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Antigenicity of Hydrolysed Casein in Goat's Milk Using Bromelain

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ABSTRACT

Milk is a major source of infant nutrition but is also a common allergen associated with cow's milk protein allergy (CMPA), which requires strict avoidance of milk proteins. Goat's milk has been proposed as an alternative due to its distinct protein composition; however, conserved casein epitopes may still trigger immune responses. While enzymatic hydrolysis is well established for reducing the antigenicity of cow's milk proteins, studies on goat's milk casein hydrolysis remain limited. This study evaluated the time-dependent hydrolysis of goat milk casein using bromelain (1:100, pH 5.5, 55 °C, 1-4 h) and its effects on protein degradation and antigenicity. The novelty of this study lies in the application of bromelain to hydrolyse goat milk casein, an area that remains less explored than the well-established hydrolysis of cow milk proteins. Degree of hydrolysis (DH), peptide content, SDS-PAGE, and ELISA were used for characterisation. Results showed increasing DH and peptide content with hydrolysis time ($p < 0.05$), with peptides mainly below 15 kDa. ELISA indicated that 1 h hydrolysis achieved the lowest antigenic reactivity. Overall, bromelain effectively reduced the antigenic reactivity of goat's milk casein under mild hydrolysis conditions, demonstrating its potential for modifying goat milk casein and supporting further investigation for specialised dairy and infant nutrition applications.

Keywords: antigenicity, bromelain, casein, ELISA, goat's milk, hydrolysis

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INTRODUCTION

Food allergies in both children and adults have increased significantly in recent years, with elimination diets remaining the primary management strategy. In Malaysia, approximately 5–15% of young children experience allergic symptoms in early life, with cow's milk protein allergy (CMPA) identified as one of the most common food allergies (Malaysian Paediatric Association & Malaysian Society of Allergy and Immunology, 2024). Major allergens such as milk, egg, fish, nuts, and cereals must be declared on food labels due to their potential to trigger hypersensitivity reactions (Food and Drug Administration, 2026). Cow's milk proteins, particularly casein and whey, are major allergens responsible for CMPA (Jensen et al., 2022). As cow's milk is often introduced early in infancy, its exclusion can lead to nutritional deficiencies, necessitating the use of alternative formulas such as extensively hydrolysed formulas (eHF), partially hydrolysed formulas (pHF), amino acid formulas (AAF), or soy-based formulas (SBF) (Ballabio et al., 2011). However, the suitability of these alternatives depends on clinical severity and patient tolerance.

Goat's milk has gained attention as a potential alternative due to its nutritional similarity to cow's milk and reportedly lower allergenicity. Some studies suggest that a proportion of CMPA patients may tolerate goat's milk, possibly due to its lower αs1-casein content (Benjamin-van Aalst et al., 2024). However, cross-reactivity between cow's and goat's milk proteins, particularly caseins, has been reported, indicating that goat's milk is not universally safe for CMPA patients (Mansor et al., 2023). Therefore, further modification may be required to reduce its antigenic reactivity.

Enzymatic hydrolysis is widely used to modify milk proteins and reduce their antigenic reactivity by breaking proteins into smaller peptides and altering antibody-recognised regions. The effectiveness of hydrolysis depends on factors such as enzyme type, pH, temperature, and reaction time, as different proteases can produce different cleavage patterns and peptide profiles (Medeiros et al., 2014). Proteases such as bromelain, papain, and alcalase have been investigated for milk protein hydrolysis, demonstrating the importance of enzyme selection in determining the extent of protein degradation and antigenic reactivity. Although enzymatic hydrolysis can reduce the antigenic reactivity of milk proteins, residual immunoreactive regions may remain (He et al., 2025).

Compared with the well-established research on cow's milk proteins, studies on the enzymatic hydrolysis of goat's milk proteins remain limited. In particular, the time-dependent hydrolysis of goat's milk casein using bromelain and its relationship with antigenic reactivity remain insufficiently characterised. Therefore, the present study advances current knowledge by evaluating the bromelain-mediated hydrolysis of goat's milk casein at different hydrolysis times and by relating the progression of hydrolysis to protein degradation and antigenic reactivity under mild hydrolysis conditions. This study aimed to evaluate the effect of bromelain hydrolysis time on the degree of hydrolysis and antigenicity of goat milk casein.

METHODOLOGY

All chemicals and reagents used in this study were of analytical grade. Sodium hydroxide (NaOH), potassium chloride (KCl), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from R&M Chemicals (Essex, UK). Sulphuric acid and sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) were obtained from Chemiz (Shah Alam, Selangor, Malaysia). Sodium sulphate anhydrous, methylene blue, glycine, and methanol were procured from Fisher Scientific (Leicestershire, UK), while ethanol was supplied by Systerm (Shah Alam, Selangor, Malaysia).

Phosphate-buffered saline (PBS) was prepared as a 10X stock solution containing 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4 \cdot 2H_2O$, and 18 mM KH_2PO_4, and adjusted to pH 7.4 using NaOH or HCl. Deionised water (18.2 MΩ·cm) was obtained from a Milli-Q Water Purification System (Merck KGaA, Burlington, MA, USA). Bovine serum albumin (BSA) was purchased from Amresco, LLC (Solon, OH, USA).

