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Original article

Comparative detection of goat's milk casein through various enzyme-linked immunosorbent assay (ELISA) approaches using caprine alpha-$_{S1}$ casein peptides and bovine casein polyclonal antibodies

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Summary Casein causes allergic reactions in cow's milk protein allergy (CMPA) individuals. Casein can cross-react with cows and goats. Hence, a rapid and highly specific technique is required for its detection. The study aims to evaluate and optimise multiple Enzyme-Linked Immunosorbent Assay (ELISA) approaches for the detection of casein isolated from goat's milk. The study utilised and optimised various ELISA approaches, including direct, indirect and sandwich with various antibodies. Several parameters of ELISA including pre-treatments of casein, blocking reagent, concentrations of antibodies and background signal were evaluated. The results showed that sandwich ELISA revealed a good signal-to-noise ratio at 1 mg mL^{-1}. Indirect ELISA showed a higher signal-to-noise ratio without any heat treatment in comparison to the direct ELISA despite utilising an equivalent concentration (i.e., 10 μg mL^{-1}) of goat's milk casein. The findings provide a theoretical foundation for obtaining a high signal-to-noise ratio for antigenicity studies of goat's milk casein to minimise its immunological response.

Keywords casein, goat's milk, immunoassay, milk allergen, peptide antibodies.

Introduction

Food allergies have become a primary issue in the realm of public health, affecting many communities worldwide. Cow's milk allergy is the most prevalent food allergy among children, characterised by an unfavourable response to the protein that exists in bovine milk. The prevalence was estimated to range from 2 to 3% among infants in the developed world, as reported by Manuyakorn & Tanpowpong (2018). Goat's milk was considered an option for those with cow's milk protein allergy (CMPA). However, goat's milk allergy prevalence is usually underreported and its high homology in an amino acid sequence with bovine milk causing a food safety concern is on the rise. Caseins, some particular constituent of milk, comprised 80% of the overall protein content (Mansor *et al.*, 2023) and were implicated as the primary allergen in milk. Currently, several methods have been employed to identify allergenic properties in food products, including chromatographic practices such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and DNA-based methods. Nevertheless, the major drawbacks of using those instruments are costly and require the use of organic solvents and skilled analysts (Costa *et al.*, 2015; Holzhauser & Röder, 2015; Villa *et al.*, 2022; Lu *et al.*, 2023). Hence, an enzyme-linked immunosorbent assay (ELISA) is a preferred method owing to its various advantages, including cost-effectiveness, user-friendly, high specificity and rapidity (Holzhauser *et al.*, 2020). ELISA has a diverse application in the detection of various antigens (Lin, 2015; Nouri *et al.*, 2018; Zhu *et al.*, 2022; Hayrapetyan *et al.*, 2023). However, until recently, limited research on the detection of the casein composition featuring α_{s1}-, α_{s2}-, β- and k-casein isolated from goat's milk has been reported. This is due to the lack of commercial availability of antibodies specifically for goat's milk casein in comparison to bovine milk. Therefore, a detection test was carried out in this study to find out the binding capacity of goat's milk casein to specific antibodies derived from caprine α_{s1}-casein peptides and anti-bovine casein polyclonal antibodies.

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In ELISA, several formats can be employed. Each of these formats, i.e., direct, indirect and sandwich has its way of detecting the antigen. Nevertheless, the superiority between the three systems for the detection of goat's milk casein is still uncertain. Meanwhile, in terms of improving the reliability and application of ELISA, the selection of antibodies by their specificity is a significant factor (He *et al.*, 2023). Furthermore, the ELISA method described by Minić & Živković (2021) emphasised the significance of several other parameters such as antibody concentration, types of blocking reagents and the specific treatments applied to the targeted antigen.

In contrast to another study that was conducted on the specific composition of goat's milk casein, α_{s1} (Mohsin *et al.*, 2023), this study aims to examine and enhance the usability of different types of ELISA systems, Fig. 1a–c, for the detection of whole casein composition from goat's, also known as caprine's milk. However, there is a limitation on the types of antibodies used. Due to the lack of commercial availability of antibodies against goat's milk casein, a polyclonal antibody specific to bovine casein along with an antibody that specifically targets goat α_{s1}-casein peptides, also known as caprine α_{s1}-peptides (N-terminal and C-terminal IgG-biotin conjugate IgGs against caprine α_{s1}-peptides) were employed for a comparative detection study on goat casein. Polyclonal antibodies specific to bovine milk casein were chosen as a result of their high degree of amino acid homology with goat's milk casein, approaching an overall of 88% (Prosser, 2021).

Materials and methods

Chemicals and reagents

N-terminal and C-terminal IgGs were produced from α_{s1}-casein peptides from goat's milk developed in rabbits, as previously reported by Mohsin *et al.* (2023). Rabbit polyclonal casein antibody with biotin-conjugate was purchased from Bioss (Woburn, MA), and rabbit polyclonal antibody was obtained from GeneTex (Irvine, CA). Streptavidin-horseradish peroxidase and peroxidase affiniPure goat anti-rabbit IgG (H + L) were attained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alpha casein from bovine (>70% electrophoresis purity) was procured from Sigma-Aldrich (St. Louis, MO). Phosphate buffer solution (PBS) was prepared in 10X stock, containing 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4.2H_2O$ and 18 mM KH_2PO_4. 1× PBS was used for all dilutions and solutions. All chemicals for PBS were obtained from R&M (Semenyih, Selangor). PBST (1X, 0.1% Tween 20) was used for washing. A 0.1 M carbonate buffer was prepared at a pH of 9.6 and 0.1 M H_2SO_4 at a pH of 1.24. Bovine Serum Albumin (BSA) was obtained from Avantor Lifescience (Missouri, TX), Pierce™ Protein-Free (PBS) (1X concentration), Superblock™ and ELISA

