HYDROLYSIS OF GOAT'S MILK CASEIN USING VARIOUS ENZYMES AT DIFFERENT CONDITIONS AND ANTIGENICITY THROUGH ENZYME-LINKED IMMUNOSORBENT ASSAY

By

NURUL IZZA NABILAH BINTI MOHD SAMSUDIN

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2025

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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January 2025

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Goat's milk has shown a rising demand among individuals with cows' milk protein allergy (CMPA) due to its lower allergenic properties than cows. However, casein, the main protein in goat's milk, showed a high homology to cow's casein, contributing to allergic reactions in CMPA patients. To mitigate the allergenic immune responses, casein, as an important epitope responsible for this problem, can be broken down through enzymatic hydrolysis, producing varying-sized peptides. Each enzyme will have its unique condition to work optimally, and there is a lack of understanding on how enzymes can break down goat's milk casein. Apart from hydrolysis conditions, there is limited research on detecting goat's milk casein and its antigenicity reduction using various enzymes. Hence, this study aims to evaluate optimal hydrolysis conditions using various enzymes for reducing antigenicity in goat's milk casein through enzyme-linked immunosorbent assay (ELISA). The hydrolysis conditions of goat's milk casein were optimised using non-gastrointestinal

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digestive enzymes (bromelain and alcalase) and gastrointestinal digestive enzymes (trypsin and pepsin), at different hydrolysis conditions, which include pH, enzyme-substrate ratio, the temperature of hydrolysis, and time. This study evaluated a series of parameters, including ELISA formats, types, and antibody concentrations to evaluate antigenicity reduction. Result showed that the optimal hydrolysis condition for bromelain (1:100, pH 5.5, 50 °C, 6 h), alcalase (1:50, pH 8.0, 55 °C, 6 h), trypsin (1:10, pH 8.0, 37 °C, 6 h) and pepsin (1:10, pH 1.5, 37 °C, 6 h) produced degree of hydrolysis (DH) (40-60%) and peptide content (ca. 2 mg/mL). Indirect ELISA format produced the highest signal-to-noise (S:N) ratio (9.80) using polyclonal antibody anti-casein (1:500 v/v) and goat anti-rabbit HRP as the secondary antibody (1:15,000 v/v). In terms of antigenicity reduction, bromelain which exhibited maximal DH of 60% and peptide content of 2.86 mg/mL, had the highest reduction of antigenicity (33.60 ± 0.014%), producing peptides below 10 kDa. This study demonstrated that hydrolysis conditions with appropriate enzymes are crucial for the effective reduction of goat's milk casein antigenicity. This discovery provides the foundational development of a hypoallergenic goat milk formula, enhancing the added value of goat milk while ensuring consumer safety.

Keywords: *Casein, goat's milk, antigenicity, ELISA, hydrolysis*

SDG: GOAL 3: Good Health and Well-Being

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

HIDROLISIS KASEIN SUSU KAMBING MENGGUNAKAN PELBAGAI ENZIM PADA KEADAAN HIDROLISIS BERBEZA DAN KESAN KE ATAS KEANTIGENAN MENGGUNAKAN UJIAN ASAI IMUNOSERAPAN TERANGKAI ENZIM

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Permintaan terhadap susu kambing semakin meningkat dalam kalangan individu yang menghidap alahan protein susu lembu (CMPA) kerana sifatnya yang kurang alahan berbanding susu lembu. Walau bagaimanapun, kasein, protein utama dalam susu kambing, menunjukkan homologi yang tinggi dengan kasein lembu, yang menyumbang kepada reaksi alahan dalam pesakit CMPA. Untuk mengurangkan tindak balas imun alahan, kasein, sebagai epitop penting yang bertanggungjawab untuk masalah ini, boleh diuraikan melalui hidrolisis enzimatik, menghasilkan peptida berbagai saiz. Setiap enzim mempunyai keadaan uniknya sendiri untuk berfungsi dengan optimum, dan terdapat kekurangan pemahaman tentang bagaimana enzim boleh menguraikan kasein susu kambing. Selain keadaan hidrolisis, terdapat sedikit kajian mengenai pengesanan kasein susu kambing and pengurangan keantigenannya dengan menggunakan pelbagai enzim. Oleh itu, kajian ini bertujuan untuk menilai keaddan hidrolisis optimum menggunakan pelbagai

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enzim untuk mengurangkan keantigenan kasein susu kambing melalui asai immunoserap terangkai enzim (ELISA). Keadaan hidrolisis kasein susu kambing dioptimumkan menggunakan enzim pencernaan bukan-gastrousus (bromelain dan alkalase) dan enzim pencernaan gastrousus (tripsin dan pepsin), pada keadaan hidrolisis yang berbeza, yang termasuk pH, nisbah enzim-substrat, suhu hidrolisis, dan masa. Pelbagai parameter, termasuk format ELISA, jenis, dan kepekatan antibodi, dinilai dalam kajian ini untuk megukur tahap keantigenan. Hasil menunjukkan keadaan hidrolisis optimum untuk bromelain (1:100, pH 5.5, 50 °C, 6 jam), alkalase (1:50, pH 8.0, 55 °C, 6 jam), tripsin (1:10, pH 8.0, 37 °C, 6 jam) dan pepsin (1:10, pH 1.5, 37 °C, 6 jam) menghasilkan tahap hidrolisis (DH) (40-60%) dan kandungan peptida (sekitar 2 mg/mL). Format ELISA tidak langsung menghasilkan nisbah isyarat-hingar (S:N) tertinggi (9.80) menggunakan antibodi poliklonal anti-kasein (1:500 *v/v*) dan antibodi kambing anti-arnab HRP (1:15,000 *v/v*). Dari sudut pengurangan keantigenan, bromelain menunjukkan DH maksimum sebanyak 60% dan kandungan peptida sebanyak 2.86 mg/mL, dan mempunyai pengurangan keantigenan tertinggi (33.60 ± 0.014%), menghasilkan peptida di bawah 10 kDa. Kajian ini menunjukkan bahawa keadaan hidrolisis dengan enzim yang sesuai adalah penting untuk pengurangan keantigenan kasein susu kambing yang berkesan. Penemuan ini

menyediakan pembangunan asas formula susu kambing hipoalergenik yang meningkatkan nilai tambah susu kambing dan seterusny memastikan keselamtan pengguna.

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Kata Kunci*: Kasein, susu kambing, keantigenan, ELISA, hidrolisis*

SDG: MATLAMAT 3: Kesihatan dan Kesejahteraan yang Baik

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science by Research. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vii
DECLARATION		ix
LIST OF TABLES		xiv
LIST OF FIGURES		xvi
LIST OF ABBREVIATIONS		xviii
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	10
2.1	Goat's milk	10
2.1.1	Health benefits and safety concerns	10
2.2.1	Cross-reactivity	12
2.2	Hydrolysis in milk	19
2.2.1	Enzyme hydrolysis	19
2.2.2	Types and specificity of enzymes	21
2.2.3	Enzyme cleavage sites in casein generating different sizes of peptide	23
2.2.4	Hydrolysis conditions	63
2.2.5	Bioactive peptide and structure-activity relationship (SAR) on goat milk peptide	66
2.3	ELISA assessment method in milk	70
3	**EFFECT OF HYDROLYSIS CONDITIONS USING GASTROINTESTINAL DIGESTIVE AND NON-DIGESTIVE ENZYMES**	75
3.1	Introduction	75
3.2	Materials and Methods	76
3.2.1	Chemicals, reagents, and instrument	77
3.2.2	Casein hydrolysis	77
3.2.3	Determination of degree of hydrolysis	82
3.2.4	Determination of peptide content	83
3.2.5	Statistical analysis	84
3.3	Results and Discussion	84
3.3.1	Evaluation of hydrolysis conditions	85
	3.3.1.1 Hydrolysis using alcalase	89
	3.3.1.2 Hydrolysis using bromelain	91
	3.3.1.3 Hydrolysis using trypsin	96
	3.3.1.4 Hydrolysis using pepsin	102
	3.3.1.5 Comparison between various enzymes	107

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3.4 Conclusion 111

4 DETECTION OF GOAT'S MILK CASEIN THROUGH VARIOUS ENZYME-LINKED IMMUNSORBENT ASSAY (ELISA) APPROACHES USING CAPRINE ALPHA-S1 CASEIN PEPTIDES AND BOVINE CASEIN POLYCLONAL ANTIBODIES 113

