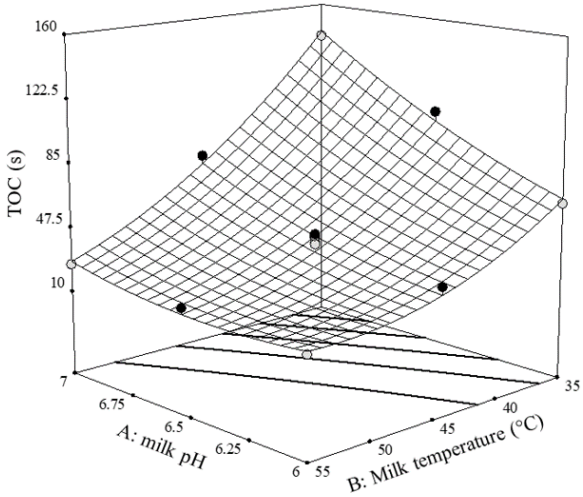

Figure 2: Response surface plot for TOC

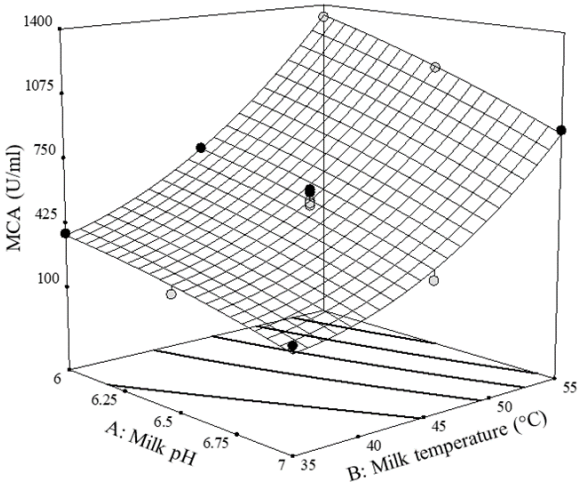

Figure 3: Response surface plot for MCA

Table 2: Recovery profile of ginger rhizome protease

Purification step	Total CA (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
CE	75.77	1.56	48.57	1	100
$(NH_4)_2SO_4$ precipitation (50%)	64.98	0.82	79.44	1.64	85.76

Table 3: Central composite design with experimental TOC and MCA values

Run	Factor 1 A: Milk pH	Factor 2 B: Milk temperature (°C)	Response 1 TOC (s)	Response 2 MCA (U/ml)
1	6	35	64	375

2	6.5	45	44	545.45
3	6.5	35	104	230.77
4	7	35	140	171.43
5	6	55	18	1333.33
6	7	45	76	315.79
7	6.5	45	40	600
8	6.5	55	21	1142.86
9	6.5	45	39	615.38
10	6.5	45	43	558.14
11	6	45	34	705.88
12	7	55	26	923.08
13	6.5	45	45	533.33

Table 4: ANOVA for TOC quadratic model

Source	**Sum of squares**	**df**	**Mean square**	**F-value**	**p-value**
Model	14615.28	5	2923.06	134.80	< 0.0001
A	2646.00	1	2646.00	122.02	< 0.0001
B	9841.50	1	9841.50	453.84	< 0.0001
AB	1156.00	1	1156.00	53.31	0.0002
A^2	112.40	1	112.40	5.18	0.0569
B^2	532.04	1	532.04	24.54	0.0017
Residual	151.79	7	21.68		
Lack of fit	124.99	3	41.66	6.22	0.0549
Pure error	26.80	4	6.70		

Cor Total	14767.08	12

Table 5. ANOVA for MCA quadratic model

Source	Sum of squares	df	Mean square	F-value	p-value
Model	1392000	5	278400	160.15	< 0.0001
A	168000	1	168000	96.64	< 0.0001
B	1146000	1	1146000	659.24	< 0.0001
AB	10679.66	1	10679.66	6.14	0.0423
A^2	1608.49	1	1608.49	0.9254	0.3681
B^2	63680.66	1	63680.66	36.64	0.0005
Residual	12167.25	7	1738.18		
Lack of fit	7120.96	3	2373.65	1.88	0.2737
Pure error	5046.29	4	1261.57		
Cor Total	1404000	12			

Table 6: Different parameters for optimization

Parameters	Goal	Lower limit	Upper limit
Milk pH	Target = 6.5	6	7
Milk temperature (°C)	In range	35	55
TOC (s)	In range	18	140
MCA (U/ml)	In range	171.43	1333.33

The optimal combinations were found to be 6.5 milk pH and 50°C milk temperature, having desirability 1. Under this optimum condition, the TOC and MCA of ginger protease were 27.25 s and 817 U/ml respectively.