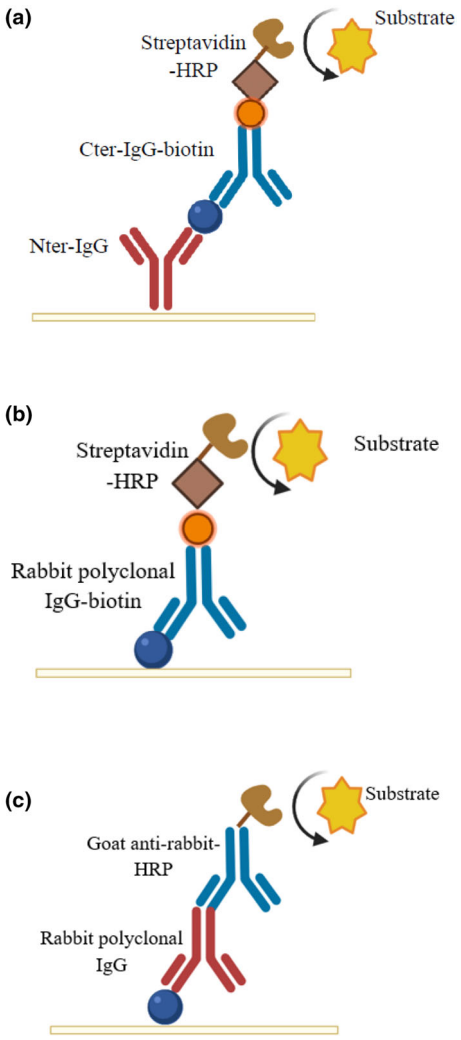

Figure 1 (a) Capture antibody, Nter-IgG, was initially subjected to a pre-incubation process before binding to goat's milk casein. The secondary antibody, Cter-biotin IgG bound to the casein before undergoing a chemical reaction of streptavidin-HRP with TMB, resulting in the formation of coloured products. The reaction was halted with 0.1 M H_2S0_4, and the resulting signal was analysed at Abs 450 nm. (b) Casein was first diluted in PBS before applying rabbit polyclonal IgG-biotin and streptavidin-HRP before undergoing a chemical reaction with TMB, resulting in the formation of coloured products. The reaction was halted with 0.1 M H_2SO_4, and the resulting signal was analysed at Abs 450 nm. (c) Goat's milk casein was first diluted in PBS before applying rabbit polyclonal IgG and goat anti-rabbit-HRP before undergoing a chemical reaction with TMB, resulting in the formation of coloured products. The reaction was halted with 0.1 M H_2SO_4, and the resulting signal was analysed at Abs 450 nm.

substrate (1-Step Ultra TMB), as well as Maxisorp 96-well plate, were acquired from ThermoFisher Scientific (Waltham, MA).

Sample collection

Raw and unpasteurised Saanen goat's milk (6–8 L) was collected from bulk at Ladang Basung Semenyih, Selangor, Malaysia. Samples were kept in sterile glass bottles and transported to the laboratory in an icebox with the temperature sustained at 4 °C. Subsequently, the samples were kept frozen at −20 °C for further investigations.

Casein isolation

Isolation casein from goat's milk was conducted as defined by Hodgkinson *et al.* (2012) with some modifications on the washing of precipitate. Goat's milk was first thawed at 36 °C in a water bath and cooled at temperature (24 °C). Then, it was subjected to centrifugation (Sartorius, Gottingen, Germany) at 5232 ***g*** for 30 min while maintaining a temperature of 4 °C. Subsequently, the fat layer was removed and pH of the solution was attuned to 4.2 by the addition of 1 M HCl to induce precipitation of the casein. The skimmed milk underwent a stirring process for 30 min, followed by subsequent centrifugation at 5232 ***g*** for 15 min at 20 °C to retrieve the casein precipitate. Then, the residue was subjected to washing by suspending it in purified water at ten times the volume of milk (100 mL:1 L) (v/v). The suspension was then stirred at ambient temperature (24 °C), for 1 h prior to undergoing re-centrifugation under similar conditions. The isolated casein was freeze-dried (Labconco, USA) under a vacuum of 175×10^{-3} mBar at a temperature of −46 °C and lyophilised goat's milk casein was stored at −20 °C until further investigation.

Sandwich ELISA

Immunoassay was conducted using a method established by Mohsin *et al.* (2023). N-terminal and C-terminal-biotin antibodies were produced by both N-terminal and C-terminal peptides of goat α_{s1}-casein. These antibodies were produced in rabbits. Nter-IgG, serving as the capture antibody, was dissolved in 0.1 M carbonate buffer at a pH value of 9.6 to be coated on the 96-well plate and incubated at 4 °C overnight (16 h). To prevent any remaining non-specific binding, wells were treated with 250 µL blocking reagent. This blocking process took place for 2 h at ambient temperature (24 °C), with a shaker set at 150 rpm. After blocking, 100 µL of lyophilised caseins were added into each well at 1 mg mL^{-1} in 1× PBS. The plate was then subjected to incubation for 60 min at ambient temperature (24 °C) in shaking at 150 rpm. Subsequently, Cter-IgG biotin-conjugate was added to the wells and incubated for 1 h at similar conditions. Next, the wells were filled with streptavidin-horseradish peroxidase (streptavidin-HRP) and incubated at similar conditions. The mixture was then incubated for an hour under identical conditions. Following each incubation stage, the plates underwent three washes using 250 µL of 0.1% PBST. Following the incorporation of 100 µL of 1-Step ultra TMB-ELISA substrate solution, the ELISA plate was positioned in the dark, and the development of a blue colour was monitored. The reaction was halted by the incorporation of 100 µL of 0.1 M H_2SO_4. Finally, absorbance values were acquired at a wavelength of 450 nm via Multiscan FC (ThermoFisher Scientific, Waltham, MA).