4.1 Introduction 113
4.2 Materials and Method 116
4.2.1 Chemicals and reagent 116
4.2.2 Sample collection 116
4.2.3 Protein determination (Kjeldahl) 117
4.2.4 Casein isolation 120
4.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis 121
4.2.6 Sandwich ELISA 122
4.2.6.1 Antibody concentration of caprine alpha-s1 casein peptide 122
4.2.6.2 Blocking reagent 122
4.2.6.3 Casein pre-treatment 122
4.2.7 Bovine casein polyclonal antibodies 123
4.2.7.1 Direct ELISA 123
4.2.7.2 Indirect ELISA 123
4.2.8 Statistical analysis 124
4.3 Result and Discussion 125
4.3.1 Isolation of casein 127
4.3.2 Sandwich ELISA 127
4.3.2.1 Background signal 127
4.3.2.2 Blocking reagent 132
4.3.2.3 Antibody concentration and pre-treatment 138
4.3.3 Direct ELISA 138
4.3.4 Indirect ELISA 141
4.4 Conclusion 144

5 ANTIGENICITY OF GOAT'S MILK CASEIN FROM GI DIGESTIVE AND GI NON-DIGESTIVE ENZYME HDYROLYSATE AND PEPTIDE SIZE 145

5.1 Introduction 145
5.2 Material and Methods 145
5.2.1 Chemicals, reagents, and instrument 147
5.2.2 Bradford assay 147
5.2.3 Indirect ELISA 148
5.2.4 Fractionation of goat's milk casein hydrolysate using viva spin column 149
5.2.5 SDS-PAGE 149
5.2.6 Statistical analysis 150
5.3 Results and Discussion 153
5.3.1 Protein concentration 153

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5.3.2 Antigenicity reduction using various enzymes 155
5.3.3 Effect of peptide size on antigenicity 155
5.4 Conclusion 160

6 SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH 161

REFERENCES 164
APPENDICES 185
BIODATA OF STUDENTS 187
LIST OF PUBLICATIONS 188

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LIST OF TABLES

Table		Page
2.0	Casein sequence on goat and other types of ruminants' milk casein using Uniprot database	13
2.1	Enzymatic cleavage sites of different enzymes for goat's milk casein sequence	23
2.2	Condition of enzymatic hydrolysis conditions from different ruminants using various enzymes	64
2.3	Development of ELISA using various formats for detection of different milk protein	69
2.4	Commercial kits of ELISA for detection of different targeted milk proteins	73
3.0	A mixture of different types of solution to make ortho-phtalaldehyde solution	83
3.1(a)	Degree of hydrolysis and peptide content at different hydrolysis time and substrate: enzyme (E:S) ratios using alcalase	88
3.1(b)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and pH using alcalase	89
3.1(c)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and temperatures using alcalase	90
3.2(a)	Degree of hydrolysis and peptide content at different hydrolysis times and substrate: enzyme (E:S) ratios using bromelain	93
3.2(b)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and pH using bromelain	94
3.2(c)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and temperatures using bromelain	95

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3.3(a)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and substrate: enzyme (E:S) ratios using trypsin	99
3.3(b)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and pH using trypsin	100
3.3(c)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and temperatures using trypsin	101
3.4(a)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and substrate: enzyme (E:S) ratios using pepsin	104
3.4(b)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and pH using pepsin	105
3.4(c)	Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and temperatures using pepsin	106
3.5	Comparison of degree of hydrolysis and peptide content using different types of enzymes	110
4.0	Background reading of streptavidin-HRP in phosphate-buffered saline (PBS) at different dilutions	129
4.1	Background reading of C-terminal-biotin IgG together with streptavidin-HRP (1:15, 000, v/v) analysed in three different days	130
4.2	Background reading of sandwich ELISA in different solutions without casein using different dilutions of Cter-biotin IgG	131
5.0	Total soluble protein of unhydrolysed and hydrolysed casein	152

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LIST OF FIGURES

Figure		Page
1.0	Overview of the study	9
2.0	Structure of casein (adapted from Hristov *et al.,* 2015)	11
2.1	Illustration on reduction of antigenicity and allergenicity of milk protein using enzymatic hydrolysis	18
3.0(a)	Non-gastrointestinal digestive enzymes and hydrolysis conditions used for hydrolysis of goat's milk casein	80
3.0(b)	Gastrointestinal digestive enzymes and hydrolysis conditions used for hydrolysis of goat's milk casein	81
3.1(a)	A comprehensive workflow of non-gastrointestinal digestive enzymes after selection of hydrolysis conditions used for hydrolysis of goat's milk casein	108
3.1(b)	A comprehensive workflow of gastrointestinal digestive enzymes after selection of hydrolysis conditions used for hydrolysis of goat's milk casein	109
4.0	Different formats of ELISA; (a) sandwich (b) direct (c) indirect	115
4.1	Separation of goat's milk protein based on size through SDS-PAGE. Protein marker. Lanes 3-6: Casein bands at 25-32 kDa	127
4.2	Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA using different types of blocking reagents.	133
4.3	Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein using sandwich ELISA after heating and addition of denaturing agent 0.1% SDS. Dilution used in this experiment were 1:1,000 (v/v), 1:1,000 (v/v) Cter biotin-IgG, and 1:15,000 (v/v) streptavidin-HRP.	135
4.4	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 1:1,2500 (v/v) primary antibody, 1:1,000 (v/v) secondary antibody, and 1:15,000 (v/v) streptavidin-HRP	135

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4.5	Absorbance and signal-to-noise ratio of goat's milk casein in direct ELISA using capture antibody and streptavidin-HRP, with 1:500 and 1:7,500 (v/v), respectively, after heating and addition of 0.1% SDS.	139
4.6	Signal-to-noise ratio and average absorbance of goat's milk casein in direct ELISA after heating and addition of 0.1% SDS, using a 1:1,000 capture antibody and a 1:7,500 (v/v) of streptavidin-HRP.	140
4.7	Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein and bovine standard using different concentrations of primary and secondary antibody in indirect ELISA.	142
4.8	Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein and bovine standard in indirect ELISA after heating and addition of 0.1% SDS, using 1:500 (v/v) of primary antibody and 1:7,500 (v/v) of secondary antibody.	143
5.0	Antigenicity reduction by various enzymes	154
5.1	Protein separation of goat's milk casein hydrolysate using bromelain on SDS PAGE at Lane 2.	156
5.2	Effect of peptide size on antigenicity	158

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LIST OF ABBREVIATION

BSA	Bovine serum albumin
CMPA	Cow milk protein allergy
α-LA	Alpha-lactalbumin
β-LG	Beta-lactoglobulin
Cter	C-terminal
DH	Degree of hydrolysis
ELISA	Enzyme-linked immunosorbent assay
HCl	Hydrochloric acid
IgE	Immunoglobulin E
IgG	Immunoglobulin G
Nter	N-terminal
OPA	O-phthaldialdehyde
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline Tween 20
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

The global goat's milk market is expected to grow at 3.8% from 2019 to 2026, from $8.5 billion in 2018 to $11.4 billion in 2026 (Kadam *et al.,* 2019). According to the Food and Agriculture Organisation (2022), around 1.11 billion goats worldwide produce over 20.7 million tons of milk annually. Asia contributes 56% of this output, with Africa at 24%, Europe at 15%, the Americas at 4%, and Oceania at 1%. According to Statistica (2024), the production of Malaysian goat's milk is expected to expand from 2022 to 2026 at a rate of 7.8%. Malaysian fresh raw milk production was expected to grow annually by 9.04% from 2024 to 2029. The growth is driven by increasing consumer demand for alternative infant feeding that may reduce allergenicity. The growing popularity of goat's milk is also linked to cultural practices, particularly its use as a sunnah meal among Muslims in Malaysia, who appreciate its health benefits, digestibility, and nutritional composition (Zulkifli *et al.*, 2023). Paskas *et al.* (2020) discovered that goat's milk consumers are inclined to pay a premium value for the product, owing to its superior nutritional benefits.