3.2.3 Model verification

In order to confirm the adequacy of model regression equations, three experimental runs were performed. The results of the confirmatory tests are summarized in Table 7.

Since the mean observed values were close to the predicted values, showing less than 5% deviation, this regression model is accurate enough for the prediction of optimal values. Hence, milk pH of 6.5 and milk temperature of 50°C were chosen for fresh cheesemaking. Gagaoua et al. (2015) on characterization of zingibain (ginger protease) also expressed the similar outcomes.

3.3 Physicochemical characteristics of fresh cheeses

The physicochemical properties of fresh cheeses manufactured using chymosin (A) and partially purified ginger protease (B) are presented in Table 8.

Table 8. Physicochemical characteristics of prepared fresh cheeses

Parameters	Cheese A	Cheese B
Moisture (%)	$48.96^{b} \pm 0.1$	$52.01^{a} \pm 0.02$
Fat % (wb)	$27.85^{a} \pm 0.34$	$24.96^{b} \pm 0.28$
Protein % (wb)	$19.93^{a} \pm 0.05$	$19.34^{a} \pm 0.09$
Ash % (wb)	$2.66^{b} \pm 0.01$	$3.11^{a} \pm 0.03$
pH	$5.68^{b} \pm 0.01$	$6.42^{a} \pm 0.03$
Acidity (% lactic acid)	$0.21^{a} \pm 0.01$	$0.15^{b} \pm 0.01$
Calcium (mg/100 g)	$632.73^{a} \pm 5.49$	$624.66^{a} \pm 6.42$
Yield (%)	$12.96^{b} \pm 0.01$	$14.61^{a} \pm 0.04$

Note. Values represent mean ± SD of three determinations. Values in the same row with different superscript differ significantly at 5% level of significance.

The type of protease utilized to manufacture the fresh cheese exhibited a significant ($p<0.05$) impact on moisture content. The lower moisture content was observed in cheese A. Nawaz et al. (2011) and Khan & Masuq (2013) also reported higher moisture content in the cheese made from *Withania coagulans* extract as compared to rennet cheese. Similar percentage of moisture in the cheese made by plant proteases was mentioned by Mahajan & Chaudhari (2014). Cheese with a higher level of moisture destabilizes the network of protein, producing a smooth and soft texture in cheese manufactured from plant protease (Maskey & Shrestha, 2020). The difference observed in the fat content of the cheeses made by two coagulants was significant ($p<0.05$). Greater value of fat content was observed in cheese A. Similar findings in the cheeses manufactured by using chymosin and kiwifruit protease were expressed by Maskey & Karki (2022). The loss of fat during whey drainage might be the reason for the lower fat level in cheese B. Plant proteases having higher caseinolytic activity hydrolyze the casein network, leading to greater fat loss in whey (Nuñez et al., 1991). One of the most vital components in establishing the cheese body, texture as well as flavor is fat (Abd El-Gawad Mona et al., 2007). No significant difference ($p>0.05$) was found between the protein contents of both cheeses. However, the higher protein content was recorded in cheese A. This variation might be due to accelerated hydrolysis of β-casein by the coagulating enzyme (Kim et al., 2004). Previous studies by Khan & Masuq (2013) and Maskey & Shrestha (2020) also reported higher protein content in chymosin cheese. However, Hashim et al. (2011) reported a slightly higher protein content in Peshawari cheese made using ginger protease as compared to rennet cheese, which might be due to the quality of milk as well as processing methods. The difference found in the ash content between the cheeses made by two coagulants was significant ($p<0.05$). Higher ash content was observed in cheese B, which is in accordance with the findings of Kheir et al. (2011), Khan & Masuq (2013) and Maskey & Karki (2022). The ash content of cheese, which is represented by its mineral content, depends on the concentration of salt used during cheese brining. Significant difference ($p<0.05$) was observed in the pH of cheeses A and B. Higher pH was detected in cheese B, which might be due to the variation in the optimum pH of both coagulants for their respective activities. Similar outcomes were also revealed by Maskey & Shrestha (2020). There was a significant difference ($p<0.05$) in the acidity between both cheeses. Higher acidity was detected in cheese A, which is in consistent with the outcomes of Nuñez et al. (1991). This variation might be attributed to the formation of lactic acid from the lactose present in the cheese. No significant difference ($p>0.05$) was found in the calcium content of two cheeses A and B. However, cheese A had higher calcium content than cheese B, which is in line with the findings of Maskey & Shrestha (2020) and Adhikari et al. (2021). The caseins become more hydrated when the bound calcium content in milk decreases (Joshi et al., 2004). Hence, the calcium content of the cheese is inversely related to its moisture content. The cheese B had significantly ($p<0.05$) higher yield than cheese A, which might be associated with the higher moisture content in cheese B (Johnson et al., 2001).