Optimisation of sandwich ELISA

Antibody concentration

Nter-IgG at 1:1000 (v/v) and Cter-biotin-IgG was used at (1:1000, 1:2500) (v/v) and streptavidin-horseradish peroxidase (Streptavidin-HRP) was applied at ratios of (1:7500, 1:10 000; 1:15 000) (v/v) in phosphate-buffered saline (PBS). All reagents were prepared in 1× PBS, pH 7.4.

Blocking reagent

To prevent any remaining non-specific binding, wells were treated with 250 µL blocking reagents at 2 h incubation at ambient temperature (24 °C), consisting of 2.5% bovine serum albumin (BSA) and Protein-Free (PBS) (1×) (PierceTM) and Superblock™ (Pierce) blocking buffer performed directly to each well.

Casein pre-treatment

Lyophilised goat's milk casein was diluted in PBS and treated with heat in a water bath at 80 and 90 °C, with the addition of a denaturing agent, 0.1% SDS for 2 min.

Direct ELISA

Direct ELISA was done as described by Liang *et al.* (2020) with some amendments. Firstly, a Maxisorp 96-well plate was subjected to an overnight coating process at 4 °C for 16 h. The coating was achieved by applying untreated casein at 10 µg mL^{-1} in 1X PBS. Casein was diluted in PBS as well as treated with a denaturing agent of 0.1% SDS and heated at 80 and 90 °C in a water bath for 2 min. Then, the plate underwent three washes using PBS. Following this, the plate was blocked with a solution containing 2.5% BSA for 2 h at ambient temperature (24 °C) and placed on an orbital shaker at 150 rpm. Subsequently, the plate was washed with PBST thrice and was

thereafter incubated with anti-casein rabbit-biotin conjugate polyclonal antibody (100 μL; diluted at 1:1000, 1:500 [v/v]) in carbonate buffer, pH 9.6 for 1 h at ambient temperature (24 °C). The plate underwent three additional washing steps before the addition of streptavidin-HRP 1:7500 (v/v) in PBS and incubated at the same condition. After the washing step, a solution of 1-Step ultra TMB-ELISA substrate solution (50 μL) was administered and subsequently incubated for 20 min at ambient temperature (24 °C) in the absence of light. The reaction was halted by incorporating 50 μL of 0.1 M H_2SO_4. The absorbance value was measured at 450 nm.

Indirect ELISA

Indirect ELISA utilised primary antibody, anti-casein rabbit polyclonal (100 μL; diluted at 1:500, 1:1000 (v/v) in carbonate buffer, pH 9.6) and secondary antibody, goat anti-rabbit-HRP (100 μL: 1:7500, 1:10 000,1:15 000) (v/v) as well as streptavidin-horseradish peroxidase (Streptavidin-HRP) at varying dilution ratios of (1:7500, 1:10 000; 1:15 000) (v/v). All reagents were prepared in 1X PBS. Casein was diluted in PBS treated with a denaturing agent of 0.1% SDS and heated at 80 and 90 °C in a water bath for 2 min.

Statistical analysis

All ELISA was conducted in triplicates ($n = 3$). Absorbance data were analysed using mean and standard deviation. Minitab (Pty Ltd, Sydney, Australia) statistical software Version 21 was applied for the analysis of variance (ANOVA). Post-hoc Tukey test was then made for comparison between pairs of mean from treatment levels. Significant differences were calculated at $\alpha = 0.05$ (95% confidence interval).

Results and discussion

Optimisation of sandwich ELISA

Concentrations of secondary antibody (streptavidin-HRP) and capture antibody (Cter-biotin IgG)

Streptavidin-biotin binding affinity is frequently used in various types of applications, including immunosorbent assays. Streptavidin is a protein with a solid binding affinity for biotin at a dissociation constant of 1×10^{-15} M^{-1} (Luong & Vashist, 2019). The result in Table 1 showed the absorbance value of the background reading of HRP-conjugated streptavidin in a sandwich ELISA. The result demonstrates no significant difference ($P > 0.05$) among the concentrations used (1:7500, 1:10 000 and 1:15 000) (v/v), indicating streptavidin-HRP had minimal binding on the plate surface, with an absorbance value of less than 0.1. This implies the absence of background noise and these results were acceptable and consistent signifying the stability of the system. Moreover, incorporating additional incubation and washing processes can result in a reduced background, which was corroborated by Savini *et al.* (2023). The implementation resulted in minimal background values, approximately in Abs 0.05, reflecting a reduced binding of HRP-streptavidin to the plate. Furthermore, minimal binding of streptavidin-HRP to the plate surface was caused by the polystyrene material of the NUNC Maxisorp ELISA, which specifically targeted antibodies (glycoprotein) immobilised through the COOH-link on the polystyrene surface (Lakshmipriya *et al.*, 2016). Due to the absence of any interaction between streptavidin-HRP and biotinylated molecules, the absorbance reading in the immunoassay is significantly reduced.