Goat's milk is considered superior to other ruminant milk due to its abundant high-value nutrients and functional food benefits. It contains bioactive peptides exhibiting antimicrobial, antioxidant, anticancer, and therapeutic properties (Dagnaw *et al.* 2016; Yadav *et al.*, 2016; Chauhan *et al.*, 2021; Al-Kaisy *et al.,*

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2023). In terms of milk composition, goat's milk possesses a higher fat content (3.8%) than cow's milk, making it a thicker and creamier texture, and has more protein (3.5%) while containing less lactose (Akshit *et al.,* 2024). These properties make goat's milk ideal for dairy products, including cheese, ice cream, buttermilk, butter and yoghurt (Nayik *et al.,* 2021) and suitable for those with lactose sensitivity. Additionally, research indicates that goat's milk, characterised by smaller fat globules and high concentration of short and medium-chain fatty acids, as well as vitamins and minerals with a superior absorption rate than a cow, is easier to digest and absorb (Turkmen, 2017; Jung *et al.*, 2016). As a result, the demand for goat's milk has been rising globally due to its numerous health-promoting benefits.

Despite its growing popularity, there are still concerns regarding the safety issues of goat's milk and its products, especially allergenicity. Like cow's milk, goat's milk consists of 80% casein (alpha-$_{s1}$-, alpha-$_{s2}$-, beta-, and kappa-) and 20% whey casein. Casein was reported to be the most predominant allergen in goat's milk (Aalst *et al.,* 2024). Historically, 24 out of 26 children had a positive test on the skin prick test, double-blind placebo, and oral food challenges with goat's milk and cow's milk (Bellioni, 1999).

Although cases of goat's milk allergy in individuals who are tolerant to cow's milk are rare, some studies have found that goat's milk is not a replacement for cow's milk due to the high degree of similarity in their casein proteins, which may cause cross-reactivity (Mansor *et al.,* 2023; Prosser, 2021). For instance, over 90% of patients who suffer from CMPA cow's milk protein patients

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exhibited similar allergy symptoms for goat's milk (Levi *et al.,* 2014). As a result, goat's milk is not recommended as an alternative to cow's milk for infants with IgE-mediated cow's milk allergies (Aalst *et al.,* 2024). To address these allergenic concerns, hydrolysis of goat's milk proteins can offer a promising solution. Various hydrolysis approaches, including physical methods, acid treatment, and enzymatic hydrolysis (Maryniak *et al*. 2022) have been used to break down proteins into smaller peptides. By far, enzymatic hydrolysis is a reliable processing technique that decreases the allergenic characteristics of milk proteins (Liang *et al.,* 2020). This is because enzyme hydrolysis breaks down the linear and confirmational epitopes of the casein. Enzymes specifically cleave the peptide bonds of casein that are responsible for the allergic reaction, resulting in smaller peptides with shorter amino acid sequences (Corredig *et al.,* 2019). These smaller peptides are less likely to be recognised by the antibodies, thus reducing their allergenic potential. Enzymes used for this hydrolysis can derived from different sources, including the gastrointestinal tract (pepsin and trypsin) and plant-based, such as bromelain, and through microbial fermentation, such as alcalase (Fu *et al.,* 2021). Different enzymes exhibit varying specificity and proteolytic activity, each functioning optimally under specific conditions. Given the limited understanding of goat's milk casein hydrolysis, this study aims to evaluate hydrolysis conditions to reduce the antigenicity of goat's milk.

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1.1 Problem statement

Cow’s milk is the most widely consumed milk globally, but there is increasing interest in goat’s milk as an alternative, especially for individuals with cow’s milk protein allergies. Due to its nutritional benefits, goat’s milk is often considered a safer option. However, the main issue is that substituting goat’s milk for cow’s milk presents significant allergenic concerns, particularly for individuals with IgE-mediated cow’s milk allergies. The amino acid sequences of casein proteins in goat and cow milk show significant homology, resulting in cross-reactivity. Consequently, goat's milk is not advised as a replacement for individuals with IgE-mediated cow’s milk allergies. Casein serves as the principal allergen implicated in IgE-mediated allergic responses to both cow's and goat's milk. The significant amino acid sequence similarity between casein proteins in both types of milk elevates the risk of cross-reactivity, rendering goat’s milk inappropriate for infants with IgE-mediated cow’s milk allergies. The allergenic potential of cow’s milk casein is well characterised; however, the casein in goat’s milk is less studied, highlighting a significant gap in understanding its antigenicity and the necessity for strategies to mitigate its allergenicity. Enzyme hydrolysis effectively diminishes allergenicity by degrading the epitopes present in milk casein. Hydrolysis using individual enzymes presents challenges and can be complex.

Enzymes demonstrate diverse specificities and proteolytic activities, leading to inconsistencies in enzyme hydrolysis and resulting in variable peptide sequences. Understanding of the optimal enzymes and hydrolysis conditions for goat’s milk casein remain inadequate. This study integrates gastrointestinal

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(GI) and non-gastrointestinal (non-GI) digestive enzymes to assess their efficacy in diminishing the allergenic potential of goat's milk casein. Gastrointestinal digestive enzymes, including pepsin and trypsin, replicate the natural digestion process by specifically targeting peptide bonds during *in vivo* digestion. These enzymes are significant for elucidating the degradation of casein in the digestive system and its potential in decreasing milk allergenicity.

Non-GI digestive enzymes, such as bromelain and alcalase, exhibit unique proteolytic activities. These enzymes may provide benefits in the degradation of allergenic epitopes by targeting distinct peptide bonds or functioning under varying conditions. Their application may enhance the hydrolytic actions of gastrointestinal enzymes, offering a more efficient approach to decreasing allergenicity. This study compares the efficacy of GI and non-GI digestive enzymes to identify the most effective enzymes for hydrolysing goat's milk casein into smaller, less allergenic peptides. This dual approach will facilitate the assessment of hydrolysis conditions, thereby promoting the advancement of safer dairy products for individuals with milk allergies. The lack of standardised methods for assessing the allergenic potential of milk products presents a considerable challenge in addressing allergenicity.

Current methodologies, including skin prick tests and double-blind placebo tests, offer restricted understanding of the allergenic potential of hydrolysed proteins. Method such as ELISA, are primarily optimised for cow's milk and demonstrate reduced efficacy in detecting goat's milk casein, attributable to structural and antigenic differences. This study seeks to address significant

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knowledge gaps by enhancing the understanding of the hydrolysis of goat's milk protein. The study aims to enhance the safety of dairy products for individuals with milk allergies by determining optimal enzyme hydrolysis conditions and developing more precise methods for evaluating antigenicity in goat's milk.

1.3 Research objectives

The main goal of this study was to evaluate hydrolysis conditions for the reduction of antigenicity of goat's milk. To achieve the main goal, this study aimed;

1. To evaluate hydrolysis conditions on goat's milk casein using gastrointestinal digestive and non-gastrointestinal digestive enzymes.
2. To evaluate and optimise multiple approaches of ELISA for detecting goat's milk casein.
3. To determine the antigenicity of goat's milk casein hydrolysate of different enzymes and peptide sizes.

1.4 Significance of study

Effective mechanisms to reduce the allergenicity of goat's milk casein remain underexplored. This study provides valuable data and methodologies on enzyme hydrolysis and antigenicity, offering insights that advance research in this field. In the immediate future, these findings will facilitate subsequent research and development, especially in doing *in vivo* tests to validate the hypoallergenicity of hydrolysed goat's milk. The enzymes utilised in this research may also be employed on alternative protein hydrolysates, enhancing

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the comprehension of antigenicity. This facilitates further research opportunities, encompassing peptide sequence discovery, characterisation, and epitope mapping—essential processes for assessing immune responses and enhancing the safety of dairy products. The produced data will function as a significant reference in dairy science and food safety, offering researchers critical insights to progress the discipline. In the long run, findings of the study can facilitate the development of hypoallergenic goat's milk-based formulas. For consumers, the availability of safer food products enhances confidence in product safety and quality, leading to greater satisfaction and improved health management.

1.5 Limitation of the study

The limitation of this study includes that the assessment of antigenicity was conducted solely through *in vitro* ELISA-based methods, without *in vivo* allergenicity testing. Due to ethical constraints and the unavailability of blood serum from individuals specifically allergic to goat's milk casein, direct allergenicity validation could not be performed. Additionally, the absence of commercially available ELISA kit specifically designed for goat's milk casein posed a challenge, making it difficult to achieve optimal assay conditions for antigenicity assessment. However, multiple ELISA approach and different parameters were tested to confirm the substance of ELISA used in this study. The lack of a specific antibody for goat's milk casein also limits the precision of our antigenicity assessments. Furthermore, protein recovery in this study was relatively low, and the protein was not subjected to purification, which may have influenced the characterisation of antigenic peptides. Despite these

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limitations, the findings provide valuable insights into antigenicity assessment and serve as a foundation for further research.