DL-dithiothreitol (DTT), β-mercaptoethanol, ammonium persulfate (APS), sodium dodecyl sulfate (SDS), Tris, Bradford reagent, 30% acrylamide/bis-acrylamide solution were obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Bromelain enzyme, o-phthaldialdehyde (OPA), and L-serine were purchased from Acros Organics (St. Louis, MO, USA). Tween 20, acetic acid, sulphuric acid, hydrochloric acid, Immobilon®-P PVDF membrane, N, N, N′, N′-tetramethylethylenediamine (TEMED), and NBT-BCIP were obtained from Merck (Kenilworth, NJ, USA). Bromophenol blue, boric acid, selenium dioxide, and Coomassie Brilliant Blue G-250 were purchased from Merck (Darmstadt, Germany). The 1-Step Ultra TMB-ELISA substrate was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

The N-terminal (Nter) and C-terminal (Cter) immunoglobulin G (IgG) antibodies were generated against αs1-casein peptides derived from goat milk and produced in rabbits, as previously reported by Mohsin et al. (2020). Horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-rabbit IgG (H + L) (streptavidin-HRP) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Isolation of Casein (CSN)

Goat milk was obtained from a single bulk batch collected from Saanen goats. Isolation of casein (CSN) from goat milk was performed as described by Hodgkinson et al. (2012), with minor modifications to the precipitate-washing step. Frozen goat milk samples were thawed and pooled at room temperature (24 °C), followed by heating to 45 °C. The milk was then centrifuged using a Sartorius 3-18K centrifuge (Göttingen, Germany) at 6000 rpm for 30 min at 4 °C.

After centrifugation, the fat layer was carefully removed, and the pH of the skimmed milk was adjusted to 4.2 using 1 M HCl to precipitate casein. The acidified skim milk was

stirred for 30 min and centrifuged again at 6000 rpm for 15 min at 20 °C to collect the casein precipitate.

The resulting precipitate was washed by resuspending it in purified water at ten times the initial milk volume and stirred for 1 h prior to re-centrifugation under the same conditions. The isolated casein was subsequently lyophilised and stored at −20 °C until further analysis.

Hydrolysis of Casein

Enzymatic hydrolysis of casein was performed as described by Zarei et al. (2015), with modifications to the time intervals and pH conditions. Lyophilised casein was hydrolysed using bromelain (EC 3.4.22.32; stem-derived; ≥2500 GDU/g; ≥5.0 FIP U/mg) at a substrate-to-enzyme ratio (S: E) of 100:1. The bromelain enzyme was used according to the activity specified by the manufacturer and stored under the recommended conditions prior to use. The hydrolysis conditions (pH 5.5 and 55°C) were chosen based on the optimal catalytic activity of bromelain reported in previous studies, enabling efficient hydrolysis while maintaining enzyme stability.

Briefly, 1.4 g (1 mM) of lyophilised casein was dissolved in 50 mM acetate buffer (45 mM sodium acetate and 5 mM acetic acid) at pH 5.5 and incubated at 55 °C. The casein solution was pre-heated in a water bath (Memmert GmbH + Co. KG, Buchenbach, Germany) at 80 °C for 20 min to denature the protein. The solution was subsequently cooled to 55 °C before adding bromelain (powdered form, 15 mg, 0.01 mM) to initiate hydrolysis.

Aliquots (2 mL) were withdrawn at 1-h intervals (1-4 h) into 2-mL microcentrifuge tubes to monitor the hydrolysis process. Enzyme activity was terminated by heating the samples in boiling water for 10 min. The samples were then centrifuged at 10,000 rpm for 10 min at 4 °C, and the resulting supernatant was collected and stored at −20 °C for further analysis.

Protein Quantification

Determination of Crude Protein

The crude protein content of each sample was determined using the Kjeldahl method in accordance with AOAC International Official Method 992.10 (2023). Milk samples (0.15 g) were weighed in triplicate (n = 3) and mixed with a catalyst mixture containing 96% sodium sulphate anhydrous, 3.5% copper sulphate, and 0.5% selenium dioxide.

Subsequently, 2.5 mL of concentrated sulphuric acid was added, and the mixture was digested using a protein digester (Turbotherm digestion unit, Gerhardt GmbH & Co. KG, Königswinter, Germany) for 30 min until the solution turned blue-green. After cooling for 1 h, 10 mL of distilled water and 10 mL of 45% NaOH were carefully added to the digested mixture.

Boric acid solution was prepared by mixing 10 mL of 2% boric acid with two drops of indicator solution (0.2% methyl red and 0.1% methylene blue in 96% ethanol). The digested sample was then steam-distilled into the boric acid solution using a distillation unit (Gerhardt GmbH & Co. KG, Königswinter, Germany) for 3 min.