Table 1 Background reading of streptavidin-HRP in phosphate-buffered saline (PBS) at different dilutions

	Streptavidin-HRP dilutions (v/v)		
Day	**1:7500**	**1:10 000**	**1:15 000**
1	0.089 ± 0.009^{Aa}	0.093 ± 0.007^{Aa}	0.078 ± 0.006^{Ab}
2	0.071 ± 0.003^{Ba}	0.077 ± 0.003^{Ba}	0.067 ± 0.007^{Aa}
3	0.073 ± 0.006^{Ba}	0.067 ± 0.002^{Ba}	0.060 ± 0.002^{Aa}
4	0.051 ± 0.002^{Ca}	0.051 ± 0.002^{Ca}	0.050 ± 0.002^{Ba}
Relative standard deviation	5.63%	4.86%	6.67%

Values are represented by mean ± SD values of three readings ($n = 3$). Values within the same days at various dilutions, with different uppercase superscripts are significantly different ($P < 0.05$), while different lowercase superscripts signify significant differences ($P < 0.05$) among different days within the respective dilutions. Absorbance of the well without goat's milk casein was detected at 450 nm.

Following that, background reading of Cter-biotin IgG with streptavidin-HRP was evaluated at two different dilution ratios, (1:1000; 1:2500) (v/v), as shown in Table 2. The results showed an increase in absorbance value after the addition of Cter-biotin IgG, indicating an interaction between streptavidin-HRP. This indicates that Cter-biotin IgG has moderate binding to the plate due to streptavidin-biotin affinity and the ability to immobilise on the surface of the plate as a polyclonal antibody. These results align with a study reported by Lin *et al.* (2008), as streptavidin-HRP can attach to four biotin molecules, to hapten, antigen, or HRP, leading to an increased signal in immunoassay.

In other studies, by Balieiro Neto *et al.* (2023), a significant concentration of biotin can lead to highly saturated binding sites that hinder the formation of a secondary antibody to form a connection with streptavidin, resulting in inaccurate positive results or

Table 2 Background reading of C-terminal-biotin IgG together with streptavidin-HRP (1:15 000, v/v) analysed in three different days

Cter-Biotin IgG (v/v)	Streptavidin-HRP	Day 1	Day 2	Day 3
1:1000	1:15 000	0.607 ± 0.075^{bA}	0.527 ± 0.050^{aA}	0.494 ± 0.081^{cB}
1:2500	1:15 000	0.309 ± 0.085^{cA}	0.095 ± 0.012^{cA}	0.169 ± 0.005^{cA}

Values are represented by Mean ± SD values of three readings ($n = 3$). Values within the same days, with different lowercase superscripts are significantly different ($P < 0.05$), while different uppercase superscripts signify significant differences ($P < 0.05$) among different days within the same dilutions. Absorbance of the well without casein proteins was detected at 450 nm.

increased background signals. Nevertheless, this excessive binding can be minimised by increasing the frequency of washing and including blocking measures. However, a different result was stated by Samarasinghe *et al.* (2017) who found low signal due to biotin interference in sandwich ELISA. Many researchers have extensively investigated the use of biotin-streptavidin in various applications (Guo *et al.*, 2016; Cheah & Yamada, 2017; Yang *et al.*, 2021) due to its remarkable binding strength.

Subsequently, background readings of a complete sandwich ELISA in different solutions using Cter-biotin IgG at ratios of 1:2500 (v/v) and 1:1000 (v/v) were evaluated in Table 3. Results indicated negligible background value was observed regardless of different dilution rations of Cter-biotin IgG. Hence, PBS was chosen for all dilutions of goat's milk casein and antibodies due to its lowest absorbance reading. Meanwhile, 2.5% BSA can be used as a blocking reagent to avoid non-specific binding, and PBST can be used for regular washing steps. These choices were made since they were acceptable with low-background readings due to minimal non-specific bindings, hence improving the specificity of the assay.

Table 3 Background reading of sandwich ELISA in different solutions without casein using different dilutions of Cter-biotin IgG

Dilutions of Cter-biotin IgG (v/v)	Solution	Absorbance at 450 nm
1:1000	PBS, pH 7.4	0.163 ± 0.030^{cC}
	0.1% PBST	0.277 ± 0.007^{bB}
	2.5% BSA	0.281 ± 0.003^{aA}
1:2500	PBS, pH 7.4	0.148 ± 0.003^{bC}
	0.1% PBST	0.244 ± 0.003^{aB}
	2.5% BSA	0.270 ± 0.010^{aB}

Values are represented by mean ± SD values of three readings ($n = 3$). Values within the same dilution, with lowercase superscripts, are significantly different ($P < 0.05$), while uppercase superscripts signify significant differences ($P < 0.05$) among different dilutions. No casein was added for the assessment of background reading with Nter-IgG 1:1000 (v/v) and streptavidin-HRP 1:15 000 (v/v) The absorbance was detected at 450 nm.

Blocking reagents

In regards to blocking, a comparison with several types of blocking reagents was carried out in a complete ELISA system consisting of protein-free blocking buffers, superblock (Guven & Azizoglu, 2023) and bovine serum albumin (BSA). The system detected casein at 1 mg mL^{-1} with the utilisation of primary (Nter-IgG) and secondary antibodies (Cter-biotin IgG). BSA is frequently employed as a way of blocking agents in many studies (Panebra *et al.*, 2021). The selection of a 2.5% BSA was based on its demonstrated efficacy in blocking (Du *et al.*, 2019) and its cost-effectiveness. This aligns with our results showing that 2.5% BSA yielded the highest signal-to-noise ratio in detecting target samples compared to other blocking buffers as shown in Fig. 4, highlights the efficacy of this blocking buffer in preventing non-specific binding. Hence, a blocking reagent of 2.5% BSA was chosen as a blocking reagent due to its owing to favourable compatibility with ELISA reagents and cost-effectiveness. Meanwhile, the specific protein used in protein-free blocking buffer was not specified in the manufacturer's data, and Superblock was not chosen due to its high cost. To prevent false positive results, it is crucial to avoid insufficient blocking.