1.6 Overview of the Study

The overall study was summarised in **Figure 1.0.** This chapter evaluated various parameters, including different formats of ELISA, pre-treatment, blocking reagent, and concentration of antibodies. The hydrolysis conditions of goat's milk casein were optimised through different enzymes. i.e. bromelain, alcalase, trypsin and pepsin, pH, time and temperature of hydrolysis, and enzyme-substrate ratio. Parameters on enzyme-to-substrate ratio, pH, time, and temperature were optimised to obtain the optimal condition for casein hydrolysate. The optimal hydrolysis conditions were then used to analyse the antigenicity reduction using optimised ELISA. These findings provide a basis for developing a hypoallergenic goat's milk formula that increases the added value of goat's milk manufacturers, safeguarding consumer's health.

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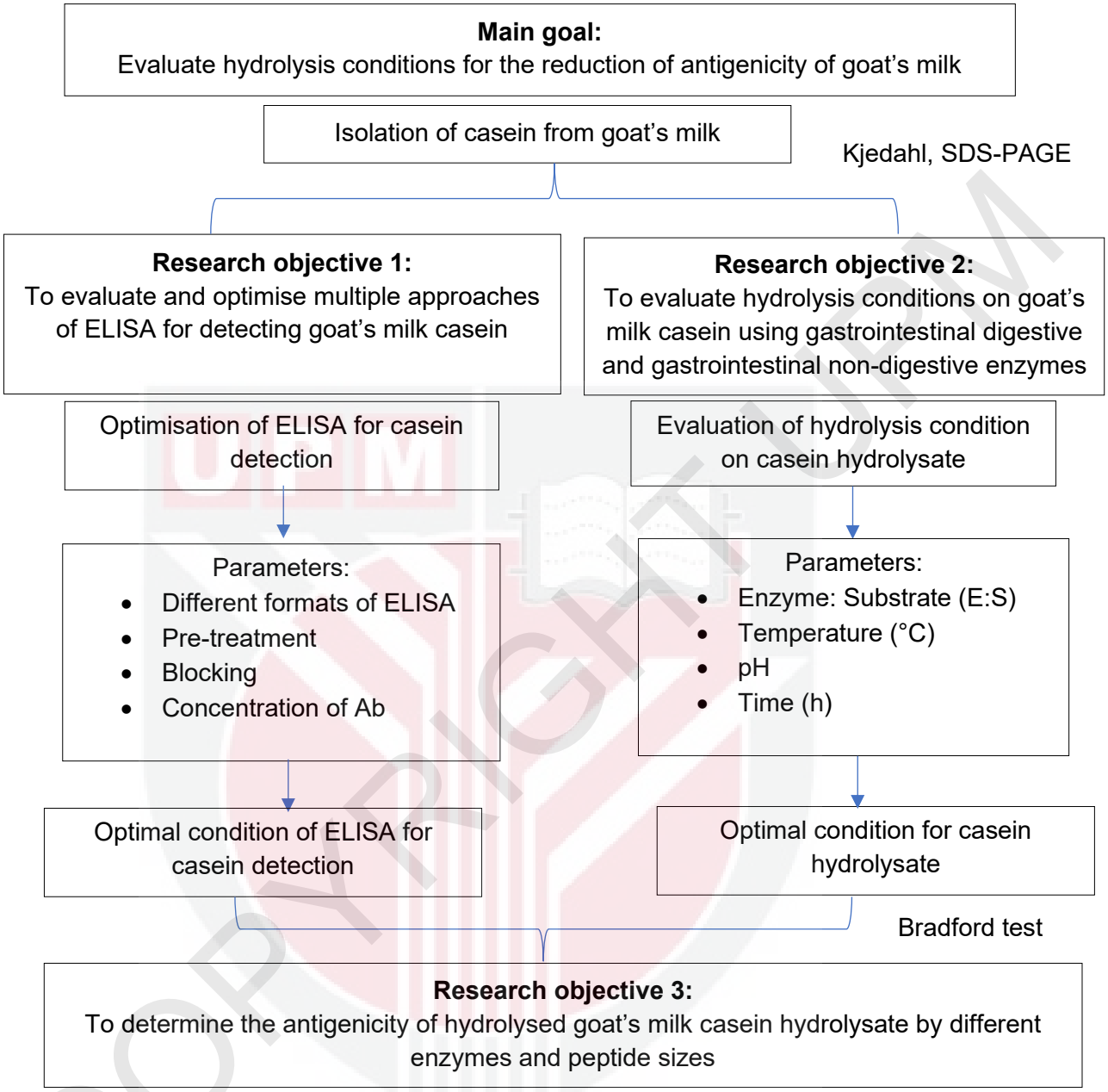

Figure 1.0: Overview of the study

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CHAPTER 2

LITERATURE REVIEW

2.1 Goat's Milk

2.1.1 Health benefits and safety concerns

Goat's milk is often superior to ruminant milk due to its rich nutritional profile and multiple functional benefits. It contains approximately 3.8% fat, resulting in a thicker and creamier consistency compared to other milk varieties. It includes approximately 3.5% protein and reduced levels of lactose, rendering it more appropriates for individuals with lactose intolerance (Akshit *et al.,* 2024). Goat's milk possesses properties that render it an ideal foundation for various dairy products, such as cheese, ice cream, buttermilk, butter, and yoghurt (Nayik *et al.,* 2021). Additionally, goat's milk offers several benefits compared to cow's milk, attributed to its smaller fat globules and elevated levels of short and medium-chain fatty acids. These factors enhance digestibility and improve the absorption of essential vitamins and minerals (Turkmen, 2017; Jung *et al.,* 2016). The distinctive composition of goat's milk renders it a versatile ingredient in dairy products and a preferable alternative for consumers aiming for improved digestive tolerance and enhanced nutritional benefits. Although goat's milk possesses significant nutritional benefits and is in high demand, concerns regarding its allergenic potential have increased. Levi *et al.* (2014) report that over 90% of patients with cow's milk protein allergy show cross-reactivity to goat's milk.

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Consumers with a cow's milk protein allergy may display comparable allergic reactions, including hives, eczema, itching, diarrhoea, vomiting, respiratory difficulties, or severe allergic responses such as anaphylaxis, which can be fatal (Edwards, 2023; Martorella-Aragones *et al.,* 2015; Hochwallner *et al.,* 2017). Only a rare case of specific allergy reactions to goat's milk in cow's milk tolerant individuals have been reported (Goh *et al.,* 2019). **Figure 2.0** shows the structure of casein micelle, consisting of alpha-s1, alpha-s2, beta-, and kappa- casein joined together within a calcium phosphate.

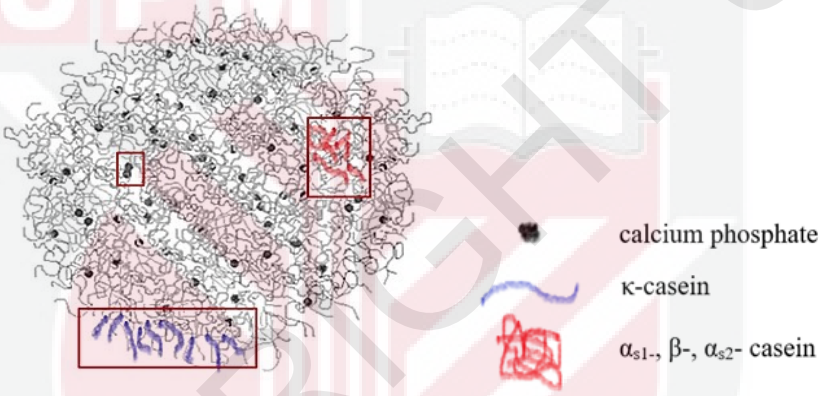

Figure 2.0: Structure of casein (adapted from Hristov *et al.,* 2015)

2.1.2 Cross-reactivity

Casein serves as a significant allergen in milk and plays a main role in cross-reactivity among milk from ruminant species (Naito *et al.,* 2020). Cross-reactivity could arise when amino acid sequences are shared or molecular structures are similar between different kinds of milk allergens, enabling them to attach to specific antibodies (Negi & Braun, 2016). Immunoglobulin (IgE) epitopes determine the primary factors behind cow's milk protein allergy, and the amount and durability of these epitopes determine food allergy reaction severity among cow's milk protein allergy individuals as discovered by He *et*

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al., (2023). Bioactive molecules, i.e. histamine, eosinophile chemokines, and 5-hydroxytryptamine may be released when it binds specifically to the IgE antibodies on activated basophils and mast cells, triggering adverse allergy symptoms in humans (Kashiwakura *et al.*, 2019). A study by Breitenede (2018) revealed that most IgE epitopes in food allergens are linear and conformational. Linear epitopes can be easily identified using peptide microarrays, meanwhile, conformational epitopes are more complex to identify since they are formed by the folding of proteins, which brings amino acid residues that are far apart into close spatial proximity. IgE-mediated responses occur immediately after consumption, but non-IgE-mediated reactions, such as IgG antibodies, are also generated in milk allergies, although the immunological response is delayed (Anvari *et al.,* 2019).