The borate formed during distillation was titrated with 0.05 N sulphuric acid until the endpoint colour changed from green to purple. A blank sample was analysed using the same procedure. The percentage of nitrogen was calculated using the formula as shown in Equation 1:

$$\text{Nitrogen (\%)} = \frac{(Vs - Vb) \times N \times 1.4}{W} \quad [1]$$

Where:
Vs = Volume of H_2SO_4 used to titrate H_3BO_3 (sample)
Vb = Volume of H_2SO_4 used to titrate blank
N = Normality of H_2SO_4
W = Weight of sample (g)

The percentage of nitrogen obtained was multiplied by the dairy conversion factor (6.38) to determine the percentage of crude protein in the goat milk sample.

Bradford Assay

The Bradford protein assay (microplate method) was performed according to the protocol provided by Bio-Rad Laboratories to quantify total soluble protein in milk samples, lyophilised CSN, and hydrolysed CSN.

Serial dilutions of each sample (1:5, 1:10, 1:50, and 1:100) were prepared in phosphate-buffered saline (PBS). Aliquots (10 µL) of each diluted sample were pipetted into individual wells of a 96-well microtiter plate in triplicate (n = 3). Bradford reagent was diluted 1:4 (v/v) with deionised water, and 200 µL was added to each well. The contents were gently mixed to avoid bubble formation and allowed to react for 2 min at room temperature (24 °C).

Absorbance was measured at 595 nm (A595) using a microplate reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Protein concentrations were calculated from a linear standard curve generated using bovine serum albumin (BSA) at concentrations of 0.125, 0.250, 0.375, 0.500, and 0.750 mg/mL.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Lyophilised CSN, purified αs1-CSN, and hydrolysed CSN samples were analysed by SDS-PAGE according to the method of Laemmli (1970).

A 12% separating gel was prepared by mixing 6.4 mL of 30% acrylamide/bis-acrylamide, 4 mL of 1.5 M Tris-HCl (pH 8.8), 160 µL of 10% SDS, 160 µL of 10% APS, 16 µL of TEMED, and 5.3 mL of deionised water.

Sample buffer consisted of 3.55 mL deionised water, 1.25 mL 0.5 M Tris-HCl (pH 6.8), 2.5 mL glycerol, 2.0 mL 10% SDS, and 0.2 mL 0.5% (w/v) bromophenol blue. Samples (10 µg/mL) were mixed with sample buffer at a 1:1 (v/v) ratio and heated at 95 °C for 4 min. A 10 µL volume per sample was loaded onto the gel and electrophoresed at 110 V for 80 min.

Gels were stained overnight using staining solution containing 0.05% (w/v) Coomassie Brilliant Blue G-250, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid, followed by destaining for 2 h using 40% (v/v) methanol and 7% (v/v) acetic acid. Protein bands were identified using Precision Plus Protein Dual Colour Standards (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Determination of Degree of Hydrolysis Using o-Phthaldialdehyde (OPA)

To determine complete hydrolysis, 0.5 g of casein was mixed with 15 mL of 6 N HCl and incubated at 110 °C for 24 h in an oven (Memmert UFE400 oven, Memmert GmbH + Co. KG, Germany). After cooling to room temperature (24 °C), samples were centrifuged at 5000 rpm for 10 min at 4 °C, and the supernatant was collected.

OPA reagent was freshly prepared from three solutions:

i. Solution A: 200 mg SDS and 7.62 g sodium tetraborate dissolved in 150 mL deionised water
ii. Solution B: 160 mg OPA dissolved in 4 mL of 96% ethanol
iii. Solution C: 400 µL β-mercaptoethanol diluted to 50 mL with deionised water

In a 96-well Maxisorp plate (Nunc, Thermo Scientific, Waltham, MA, USA), 36 µL of hydrolysed sample was mixed with 270 µL OPA solution. After 2 min incubation in the dark at room temperature, absorbance was measured at 340 nm using a microplate reader (Millipore, Darmstadt, Germany).

The degree of hydrolysis (DH, %) was calculated as in Equation 2:

$$\text{DH (\%)} = \frac{h_{hydrolysed} - h_{substrate}}{h_total} \times 100 \quad [2]$$

Where:

$h_h$ydrolysed = absorbance of hydrolysed casein
$h_s$ubstrate = absorbance of non-hydrolysed casein
$h_t$otal = absorbance after complete hydrolysis

An L-serine standard curve ($y = 3.9362x + 0.0009$; $R^2 = 0.9998$) was used to determine peptide concentration.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antigenicity of hydrolysed casein at different hydrolysis times was evaluated using an in-house sandwich ELISA as described by Mohsin et al. (2023). Capture (Nter-IgG) and detection (Cter-IgG) antibodies were previously developed in rabbits (Mohsin et al., 2020).

Nter-IgG was diluted (1:1000 v/v) in carbonate buffer (pH 9.6) and coated onto a Nunc Maxisorp 96-well plate overnight (16 h) at 4 °C. Wells were blocked with 2.5% BSA in PBS for 2 h at room temperature using an orbital shaker at 150 rpm.

Samples (100 µL) were added and incubated for 1 h. Cter-IgG (1:2,500 v/v in PBS) was then added and incubated for 1 h. Streptavidin-horseradish peroxidase (Strep-HRP; 1:15,000 v/v in PBS) was added and incubated for 1 h under similar conditions. Between steps, wells were washed three times with 250 µL of 0.05% Tween 20 in 1X PBS for 1 min with agitation.