According to Mohsin *et al.* (2023), there was no significant difference ($P > 0.05$) observed in the incubation duration of 2 and 3 h using 2.5% BSA. Therefore, a blocking period of 2 h was selected to maintain the efficiency of the system. Nevertheless, our research exhibits a reduced signal-to-noise ratio of 2.29, as displayed in Fig. 2, in comparison to the previous study's ratio of 5.5. The potential reason for this discrepancy may be attributed to the variation in the type of casein employed, as the previous investigation utilised a purified goat α_{s1}-casein and the antibodies were raised against both terminals of α_{s1}-casein rather than the whole casein compound, leading to a strong binding affinity and consequently an increased signal-to-noise ratio.

Antibody concentration and protein pre-treatment

A complete ELISA system was performed using Cter-biotin IgG (1:1000; 1:2500) (v/v) to detect goat's milk

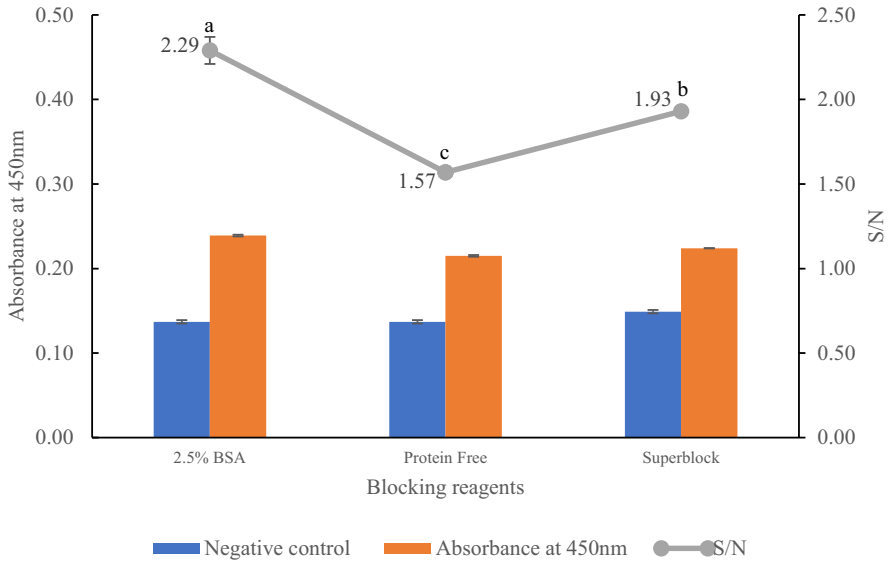

Figure 2 Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA. Caseins were used at 1 mg mL^{-1} and heated at 80 °C with the addition of 0.1% SDS using different types of blocking reagents during 2 h incubation at room temperature. Values are represented by mean ± SD values of three readings (n = 3). Values with different lowercase superscripts are significantly different ($P < 0.05$).

casein. Based on Fig. 3a, it was shown that the highest signal-to-noise ratio (2.18) was obtained when goat's milk casein was treated with a denaturing agent consisting of 0.1% SDS and heated at 80 °C. No significant difference ($P > 0.05$) was observed when treated with the same denaturing agent at 90 °C. This result contradicts the trend when goat's milk casein was heated without the addition of 0.1% SDS, with S/N ration of 1.319 and 1.353, respectively, after a dilution of 1:1000 (v/v) of Nter-IgG, 1:1000 (v/v) Cter- biotin IgG and 1:150 000 (v/v) of streptavidin-HRP. However, a lower signal-to-noise ratio when employing a more dilute antibody concentration, 1:1000 (v/v) Nter-IgG, 1:2500 (v/v) Cter-biotin IgG and 1:150 000 (v/v) streptavidin-HRP as shown in Fig. 3b.

In this system, a pre-treatment was applied to goat's milk casein owing to the antibodies used being specifically designed for goat α_{s1}- peptides. This research demonstrated that subjecting casein to temperatures exceeding 60 °C could lead to its unfolding, denaturation, aggregation and loss of its native form as reported by Corredig *et al.* (2019). This could result in alterations in their advanced structures, specifically secondary and tertiary structures, influencing IgE binding capacity Pi *et al.* (2023). This aligns with research by Sun *et al.* (2017) and Azdad *et al.* (2018) who have found that heating altered casein's secondary structure and conformational epitopes, resulting in fluctuations in its immunological features. Hence, the pre-treatment is needed to unfold the goat's milk casein structure for the antibodies to detect the casein in its denatured form.

Furthermore, the use of SDS resulted in an even higher absorbance value than heating alone. As reported by Jelińska *et al.* (2017) SDS can lead to structural changes causing denaturation and aggregation of α_{s1}-casein which in turn, affects its structural integrity. Another study by Winogradoff *et al.* (2020) reported that protein unfolding can be achieved through several methods, including temperature and SDS treatment. The protein would undergo binding with SDS molecules when exposed to SDS and heat treatment, disrupting the non-covalent interactions that were accountable for maintaining the protein's original three-dimensional structure. However, in our study, the utilisation of both heating with denaturing agents showed a diminished signal-to-noise ratio when attempting to identify the entire goat casein. This could be attributed to the specific detection of goat α_{s1}-casein by Nter-IgG and Cter-IgG, as previously reported by Mohsin *et al.* (2023).