A recent study found that goat's milk protein has a significant homology or cross-reactivity with cow's milk (Arasi *et al.*, 2022). The occurrence of cross-reactivity in milk from various ruminants is attributed to the significant resemblance in the amino acid sequence. In another study, Mansor *et al.* (2023) showed evidence of cross-reactivities of cow's casein with four variants from Jamnapari breed and two milk allergens from Saanen, α_{S1}- and β-lactoglobulin. Meanwhile, Toggenburg goat's milk exhibited reactivity solely towards one specific milk allergen, k- casein. To compare the sequence of each type of goat casein with other ruminants, a protein sequence database, Uniprot (Universal Protein Resource) was utilised and analysed as presented in **Table 2.0.**

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Table 2.0: Casein sequence of goat and other ruminant milk casein from Uniprot database (2023)

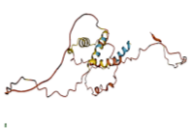
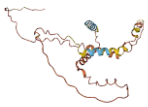
Type of casein		Goat casein protein sequence (*Capra hircus*)	Cow casein protein sequence (*Bos taurus*)	Water buffalo protein sequence (*Bubalus bubalis*)	Amino acid sequence homology
					
α_{S1}	Protein ID/Uniprot ID:	CSN1S1/>sp|P18626|CASA1_CAPHI	CSN1S1>sp|P02662|CASA1_BOVIN	CSN1S1>sp|O62823|CASA1_BUBBU	91.6% similarities of α_{s1}-casein between goat and bovine. There are 18 unsimilar letter found in cow α_{s1}-casein 82.71% similarities between α_{s1}-casein between goat and buffalo. There are 37 unsimilar letter found in buffalo α_{s1}-casein.
	Number of total amino acid	214 aa	214 aa	214 aa	
	Molecular weight	24.3 kDa	24.5 kDa	24.3 kDa	
	Amino acid sequence	MKLLILTCLV[10]AVALARPKHP[20]**I**NHRGL**SP**EV[30]**P**NENLLRF**V**V[40]APFPEVF**R**KE[50]**NI**NELSKDIG[60]SESTEDQAME[70]D**A**KQMKA**G**S**S**[80]SSSEEIVPNS[90]**A**EQK**Y**IQKED[100]VPSERYLGYL[110]EQLLRLKKY**N**[120]VPQLEIVP**K**S[130]AEEQLHSMKE[140]GNPAHQKQPM[150]IAVNQELAYF[160]YP**Q**LFRQFYQ[170]LDAYPSGAWY[180]Y**L**PLGTQYTD[190]APSFSDIPNP[200]IGSENS**G**KTT[210]MPLW	MKLLILTCLV[10]AVALARPKHP[20]**I**KHQGL**PQ**EV[30]LNENLLRF**F**V[40]APFPEVF**G**KE[50]**KV**NELSKDIG[60]SESTEDQAME[70]D**I**KQM**E**AES**I**[80]SSSEEIVPNS[90]**V**EQK**H**IQKED[100]VPSERYLGYL[110]EQLLRLKKY**K**[120]VPQLEIVP**N**S[130]AEERLHSMKE[140]GIHAQQKEPM[150]IGVNQELAYF[160]YP**E**LFRQFYQ[170]LDAYPSGAWY[180]Y**V**PLGTQYTD[190]APSFSDIPNP[200]IGSENS**E**KTT[210]MPLW	MKLLILTCLV[10]AVALARPK**QP**[20]I**K**HQG**L**P**QG**V[30]**L**NENLLRF**F**V[40]**A**PFPEVF**G**KE[50]**KV**NELS**T**DIG[60]SESTEDQAME[70]D**I**KQM**EA**E**S**I**[80]SSSEEIVPI**S**[90]**V**EQK**H**IQKED[100]VPSERYLGYL[110]EQLLRLKKYN[120]VPQLEIVP**NL**[130]AEEQLHSMKE[140]G**I**H**A**Q**Q**K**E**PM[150]I**G**VNQELAYF[160]YPQLFRQFYQ[170]LDAYPSGAWY[180]Y**V**PLGTQY**P**D[190]APSFSDIPNP[200]I**G**S**ENSG**KTT[210]MPLW

Table 2.0: Continued

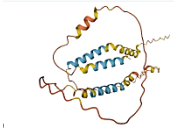
					
α_{S2}	Protein ID/Uniprot ID:	CSNIS2/>sp|P33049|CASA2_CAPHI	CSN1S2/>sp|P02663|CASA2_BOVIN	CSN1s2>tr|A0A7T6ZLP5|A0A7T6ZLP5_BUBBU	80.18% similarities of αs_2-casein between goat and bovine. There are 44 unisimilar letters found in αs_2 -casein in bovine. 76.58% similarities of αs_2-casein between goat and buffalo. There are 52 unisimilar letters found in αs_2 -casein
	Number of total amino acid	223 aa	222 aa	222 aa	
	Molecular weight	26.4 kDa	26.02 kDa	26.01 kDa	
	Amino acid sequence	MKFFIFTCLL[10]AVALAK**HK**ME[20]HVSSSEE**PIN**[30]**IFQEI**YKQEK[40]**N**MAI**HPRKEK**[50]**L****C**TT**S**C**E**EV**V**[60]**R**NANEEEYSI[70]RSSSEESAEV[80]APEEIKITVD[90]DKHYQKALNE[100]**IN**QFYQKFPQ[110]**Y**LQY**P**YQGPI[120]**V**LNPWDQVKR[1]**[30]NAGPFTPTVN**[14]	MKFFIFTCLL[10]AVALAK**NT**ME[20]HVSSSEE**SII**[30]**SQET**YKQEK**N**[40]MAI**NP****SKENL**[50]**CS**TFCKEVV**R**[60]NANEEEYSI**G**[70]SSSEESAEVA[80]TEEVKITVDD[90]KHYQKALNEI[100]NQFYQKFPQ**Y**[110]LQY**L**YQGPI**V**[120]	MKFFIFTCLL[10]AVALAKH**T**ME[20]HVSSSEE**SII**[30]**S**QET**Y**KQEK**N**[40]MAIHP**S**KE**NL**[50]**C****S**T**F**C**K**EV**IR**[60]**N**ANEEEYSI**G**[70]**S**SSEESAEV**A**[80]**T**EE**VK**ITVD**D**[90]KHYQKALNEI[100]NQFYQKFPQY[110]LQY**L**Y**Q**GPI**V**[120]LNPWD**Q**VKR**N**[1]**[30]AVPIT**PT**L**N**R[1][40]EQLSTSEEN**S**[150]KK	

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Table 2.0: Continued

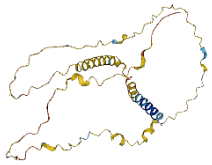
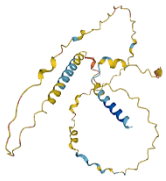
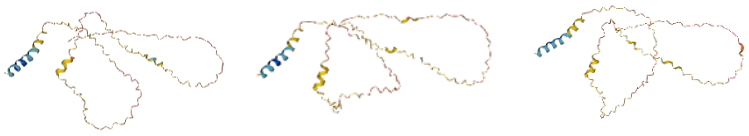
					