After adding 50 µL of 1-Step Ultra TMB ELISA substrate, colour development was monitored, and the reaction was stopped with 50 µL of 0.1 M H_2SO_4. Absorbance was measured at 450 nm using a Multiskan RC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA)

Statistical Test

All absorbance values were measured in triplicate (n = 3). Absorbance data were analysed using mean and standard deviation. Minitab (Pty Ltd, Sydney, Australia) statistical software, Version 21, was used for the analysis of variance (ANOVA). A post hoc Tukey test was then conducted to compare pairs of means across treatment levels. Significant differences were calculated at $\alpha = 0.05$ (95% confidence interval).

RESULT AND DISCUSSION

Isolation of Casein (CSN)

Casein was isolated from goat milk by acid precipitation and subsequently lyophilised for further analysis. At acidic pH (approximately pH 4.6), the surface charge of κ-casein is neutralised, resulting in destabilisation of casein micelles. The disruption of intra- and inter-chain interactions prevents the proteins from remaining in the soluble phase. Consequently, micelles aggregate and precipitate, allowing separation from whey proteins (Corredig, 2019).

In this study, the total yield of lyophilised casein was 1.65 g per 100 mL of goat milk (1.65%). The crude protein content of goat milk was determined by the Kjeldahl method in accordance with AOAC International Official Method 992.10 (2023), yielding a total of 3.87%. A previous study by Mohsin et al. (2019) reported a protein content of 4.83% in Saanen goat milk. The lower protein content observed in the present study may be attributed

to methodological differences, as Mohsin et al. (2019) quantified amino acid composition, whereas the present study estimated total protein indirectly via nitrogen determination.

The concentration of total soluble protein in the isolated casein, determined using the Bradford assay, was 1.455 mg/mL. The assay is based on the binding of Coomassie Brilliant Blue G-250 dye to specific amino acid residues, with protein concentration determined from absorbance values. However, the Bradford assay may underestimate protein concentration if the protein contains a low proportion of aromatic residues, thereby limiting dye-binding efficiency (Martine & Vojtech, 2015).

The chemical composition of goat milk varies depending on breed, age, weight, diet, lactation stage, season, and environmental temperature (Mohsin et al., 2019). In this study, individual casein subunits were not quantified. According to Tziboula-Clarke (2002), total casein content in goat milk ranges from 2.33% to 4.63%, with β-casein representing the predominant fraction. The αs1-casein content in goat milk (0-28%) is significantly lower than that in cow milk (50-53.6%). Goat milk composition is highly influenced by lactation stage, with higher levels of primary components during early lactation, followed by a decline and a subsequent increase toward the end of lactation. Additionally, genetic polymorphism of casein within the same breed can contribute to compositional variability (Yadav et al., 2016). Therefore, the relatively low total casein yield observed in this study may be attributed to differences in lactation stage and to potential casein loss during whey protein removal.

Enzyme Hydrolysis

Degree of Hydrolysis (DH)

The degree of hydrolysis (DH) represents the percentage of peptide bonds cleaved by the enzyme. Casein was hydrolysed using bromelain at pH 5.5 and 55˚C for 1, 2, 3, and 4 h. The Michaelis-Menten constant (K_M) of casein for bromelain was reported at 0.138 mM, lower than that of haemoglobin (0.165 mM) (Corzo et al., 2012), indicating that casein is a suitable substrate for bromelain. Figure 1 shows the DH of casein hydrolysate over time, quantified using the o-phthaldialdehyde (OPA) method.

The DH of casein (CSN) hydrolysate ranged from 10.64% to 26.95%, with the highest DH observed after 4 h of hydrolysis (26.95%), indicating that prolonged hydrolysis time resulted in greater casein cleavage by bromelain ($p < 0.05$). The hydrolysis rate increased linearly up to 4 h and did not reach saturation. In comparison, hydrolysis of camel milk by alcalase, chymotrypsin, and papain plateaued after 2 h (Kumar et al., 2016). Although a similar enzyme-to-substrate (E:S) ratio of 1:100 was applied (Kumar et al., 2013), differences in enzyme specificity result in varying DH values.

Shu et al. (2018) reported a maximum DH of 20.4% for bromelain-treated goat milk, with the hydrolysis rate declining after 50 min due to limited availability of cleavable