A comparative analysis was conducted utilising various forms of polyclonal antibodies with distinct ELISA systems. Nevertheless, during this investigation, the antibody was limited by the absence and unavailability of a particular antibody for goat casein. We have observed many suppliers worldwide attempting to search for an antibody specific to goat casein but

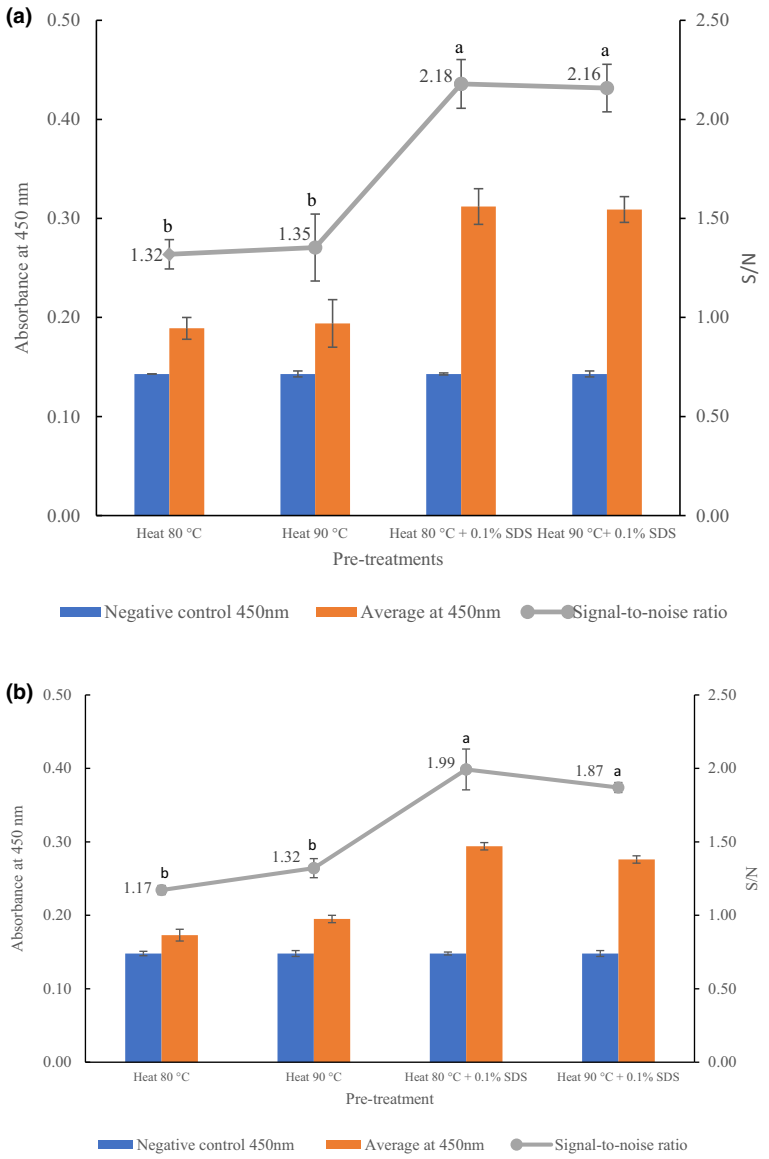

Figure 3 (a) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA after heating and addition of denaturing agent 0.1% SDS. Dilution used in this experiment were 1:1000 (v/v) Nter-IgG, 1:1000 (v/v) Cter biotin-IgG and 1:150 000 (v/v) streptavidin-HRP. Values are represented by mean ± SD values of three readings (n = 3). Values within the pre-treatments, with lowercase superscripts, are significantly different ($P < 0.05$). (b) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA after heating and addition of 0.1% SDS. Dilution used in this experiment were 1:12 500 (v/v) Nter-IgG, 1:1000 (v/v) Cter biotin-IgG and 1:150 000 (v/v) streptavidin-HRP. Values are represented by mean ± SD values of three readings (n = 3). Values within the pre-treatment, with lowercase superscripts, are significantly different ($P < 0.05$).

have been unable to identify one. Hence, due to its lack of commercial availability of antibodies specific to goat's milk casein, the choice to use an alternative, namely rabbit polyclonal antibody, specific to bovines was made for that reason. This selection was based on the fact that bovine and goat shared 88% similarities in their amino acid sequences (Prosser, 2021).

Optimisation of direct ELISA

Different concentrations of anti-casein rabbit polyclonal, biotin-conjugate with different casein pre-treatments

A rabbit polyclonal with biotin-conjugate at different dilutions 1:500 (v/v) Fig. 4a, 1:1000 (v/v) Fig. 4b together with streptavidin-HRP was used in a direct ELISA