β-	Protein ID/Uniprot ID:	CSN2/>sp|P33048|CASB_CAPHI	CSN2/>sp|P02666|CASB_BOVIN	CSN2>sp|Q9TSI0|CASB_BUBBU	88% similarities of β-casein between goat and cow. There are 28 unisimilar letters found in β-casein in cow. 89% similarities of β-casein between goat and buffalo.There are 24 unsimilar letters found in β-casein in in buffalo.
	Number of total amino acid:	222 aa	224 aa	224 aa	
	Molecular weight	24.9 kDa	25.1 kDa	25.11 kDa	
		MKVLILACLV[10]ALAIARE**Q**EE[20]LNV**VGET**VES[30]LSSSEESIT**H**[40]INKKIEKFQS[50]EEQQQTEDEL[60]QDKIHPFAQ**A**[70]QSLVYPF**T**GP[80]IPNSLPQNI**L**[90]PLTQTPVVVP[100]PFLQPE**I**MGV[110]**P**KVKE**T**MVPK[120]HKEMPFPKYP[130]VEPFTESQSL[140]TLTDVE**K**LHL[150]PLPL**V**QSWMH[160]QP**P**QPL**S**PTV[170]MFPPQSVLSL[180]SQ**P**KVLPVPQ[190]KAVPQRDMPI[200]QAFLLYQE**PV**[210]**L**GPVRGPF**PI**[220]**L**V	MKVLILACLV[10]ALA**L**ARE**L**EE[20]LNV**PGEI**VES[30]LSSSEESIT**R**[40]INKKIEKFQS[50]EEQQQTEDEL[60]QDKIHPFAQ**T**[70]QSLVYPF**P**GP[80]IPNSLPQNI**P**[90]PLTQTPVV**V**P[100]PFLQPE**V**MGV[110]**S**KVKE**A**M**A**PK[120]HKEMPFPKYP[130]VEPFTESQSL[140]TLTDVE**N**LHL[150]PLPL**L**QSWMH[160]QP**H**QPL**P**PTV[170]MFPPQSVLSL[180]SQ**S**KVLPVPQ[190]KAVP**YP**QRDM[200]**PI**QAFLLYQE[210]PVLGPVRGPF[220]**PI**IV	MKVLILACLV[10]ALA**L**ARE**L**EE[20]LNV**P**GE**I**VES[30]LSSSEESITH[40]INKKIEKFQS[50]EEQQ**M**EDEL[60]QDKIHPFAQ**T**[70]QSLVYPF**P**GP[80]IP**K**SLPQNI**P**[90]PLTQTPVVVP[100]PFLQPEIMGV[110]**S**KVKE**A**M**A**PK[120]HKEMPFPKYP[130]VEPFTESQSL[140]TLTDVE**N**LHL[150]PLPL**L**QSWMH[160]QPPQPL**P**PTV[170]MFPPQSVLSL[180]SQ**S**KVLPVPQ[190]KAVP**Y**PQRDM[200]**P**IQAFLLYQE[210]**P**VLGPVRGPF[220]**PII**V	

Table 2.0: Continued



κ-	Protein ID/Uniprot ID:	CSN3 [synonyms; CSN10, CSNK]/>sp|P02670|CASK_CAPHI	CSN3/>sp|P02668|CASK_BOVIN	>sp|P11840|CASK_BUBBU Kappa-casein	79% similarities of κ-casein in between goat and bovine. There are 40 unisimilar letters found in κ-casein in goat.
	Number of total amino acid:	192 aa	190 aa	190 aa	
	Molecular weight:	21.4 kDa	21.3 kDa	21.40 kDa	
	Amino acid sequence	MMKSFFLVVT[10]ILALTLPFLG[20]AQEQNQEQPI[30]**C**CEKDERFF**D**[40]DKIAKYIPIQ[50]YVLSRYPSYG[60]LNYYQQ**R**PVA[70]LINNQFLPYP[80]YYAKP**V**AVRS[90]PAQ**T**LQWQVL[100]**P**NTVPAKSCQ[110]**D**QPTT**L**ARHP[120]HPHLSFMAIP[130]PKK**D**QDKTE**V**[140]P**A**INTIASA**E**[150]PT**VH**STPTTE[160]**AIVN**TV**DNPE**[170]**ASS**ESIASAS[180]**ETNT AQVT**ST[190]**EV**	MMKSFFLVVT[10]ILALTLPFLG[20]AQEQNQEQPI[30]**R**CEKDERFF**S**[40]DKIAKYIPIQ[50]YVLSRYPSYG[60]LNYYQQ**K**PVA[70]LINNQFLPYP[80]YYAKP**A**AVRS[90]PAQ**I**LQWQVL[100]**S**NTVPAKSCQ[110]**A**QPTT**M**ARHP[120]HPHLSFMAIP[130]PKK**N**QDKTE**I**[140]P**T**INTIAS**G**E[150]PTSTPTTE**A**V[160]**ES**TV**ATLED**S[170]**PEVI**ESPPEI[180]NTVQVTSTAV[190]	MMKSFFLVVT[10]ILALTLPFLG[20]AQEQNQEQPI[30]RCEK**E**ERFF**N**[40]DKIAKYIPI[50]**Q**YVLSRYPSY[60]**G**LNYYQQ**K**PV[70]**A**LINNQFLPY[80]**P**YYAKP**A**AVR[90]**S**PAQ**I**LQWQV[100]**L**PNTVPAKSC[110][0]**QA**QPTT**MT**RH[120]**P**HPHLSFMAI[130]**P**PK**KN**QD**K**TE[140]**IPT**INTI**VSV**[150]**E**PT**ST**PTTEA[160]**IE**NTV**ATLEA**[170]**S**SE**VIESVPE**[180]TNTAQVTST**V**[190]V	79% similarities of κ-casein in between goat and buffalo. There are 40 unisimilar letters found in κ-casein in goat

*Bold letters refer to AA difference which is differentce between *Capra Hircus* and *Bos Taurus.*

**Superscript numbers refer to amino acid sequence*

*Graphic obtained from Uniprot Database (2023). https://www.uniprot.org

2.2 Hydrolysis in milk

2.2.1 Enzyme hydrolysis

Various treatments can reduce goat milk allergy, including physical such as ultrasound (Xu *et al.,* 2020) and high pressure (Leite Júnior *et al.,* 2019), as well as enzymatic, chemical (acidic or alkaline conditions), and thermal hydrolysis such as heating (Azdad *et al.,* 2018) techniques. Endopeptidases (trypsin, pepsin, bromelain, chymotrypsin and alacalase) are used in the enzymatic approach since they have particular cleavage sites on protein or peptide, resulting in smaller peptides (Li & Zhu, 2023). This approach is frequently utilised in development of hypoallergenic infant formulas due to its specificity, and ability to preserve nutritional protein quality. Among the approaches investigated, enzymatic hydrolysis is the most effective since it preserves milk's nutritional value while reducing antigenicity and allergenicity (Fiocchi *et al.*, 2018).

According to Zhang *et al.* (2020), enzymatic hydrolysis degrades major proteins and conformational epitopes, as well as splits linear epitopes (Zeng *et al.,* 2023), resulting in reduced protein binding to IgE and IgG antibodies (Villas-Boas *et al.*, 2015). Many including the selection of appropriate proteolytic enzymes and their specificity are crucial in dissolving allergic epitopes. Epitopes, also known as antigenic determinants, refer to specific sequences of amino acids within protein allergens that can bind to IgE antibodies (Abd El-Salam and El-Shibiny, 2019). The protein contains two distinct forms of IgE-binding epitopes; i.e., linear (sequential) epitopes and

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conformational epitopes. A cleavage of these epitopes may lead to a reduction in the ability to stimulate an immune response (antigenicity) as well as their potential to cause allergic reactions (allergenicity). **Figure 2.1** illustrates the role of enzymatic hydrolysis in reducing the antigenicity and allergenicity of milk proteins.