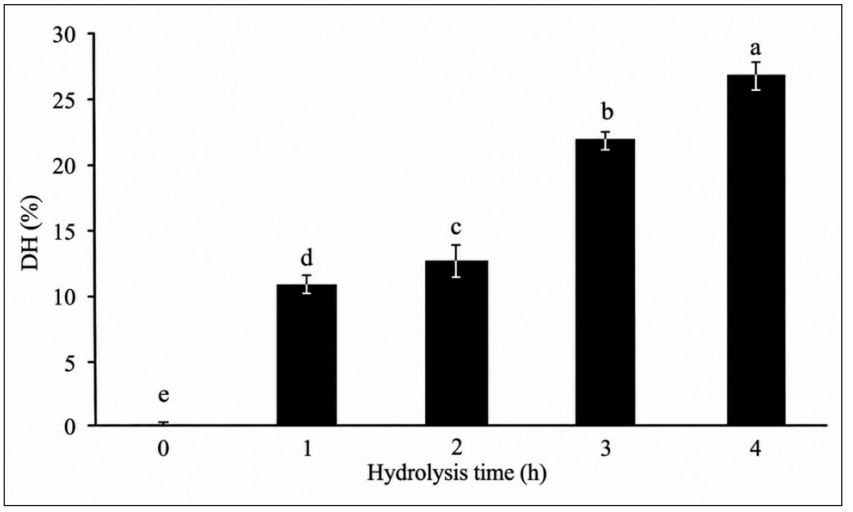

Figure 1. Degree of casein hydrolysis using bromelain. Different letters signify a significant difference ($p<0.05$) between the DH (280 nm) at different hydrolysis times. Data is presented as mean ± SD of triplicates (n=3)

peptide bonds, likely reaching saturation. They also suggested that performing hydrolysis at pH 6.0, close to the isoelectric point (pI) of caseins (4.6) and whey proteins (5.2), can promote protein aggregation, limiting peptide exposure for enzymatic cleavage.

The optimal hydrolysis conditions of the enzyme are crucial to achieving maximum activity. Bromelain exhibits optimal activity at 50°C and pH 6.5, remaining stable at pH 3-7 and 40-60°C (Mohapatra et al., 2013). Corzo et al. (2012) reported that over 90% of bromelain activity occurs at pH 6-7 with casein and sodium caseinate as substrates, and activity increases from 45°C to 59°C but declines significantly above 59°C. To enhance hydrolysis without denaturing the enzyme, this study was conducted at 55°C. For comparison, papain-treated camel casein achieved 16% DH after 5 h at 55°C and pH 6.5 (Kumar et al., 2016). This demonstrates that both optimal hydrolysis conditions and enzyme–substrate specificity influence enzymatic activity and DH.

Peptide Content in Casein Hydrolysate

Bromelain cleaves long polypeptide chains in casein into smaller peptides, releasing free amino acids that serve as an indicator of proteolytic activity. Figure 2 shows that peptide content increased with hydrolysis time, indicating that bromelain cleaves at multiple sites. Peptide concentration rose significantly ($p < 0.05$) from 0.14 to 3.90 mg/mL over the hydrolysis period, with the most substantial increase occurring during the first hour (0.14 to 1.96 mg/mL), consistent with the initial rise in DH (0.00 to 10.64%).

The results confirm that bromelain progressively hydrolyses casein, producing peptides in a time-dependent manner, with rapid cleavage occurring in the early stages of hydrolysis.

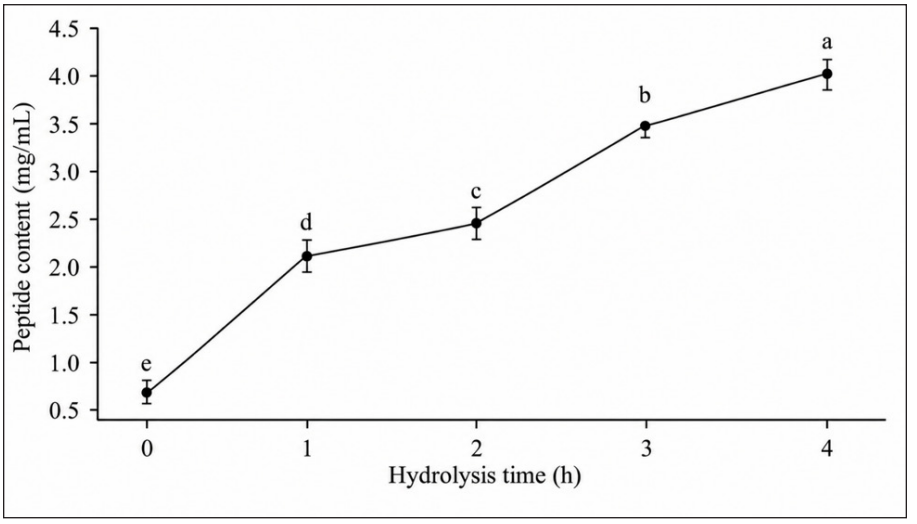

Figure 2. Peptide content of hydrolysed casein. Different letters indicate a significant difference ($p<0.05$) between the peptide content (280 nm) at different hydrolysis times

Proteolytic Activity and Molecular Weight Distribution of Hydrolysed Casein

The proteolytic activity of bromelain in this study ranged from 3 to 7 units/mg of protein. One unit of bromelain was defined as the amount of enzyme that releases 1.0 µmol of ρ-nitrophenol from N-α-Z-L-lysine ρ-nitrophenol per minute at pH 4.5 and 25°C (Medeiros et al., 2014). Bromelain cleaves peptide bonds adjacent to lysine, arginine, phenylalanine, and tyrosine residues (Mudgil et al., 2019). Medeiros et al. (2014) reported a preference for hydrolysing glutamic acid, aspartic acid, lysine, or arginine at the P1 site.