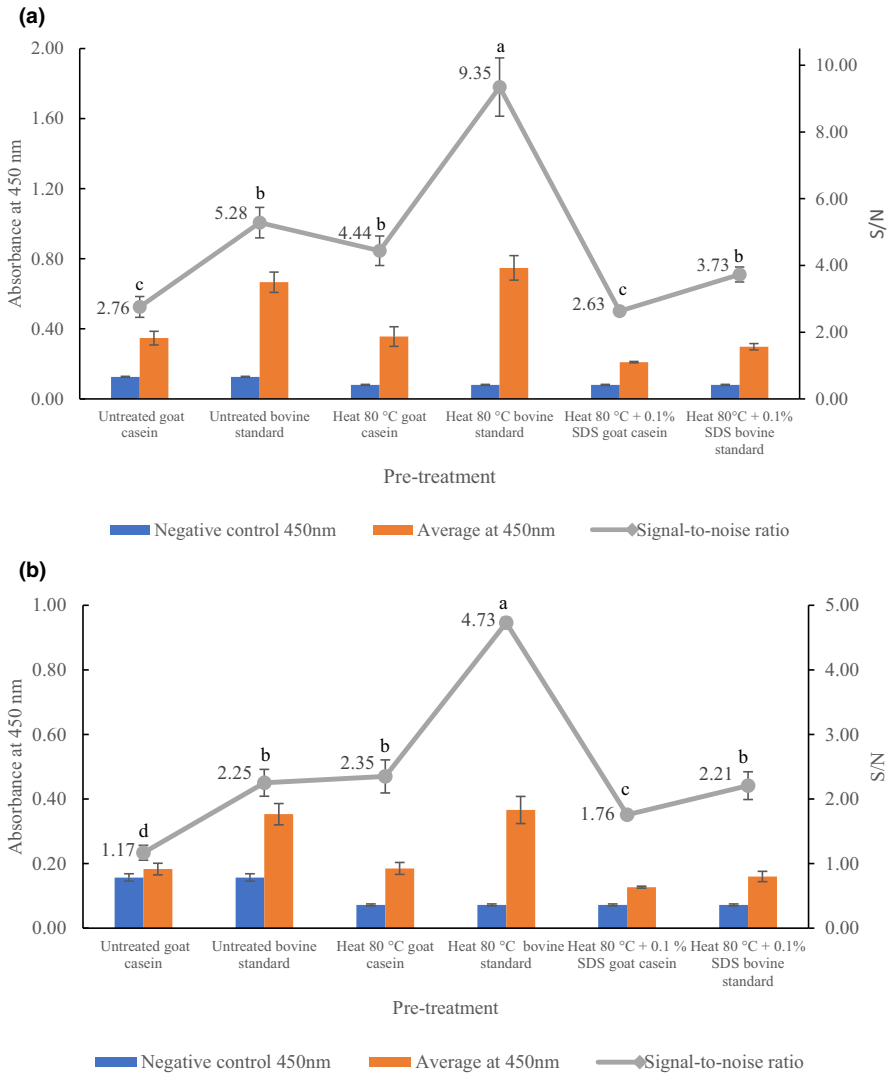

Figure 4 (a) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in direct ELISA after heating and addition of denaturing agent 0.1% SDS. Anti-bovine casein rabbit polyclonal biotin-conjugated was paired with streptavidin-HRP. Dilutions used in this experiment were 1:500 (v/v) of anti-bovine casein rabbit polyclonal antibody coupled with biotin and a 1:7500 (v/v) streptavidin-HRP. Values are represented by mean ± SD values of three readings ($n = 3$). Values of signal-to-noise ratio, with lowercase superscripts, are significantly different ($P < 0.05$). (b) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in direct ELISA after heating and addition of denaturing agent 0.1% SDS. Anti-bovine casein rabbit polyclonal biotin-conjugated was paired with streptavidin-HRP. Dilutions used in this experiment were a 1:1000 (v/v) dilution of anti-bovine casein rabbit polyclonal antibody coupled with biotin and a 1:7500 (v/v) dilution of streptavidin-HRP. Values are represented by mean ± SD values of three readings ($n = 3$). Values of signal-to-noise ratio, with lowercase superscripts, are significantly different ($P < 0.05$).

system. Results showed that a signal obtained when utilising anti-casein rabbit polyclonal antibody against lyophilised goat casein showed a significant difference ($P < 0.05$) from bovine's standard α-casein. This can be attributed to using a polyclonal rabbit casein antibody that exhibits specificity towards bovine caseins.

Heat treatment on bovine and goat's milk casein displayed a significant difference ($P < 0.05$) in comparison to untreated casein and a combination of heat and SDS. However, the result was contradicted by Fig. 3a,b, which showed a significant increase in the S/N ratio following both treatments. According to Anema (2021), the reason for this could be attributed to the thermal treatment of proteins, which could lead to hydrolysis, a chemical reaction involving the introduction of water to the covalent peptide bond. This contrasts with previous