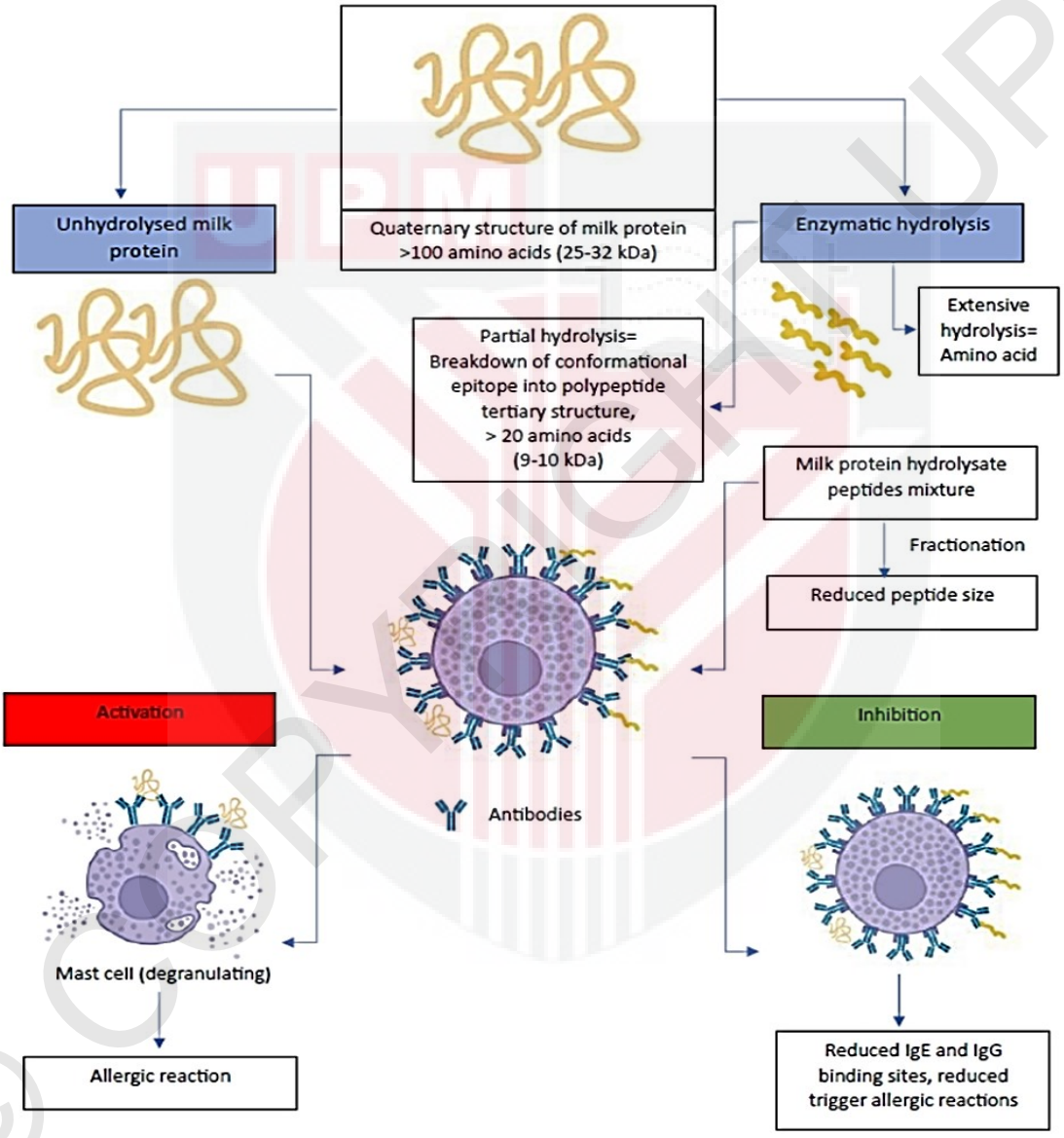

Figure 2.1: Illustration on reduction of antigenicity and allergenicity of milk protein using enzymatic hydrolysis.

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2.2.2 Types and specificity of enzymes

Different types of enzymes, including gastrointestinal digestive (trypsin, pepsin and chymotrypsin) and gastrointestinal non-digestive (bromelain and alcalase). Those enzymes have the ability to reduce antigenicity and allergenicity due to their specificity. Bromelain, belongs to cysteine protease endopeptidase and can be extracted from the pineapple stem (EC 3.4.22.32) and pineapple fruit juice (EC 3.4.22.33). The enzyme acts as a proteolytic sulfhydryl enzyme due to the presence of a free sulfhydryl group. It specifically cleaves at amino acids adjacent to lysine, arginine, phenylalanine, and tyrosine (Mudgil *et al.,* 2019).

In addition to bromelain, alcalase is another kind of enzyme known as a serine endopeptidase consisting primarily of Subtilisin A. Alcalase hydrolyzes protein substrate by breaking the peptide bond on the carboxyl side of glutamate, methionine, leucine, tyrosine, lysine residues (Cui *et al.,* 2021). Trypsin, chymotrypsin, and pepsin are classified as GI digestive enzymes due to their presence in the gastrointestinal digestive tract secreted by the pancreas (Fu *et al.,* 2021). According to research conducted by Gong *et al.,* (2015), trypsin is known to break down the peptide bonds located at the C-terminal basic amino acids, specifically lysine and arginine. Whereas, pepsin is a protease that cleaves at C-terminal phenylalanine, leucine, tyrosine, and trypsin.

Chymotrypsin, on the other hand, is another GI digestive enzyme known as chymotrypsin breaks down protein by cutting the peptide bonds at the C-terminal of specific hydrophobic amino acids, including phenylalanine,

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tryptophan, and tyrosine (Satjarak *et al.,* 2024). According to Yadav (2015), proteases are enzymes that catalyse the hydrolysis of peptide bonds in proteins, and their kinetic behaviour on the formation of enzyme-substrate complex to yield product and enzyme is often described by the Michaelis-Menten equation. The efficiency of a protease is determined by key parameters such as the maximum reaction rate (Vmax) and the Michaelis constant (Km), which represents the substrate concentration at which the reaction rate is half of Vmax. These parameters indicate the enzyme's affinity for its substrate and its catalytic efficiency.

For example, proteases like trypsin, chymotrypsin, and pepsin each have distinct optimal pH levels that significantly affect their activity and stability. Trypsin and chymotrypsin operate best in slightly alkaline conditions (pH 7.5-8.5), while pepsin is most effective in acidic environments (pH 1.5-2.0), aligning with their physiological roles in digestion. Meanwhile, alcalase maintains low Km and high kinetic efficiency at pH 8.0-8.5, ideal for rapid substrate conversion in industrial processes and bromelain shows high substrate affinity and a notable Vmax at pH 5.0-6.0, indicating optimal kinetic efficiency in moderate pH. The kinetics of these enzymes are crucial for understanding their functionality in biological systems and for optimising their use in industrial applications where precise control of proteolytic activity is required.

Research conducted by Liang *et al.* (2020) has reported that the molecular weight of cow's milk including casein, β-lactoglobulin, and β-lactalbumin hydrolysate by alcalase was mostly composed of small peptides, which is less than 3 kDa. This results in a significant decrease of over 90% in the capacity

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of IgG binding. Meanwhile, hydrolysis of αs_1- from buffalo milk using trypsin resulted in a reduction of the antigenicity to 85%, producing numerous small peptides (Ahmad *et al.*, 2015). On the other hand, chymotrypsin hydrolysates reduced antigenicity up to 39% by producing several larger peptides. In research conducted by Azdad *et al.* (2018), it was discovered that subjecting cow's casein to a combination of heating at 90°C and pepsin hydrolysis, substantial 95% resulted in a significant 95% decline in the attachment of IgE to the treated casein.

2.2.3 Enzymatic cleavage sites in casein generating different sizes of peptides

Casein's antigenicity denotes its capacity for recognition by the immune system, whereas allergenicity particularly pertains to the induction of allergic reactions, frequently via IgE-mediated mechanism. The linear epitopes of casein, generated during enzymatic hydrolysis, reduce its allergenic potential (Kashiwakura *et al.,* 2019). Minimising allergenicity and immune response can be achieved by reducing antigenicity through enzymatic hydrolysis, altering spatial conformation, and disrupting linear epitopes of significant allergens (Ding *et al.,* 2024).