Substrates suggested for bromelain hydrolysis include azocasein, azoalbumin, casein, sodium caseinate, and haemoglobin. These substrates exhibit low K_M values (azoalbumin 0.026, azocasein 0.037, sodium caseinate 0.088, casein 0.138, haemoglobin 0.165 mM) and high V_{max}/K_M ratios (azoalbumin 0.096, azocasein 0.104, sodium caseinate 0.020, casein 0.022, haemoglobin 0.014), indicating efficient enzymatic activity (Corzo et al., 2012). Saptarini et al. (2019) also reported a V_{max} of 8.11 IU/mg·min and K_M of 1.00 mg/mL for bromelain degrading casein. A low K_M reflects high substrate affinity, confirming that casein is a suitable substrate for bromelain.

SDS-PAGE analysis of hydrolysed casein. Figure 3 showed a significant decrease in molecular weight compared to whole casein. After 1 hour of hydrolysis, casein was rapidly cleaved into peptides smaller than 15 kDa. With increasing hydrolysis time, the number of low-molecular-weight peptide bands increased, indicating progressive breakdown of larger peptides. These observations are consistent with Wang et al. (2013), who reported that enzymatic hydrolysis results in the gradual disintegration of high-molecular-weight

peptides into smaller fragments. This indicates that bromelain efficiently cleaves casein into low-molecular-weight peptides, which correlates with the observed increase in the degree of hydrolysis and peptide content over time.

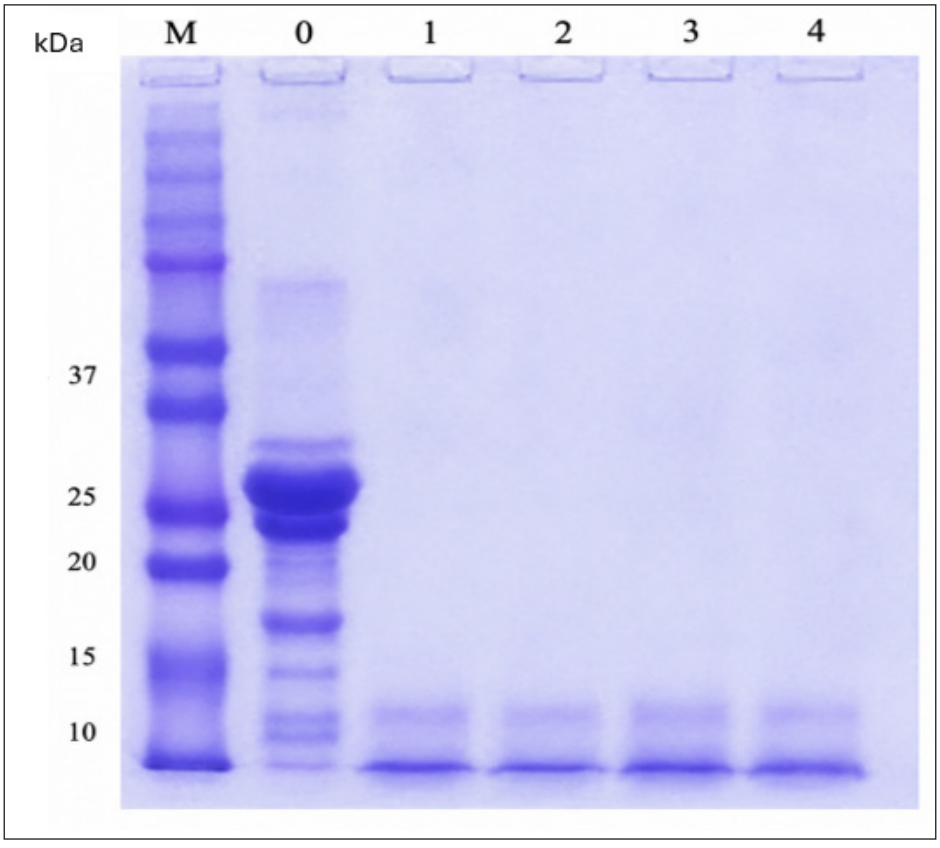

Figure 3. SDS-PAGE of casein hydrolysate. M: Marker; 0: unhydrolyzed casein; 1-4: Casein hydrolysed at 1, 2, 3, and 4 h. Marker was loaded in 10 µL. Each well was loaded with 10 ug/mL of protein. SDS-PAGE was stained using Coomasie Blue G-250

Implications of Molecular Weight on Antigenicity

Bromelain hydrolysis cleaved casein into peptides with molecular weights below 15 kDa after just 1 h. Enzymatic hydrolysis can yield either extensively or partially hydrolysed proteins, though these terms are not strictly defined. Extensively hydrolysed proteins often produce bitterness and astringency, which are undesirable in milk products. Partially hydrolysed peptides are preferred for sensory qualities; however, their antigenicity or allergenicity may persist (Lau et al., 2025).

The molecular weight of peptides is critical for immunogenicity. Peptides with lower molecular weights generally show reduced allergenic potential. For example, fractions

of whey protein concentrate (WPC) and β-lactoglobulin hydrolysates with molecular weights between 1,000 and 5,000 Da exhibited the lowest antigenicity (Svenning et al., 2000). Extensively hydrolysed casein-based formulas with molecular weights below 3,000 Da were administered to 30 CMPA patients, all of whom showed no adverse reactions, demonstrating safety for IgE-mediated cow's milk allergy (Terheggen-Largo et al., 2002).