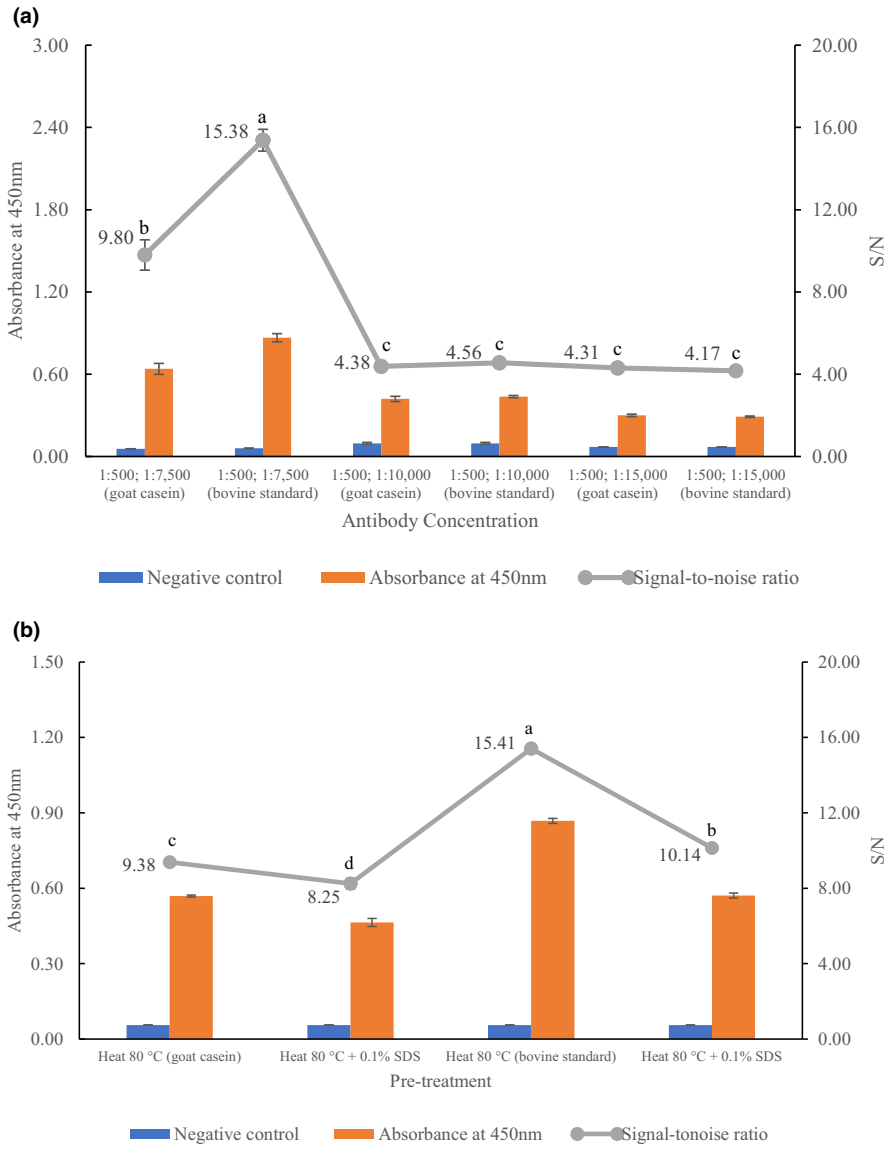

Figure 5 (a) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in indirect ELISA. Anti-bovine casein rabbit polyclonal was paired with goat anti-rabbit-HRP Dilutions used in this experiment were a 1:500 (v/v) anti-bovine casein rabbit polyclonal antibody and a 1:7500 (v/v), 1:10 000 (v/v) and 1:15 000 (v/v) of goat anti-rabbit-HRP. Values are represented by mean ± SD values of three readings (n = 3). Values of signal-to-noise ratio, with lowercase superscripts, are significantly different ($P < 0.05$). (b) Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in indirect ELISA after heating and addition of denaturing agent 0.1% SDS. Anti-bovine casein rabbit polyclonal was paired with goat anti-rabbit-HRP. Dilutions used in this experiment were a 1:500 (v/v) of anti-bovine casein rabbit polyclonal antibody and a 1:7500 (v/v) goat anti-rabbit-HRP. Values are represented by mean ± SD values of three readings (n = 3). Values of signal-to-noise ratio, with lowercase superscripts, are significantly different ($P < 0.05$).

research, which suggested that the inclusion of SDS causes the separation of casein micelles and milk gel within a specific range of SDS concentrations.

The findings of this study demonstrate that the application of direct ELISA is a reliable method in identifying the presence of goat's milk casein despite not yielding a stronger signal to bovine. Another alternative ELISA method, known as indirect, was then evaluated in this study to detect goat's milk casein, utilising anti-bovine casein rabbit polyclonal antibody without biotin-conjugate, in conjunction with goat anti-rabbit-HRP.

Optimisation of indirect ELISA

Different concentrations of anti-casein rabbit polyclonal and secondary antibody goat anti-rabbit-HRP

In this ELISA system, the results presented in Fig. 5a demonstrate that the indirect ELISA had the highest signal-to-noise ratio for goat casein (9.80) in the absence of any heat treatment when compared with Fig. 5b. The optimal dilution for the anti-casein rabbit polyclonal antibody was found to be 1:500 (v/v), whereas the goat anti-rabbit HRP exhibited the greatest results at a dilution of 1:7500 (v/v). The observed effect may be attributed to the elevated specificity, sensitivity and binding affinity levels. Indirect ELISA has been employed to detect hydrolysed bovine milk (Liang *et al.*, 2020), β-conglycinin in soy (Segura-Gil *et al.*, 2022), shrimp tropomyosin (Zeng *et al.*, 2019) and α_{s1}-casein in buffalo milk for antigenicity study. Nevertheless, subjecting casein to heat treatment leads to a decrease in value when compared to its untreated form. The reason for this occurrence might be attributed to the specific type of antibody employed, as indicated by the manufacturer, which is exclusively compatible with indirect ELISA without the requirement of heat application.

Conclusion

In summary, a complete sandwich ELISA system using Nter-IgG (1:1000) (v/v), Cter-IgG-biotin (1:1000) (v/v) and (1:15 000) (v/v) streptavidin-HRP were developed, giving a high signal-to-noise ratio at the highest concentration of goat's milk casein at 1 mg mL^{-1} after addition of heating and SDS in detection. However, the indirect ELISA method exhibited a better signal-to-noise ratio without any heat treatment in comparison to the direct and sandwich ELISA method despite utilising a similar concentration of goat's milk casein at 10 μg mL^{-1}. The three approaches, direct, indirect and sandwich ELISA, were used to detect lyophilised goat's milk casein, with the indirect method producing the highest signal-to-noise ratio. ELISA method demonstrated high specificity towards goat's milk casein, making it a valuable tool for assessing protein presence in milk and milk products. These findings provide significant interest to those in the field of food safety specifically those working on the development of new formula or improvement of the safety and quality of food dairy products, as well as general consumers.

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Author contributions

Nurul Izza Nabilah Mohd Samsudin: Investigation; writing – original draft; writing – review and editing; formal analysis; software; data curation. **Rashidah Sukor:** Conceptualization; funding acquisition; writing – review and editing; validation; methodology; project administration; supervision; resources; data curation; formal analysis. **Aliah Zannierah Mohsin:** Writing – review and editing; supervision; validation; methodology; data curation; formal analysis. **Nazamid Saari:** Supervision; validation.

Conflict of interest

The authors declare no conflicts of interest in the research presented in this publication.

Ethics approval

Ethics approval was not required for this research.

Peer review

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Data availability statement

Research data are not shared.

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