3.4 Sensory evaluation of fresh cheeses

The mean sensory scores of prepared fresh cheeses A and B are presented in Figure 3. The type of coagulant significantly ($p<0.05$) affected the texture, spreadability and aftertaste of the fresh cheeses A and B, but not significantly ($p>0.05$) affected the flavor and overall acceptance. The mean texture score was comparatively higher for cheese A, which indicated that the panelists preferred a firm texture over soft and creamy. The texture of cheese prepared with plant protease is a result of primary proteolysis by the activity of plant coagulant, rather than native enzyme and predominant microorganisms (Sousa & Malcata, 1997).

Table 7: Predicted and observed values of the responses

Response	Optimal conditions		Predicted mean value	Observed mean value	Deviation (%)
	pH	Temperature (°C)			
TOC (s)	6.5	50	27.25	28.67	4.95
MCA (U/ml)	6.5	50	816.79	837.44	2.47

The high level of proteolysis in cheese prepared with plant coagulant may hydrolyze the casein network, resulting in soft and creamy cheese (Tejada et al., 2007). The mean spreadability score was higher for cheese B, which might be attributed to its smooth and soft texture as well as higher moisture content. Maskey & Shrestha (2020) also reported the higher spreadability of cheese produced by using papain enzyme as compared to rennet cheese. The cheese B had a slightly lower score for flavor, which indicated that the panelists favored milky smell of cheese A over spicy smell of cheese B. Kheir et al. (2011) also reported the slightly higher flavor score for rennet cheese as compared to the cheese produced by using *Solanum dubium* fruit extract. The cheese B had also a lower score for aftertaste, which revealed that the mild bitter aftertaste was observed in cheese B. This bitterness is mainly caused by the formation of bitter peptides, which contain a greater amount of hydrophobic amino acid residues (Singh et al., 2003). Since no significant difference ($p>0.05$) in overall acceptance of two cheeses A and B was observed, hence the ginger protease could be utilized as a plant coagulant in the manufacturing of soft cheeses.

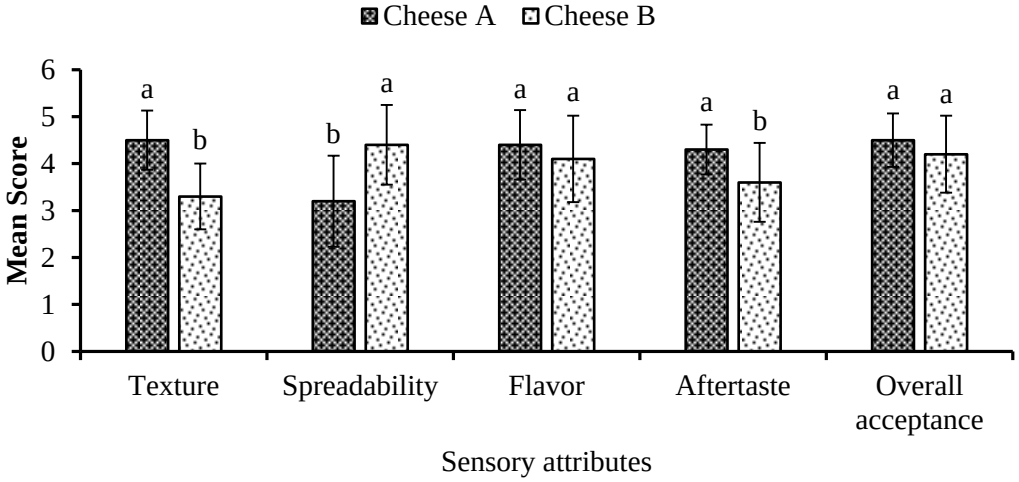

Figure 4: Graphical view of mean sensory scores of fresh cheeses. Values represent the mean ± SD (n = 10). Values having different alphabets on the top of the bars are significantly different ($p<0.05$).

4. Conclusion

Based on the findings of this research work, ginger rhizome protease can be utilized as an effective rennet substitute in manufacturing of fresh cheese. By using 50% $(NH_4)_2SO_4$ saturation (w/v), the crude ginger rhizome extract was partially purified to 1.64 purification fold and 85.76% recovery. The milk pH of 6.5 and milk temperature of 50°C were the optimum conditions for producing the cheese using partially purified ginger protease. The produced fresh cheese had physicochemical and sensory properties comparable to the rennet cheese. However, the quality analysis of ripened cheese produced by using purified ginger protease is needed for its application.

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Conflicts of Interest

The authors declare that there do not have any conflict of interest.

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