Despite enzymatic hydrolysis, residual antigenicity may remain. Papain-treated bovine α- and β-casein exhibited reduced, but not fully eliminated, antigenicity (Liu et al., 2012). Similarly, buffalo milk αs1-casein hydrolysates treated with trypsin, α-chymotrypsin, or pepsin exhibited maximum reductions of 85%, 63%, and 60%, respectively, yet infants with CMPA still reacted to these hydrolysates (Ahmad et al., 2016). These studies underscore that enzymatic treatment can reduce, but not always completely abolish, the antigenicity of casein.

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

To evaluate the antigenicity of the hydrolysed casein, the hydrolysates were tested for their binding to in-house αs1-casein polyclonal antibodies using a sandwich ELISA. In this assay, N-terminal (Nter) IgG antibodies were coated on high-binding 96-well plates to capture casein peptides. Detection was achieved using a secondary enzyme-conjugated antibody targeting a different epitope on the same antigen. This setup allows quantification of residual antigenicity in hydrolysed

Table 1 shows that ELISA coated with Cter-IgG exhibited a higher background signal compared to ELISA coated with Nter-IgG alone. This observation may be due to Cter-IgG binding directly onto the plate, resulting in non-specific binding (NSB) of antibodies. NSB can negatively affect both the accuracy and reproducibility of ELISA measurements. A sufficient blocking step is therefore essential to saturate unoccupied sites on the solid phase and minimise NSB (Jiang et al., 2021). Blocking has been identified as a major contributor to NSB; however, Güven et al. (2014) reported that extending blocking from 30 min to 1 h did not significantly reduce NSB. Common blocking solutions, such as BSA, skimmed milk, or casein, can inhibit hydrophobic and electrostatic interactions of proteins, thereby reducing background signal. These solutions may contain bovine IgG, which

Table 1
Negative controls (NC) were included in the ELISA test

Negative controls (NC)	Mean ± standard error of the mean (SEM)
NC 1[a]	0.411 ± 0.018
NC 2[b]	0.537 ± 0.001
NC 3[c]	0.569 ± 0.013

Note. Data are reported as the mean of triplicates ± standard error of the mean (SEM), calculated as SD/√n, where n = 3
[a]ELISA coated with Nter IgG, followed by PBS and Strep-HRP.[b] ELISA coated with Nter IgG, followed by PBS, Cter IgG, and Strep-HRP.[c] ELISA coated with Nter IgG, followed by bovine α-casein, Cter IgG, and Strep-HRP

can bind secondary antibodies and induce cross-reactivity. Insufficient washing with non-ionic detergents, such as Tween-20 or Triton X, can further contribute to NSB in ELISA assays (Buchwalow et al., 2011).

Table 2
Normalised absorbance values of untreated, treated, and hydrolysed caseins tested in the ELISA study

Caseins		Normalised absorbance values (mean ± SEM)
Untreated		0.464 ± 0.031^{a}
Heat treatment	60˚C	0.257 ± 0.037^{d}
	80˚C	0.315 ± 0.025^{d}
	90˚C	0.383 ± 0.022^{c}
Heat and chemical treatment	60˚C + 0.1% SDS	0.440 ± 0.016^{b}
	80˚C + 0.1% SDS	0.498 ± 0.022^{a}
	90˚C + 0.1% SDS	0.516 ± 0.019^{a}
Hydrolysed casein	1 h	0.141 ± 0.004^{g}
	2 h	0.178 ± 0.006^{f}
	3 h	0.208 ± 0.032^{f}
	4 h	0.268 ± 0.016^{e}

Note. Caseins were hydrolysed by bromelain at an S:E ratio of 100:1, pH 5.5, and 55˚C (Section 3.4). Different small letters indicate significant differences ($p<0.05$) between absorbance values at 450 nm

The antigenicity of whole casein and hydrolysed caseins was evaluated using indirect ELISA. Whole casein treated at 90˚C with 0.1% SDS produced the highest ELISA signal, indicating effective dissociation of casein micelles. Heat treatment is known to destabilise casein micelles by dissociating κ-casein (Dumpler et al., 2017), and at temperatures above 80˚C, β-lactoglobulin from whey binds to κ-casein via disulfide bonds, further destabilising micelles (Oka et al., 2022). SDS, an anionic surfactant, disrupts protein tertiary structure and cleaves disulfide bonds, thereby exposing epitopes for antibody binding (Anand et al., 2013). The combination of heat and SDS effectively dissociated casein more than heat alone.

Bromelain hydrolysis reduced the antigenic reactivity of goat milk casein, as indicated by the decreased ELISA response compared with untreated casein. ELISA results (Table 2) showed significant differences ($p < 0.05$) between the hydrolysed and untreated casein samples. Previous studies showed that the effectiveness of protein hydrolysis depends on the type of enzyme and the hydrolysis conditions. Liu et al. (2012) found that papain reduced the antigenicity of α-casein under optimised conditions of pH 5.6, 50°C, 2.5% E/S, and 90 min. Wang et al. (2013) reported that trypsin hydrolysis increased rapidly during the first 20 min and reached a DH of 45.2% after 45 min, showing active peptide formation during the early stage of hydrolysis. Medeiros et al. (2014) found that bromelain

could produce partially hydrolysed milk, while Ahmed et al. (2015) reported that different enzymes showed different levels of αs1-casein antigenicity reduction. Kumar et al. (2016) also showed that DH and hydrolysis time varied depending on the enzyme used. For goat's milk, Shu et al. (2016) reported that alcalase showed optimal hydrolysis at 150 min, 50°C, pH 8.0, and 2% E/S. Wang et al. (2023) further showed that alcalase–Neutrase hydrolysis achieved 26.45% DH and a 59.41% reduction in antigenicity. Overall, these studies show that enzyme type and hydrolysis time are important factors influencing peptide formation and the reduction of antigenicity.

Based on these findings, the present study further evaluated the effect of hydrolysis time on the antigenic reactivity of goat's milk casein using bromelain. ELISA results showed that 1 h hydrolysis produced the lowest antigenic reactivity (69.6%) among the hydrolysis times tested. The lower ELISA response suggests that bromelain hydrolysis reduced the ability of goat's milk casein to react with the antibodies. This may be due to the breakdown of protein regions recognised by the antibodies, resulting in the formation of smaller peptides. However, longer hydrolysis did not result in a further decrease in antigenic reactivity. This may be due to changes in the types of peptides formed during prolonged hydrolysis. Therefore, 1 h was identified as the most effective hydrolysis time for reducing antigenic reactivity under the conditions tested.

Affinity constants (Kd) indicated that Nter-IgG had stronger binding than Cter-IgG, while both antibodies had lower affinity for native αs1-casein compared to peptide-BSA conjugates (Mohsin et al., 2020). These observations highlight challenges in quantifying antigenicity by ELISA when both N- and C-terminal epitopes are present.

Previous studies align with these findings. Trypsin-treated buffalo αs1-casein exhibited reduced immunoreactivity, yet residual allergenicity persisted among CMPA patients (Ahmad et al., 2016). Papain-treated bovine α- and β-casein demonstrated that DH negatively correlated with binding inhibition, although high DH did not always eliminate antigenicity (Liu et al., 2012). These studies emphasise that enzyme specificity, peptide length, and epitope exposure significantly influence the observed antigenicity.

Industrial Feasibility and Practical Considerations

Although bromelain hydrolysis reduced the antigenic reactivity of goat's milk casein under the conditions investigated, several practical considerations need to be addressed before application at an industrial scale. Extensive proteolysis may generate small peptides that can contribute to bitterness or other undesirable sensory characteristics; therefore, sensory evaluation and bitterness assessment are necessary to determine consumer acceptability. In addition, the nutritional quality and digestibility of the resulting hydrolysates should be evaluated to ensure that hydrolysis does not adversely affect their nutritional value and to determine their suitability for use in specialised dairy products. From a hydrolysis

perspective, the scalability of bromelain hydrolysis should be investigated by considering enzyme dosage, reaction time, temperature control, mixing, enzyme inactivation, and batch-to-batch consistency. The economic feasibility of the process would also depend on bromelain cost, enzyme utilisation efficiency, process yield, and the costs associated with subsequent purification or drying steps. Furthermore, regulatory acceptance would require appropriate evidence of safety and efficacy for the intended application and in accordance with applicable food regulations. Therefore, while the present study provides a preliminary scientific basis for using bromelain to reduce the antigenic reactivity of goat milk casein, further sensory, nutritional, digestibility, techno-economic, scale-up, and regulatory studies are required before its suitability for commercial functional dairy products can be established.

CONCLUSION

In conclusion, bromelain hydrolysis at pH 5.5 and 55 °C effectively reduced the antigenicity of goat milk casein, as indicated by the highest degree of hydrolysis (DH) and peptide content, together with the lowest ELISA response. The antigenicity assessment in this study was performed using an in-house sandwich ELISA with rabbit IgG antibodies; therefore, the reduced ELISA response reflects reduced antigenic reactivity and does not establish clinical allergenicity or confirmed hypoallergenicity. Further investigation using clinically relevant immunological approaches is necessary to assess its relevance to specialised dairy and infant nutrition applications.

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LIST OF ABBREVIATION

AAF	Amino Acid Formula
BSA	Bovine Serum Albumin
CMPA	Cow Milk Protein Allergy
CSN	Casein

Cter	C-terminal
DH	Degree of Hydrolysis
eHF	Extensively Hydrolysed Formula
ELISA	Enzyme-Linked Immunosorbent Assay
IgE	Immunoglobulin E
IgG	Immunoglobulin G
NC	Negative Control
NSB	Non-Specific Binding
Nter	N-terminal
OPA	o-Phthaldialdehyde
PBS	Phosphate-Buffered Saline
pHF	Partially Hydrolysed Formula
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
α-LA	Alpha-Lactalbumin
αs1-casein	Alpha-s1-Casein
β-LG	Beta-Lactoglobulin

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