The bioinformatics tools are *in silico* approaches, a computational method used to predict substrate (antibody) and ligand (peptides) binding affinity. A combination of *in silico* and *in vitro* analysis to acquire insights into casein cleavage patterns and the predicted peptide sequences with their molecular weights. In this study, a more hands-on experiment to acquire insights into

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casein cleavage patterns and the consequent calculation of molecular weights before *in vitro* analysis. This required various enzymes, each with its first-letter code, as shown in **Table 2.1**. Each letter code representing its specificity was matched with the goat's casein sequence (acquired from the Uniprot database) to determine its position in the goat's milk casein. The expected peptides were analysed using a bioinformatic tool to determine their molecular weight. The subsequent discovery of amino acid sequences within the cow's milk IgG and immunoglobulin (IgE) regions yielded vital insights into probable allergenic epitopes of goat's milk

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Table 2.1: Enzymatic cleavage sites of various enzymes targeting goat's milk casein, generating peptides of different sizes linked to IgE and IgG epitopes

Bromelain	Cleavage sites	Tyr (Y)	References
	Amino acid position	α_{S1}=95,106,109,119,158,161,169,174,180,181,188 α_{S2}=36,94,105,111,114,116,186,187,195,219,222 β=75,129,206 κ=46,51,56,59,63,64,79,81,82	
	Expected peptides size	α_{S1}=AA95-188 α_{S2}=AA36-222 β=AA75-206 κ=AA46-82	
	Molecular weight (<20 kDa)	α_{S1}=96 residue sequence, 11.37 kDa α_{S2}= 187 residue sequence, 22.25 kDa β=132 residue sequence, 14.86 kDa κ=37 residue sequence, 4.58 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA173-194 AA171-185 AA91-100 AA111-120 AA123-132	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002)

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Table 2.1: Continued

		AA 157-166 α_{S2}= AA 33-42 AA87-96 κ=AA 47-68	
	IgG epitopes (<20 kDa)	α_{S1}= AA93-98 AA161-175 β=AA121-140 κ=AA55-80	Spuergin *et al.* (1996) Cong *et al.* (2013) Cercedo *et al.* (2008) Chatchatee *et al.* (2001)
Bromelain	Cleavage sites	Arg (R)	References
	Amino acid position	α_{S1}=16,24,37,48,105,115,166 α_{S2}=47,61,71,130,141,176,221 β=16,196,215 κ=37,55,67,89,118	
	Expected peptides size	α_{S1}=152 residue sequence, 17.44 kDa α_{S2}=187 residue sequence ,22.25 kDa β=132, residue sequence, 14.86 kDa κ=37 residue sequence, 4.58 kDa	

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Table 2.1: Continued

Molecular weight (<20 kDa)	α_{S1}=AA16-166 α_{S2}=AA47-221 β=AA16-215 κ=AA37-118	
IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA 69-78 AA83-92 AA135-144 AA6-20 AA11-25 AA126-140 AA39-48 AA91-100 AA111-120 AA123-132 AA 157-166 α_{S2}= AA32-42 AA87-96 AA145-153 AA159-168 AA171-180	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002)

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Table 2.1: Continued

	κ= AA21-44 AA67-78 AA95-116	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA28-50 AA73-92 AA135-144 AA19-30 AA93-98 AA141-150 AA 21- 35 AA 56 -70 AA 161- 175	Cerecedo *et al.* (2008) Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002)
	β= AA25-50 AA52-74 AA121-140 AA 154-173	

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Table 2.1: Continued

Bromelain	Cleavage sites	Phe (F)	References
	Amino acid position	α_{S1}=43,47,160,165,168,194 α_{S2}=3,4,6,104,108,135,163,179,190 β=67,77,102,126,134,172,203, 218 κ=5,6,18,38,39,76,126	
	Expected peptides size	α_{S1}= AA43-194 α_{S2}= AA3-190 β= AA67-218 κ= AA5-126	
	Molecular weight (< 20 kDa)	α_{S1}=152 residue sequence, 17.54 kDa α_{S2}= 188 residue sequence, 22.15 kDa β=60 residue sequence, 6.67 kDa κ=122 residue sequence, 14.16 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA 83-92 AA135-144 AA173-194 AA39-48 AA91-100 AA111-120	Chatchatee *et al.* (2001) Jarvinen *et al.* (2002) Cong *et al.* (2013) Cercedo *et al.* (2008)

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Table 2.1: Continued

	AA123-132
	AA 157-166
	AA 126-140
	AA171-185
	α_{S2}=
	AA 33-42
	AA87-96
	AA145-153
	AA159-168
	AA171-180
	β=
	AA 149-164
	AA 167-178
	AA 173-184
	AA167-178
	κ=
	AA9-26
	AA21-44
	AA67-78
	AA111-126
	AA 137-148
	AA 149-166
IgG epitopes (<20 kDa)	α_{S1}=
	AA16-35
	AA28-50

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Table 2.1: Continued

		AA73-92 AA21-35 AA56-70 AA161-175 AA141-150 β= AA25-50 AA52-74 AA121-140 A154-173 κ= AA15-24, AA55-80 AA105-116	
Alcalase	Cleavage sites	Glu (E)	References
	Amino acid position	α_{S1}=29,33,45,54,62,65,70,84,85,92,99,104,111,125,132,133,140,156,204 α_{S2}=20,26,27,34,39,49,57,58,65,66,67,75,76,83,84,100,142,148,149,158,161,171,172,173 β=17,19,20,26,29,35,36,46,51,52,57,59115,123,132,146,208 κ=23,27,33,36,139,150,160,170,180,191	

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Table 2.1: Continued

Expected peptide size	α_{S1}= AA29-204 α_{S2}= AA20-173 β= AA17-208 κ= AA23-191	
Molecular weight (<20 kDa)	α_{S1}=176 residue sequence, 20.13 kDa α_{S2}=152 residue sequence,18.01 kDa β=192 residue sequence, 21.75 kDa κ=169 residue sequence, 18.92 kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA69-78 AA83-92 AA135-144 AA173-194 AA126-140 AA171-185 AA39-48 AA91-100 AA123-132 AA157-166 α_{S2}= AA 33-42 AA87-96	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cerecedo (2008). Chatchatee *et al.* (2021)

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Table 2.1: Continued

	AA145-153 AA159-168 AA171-180 β= AA 149-164 AA 167-178 AA 173-184 κ= AA 9-26 AA 21-44 AA 47-68 AA 67-78 AA 95-116 AA 111- 126 AA 137-148 AA 149-166	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA 28-50 AA73-92 AA 135-144 AA 183-208 AA93-98	(Cerecedo *et al.* 2008) Chatchatee *et al.* (2001) Spuergin *et al.* (1996)

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Table 2.1: Continued

		AA141-150 AA 21- 35 AA 56 -70 AA 161- 175 β= AA25-50 AA52-74 AA121-140 AA 154-173 κ= AA 15-24 AA 55-80 AA 105-116	Cong *et al.* (2013)
Alcalase	Cleavage sites	Met (M)	References
	Amino acid position	α_{S1}=1,69,75,138,150,211 α_{S1}=1,42,206 β=1,108,117,124,159,171,198 κ=1,2,127	
	Expected peptides size	α_{S1}= AA1-211 α_{S2}= AA1-206	

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Table 2.1: Continued

	β= AA1-198 κ= AA1-127	
Molecular weight (<20 kDa)	α_{S1}=211 residue sequence, 23.9kDa α_{S2}=206 residue sequence, 24.34kDa b=198 residue sequence, 22.23kDa k=127 residue sequence, 14.77kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA 1-16 AA 83-92 AA 135-144 AA 69-78 AA173-194 AA 126- 140 AA 6- 20 AA 171-185 AA 11- 25 AA39-48 AA91-100 AA111-120 AA123-132 β= AA 149-164 AA 167-178	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cercedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

	k= AA 9-26 AA 67-78 AA 95-116 AA 111- 126	
IgG epitopes (<20 kDa)	α_{S1}= AA 135-144 AA 183-208	Chatchatee *et al.* (2001) Cerecedo *et al.* (2008)
	AA16-35 AA 28-50 AA73-92	Spuergin *et al.* (1996) Cong *et al.* (2013)
	AA 19-30 AA93-98 AA141-150	
	AA 21- 35 AA 56 -70 AA 161- 175	
	β= AA25-50 AA52-74	

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Table 2.1: Continued

		AA121-140 AA 154-173	
Alcalase	Cleavage sites	Tyr (Y)	References
	Amino acid position	α_{S1}=95,106,109,119,158,161,169,174,180,181,188 α_{S2}=36,94,105,111,114,116,186,187,195,219,222 β=75,129,206 κ=46,51,56,59,63,64,79,81,82	
	Expected peptides size	α_{S1}= AA95-188 α_{S2}= AA36-222 β= AA75-206 κ= AA46-82	
	Molecular weight (<20 kDa)	α_{S1}=96 residue sequence, 11.37 kda α_{S2}=187 residue sequence,22.25 kDa β=132, residue sequence, 14.86 kDa κ=37 residue sequence, 4.58 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA173-194 AA171-185 AA91-100 AA111-120 AA123-132	Chatchatee *et al.* (2001) Chong *et al.* (2013)

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Table 2.1: Continued

		AA 157-166 α_{S2}= AA 33-42 AA87-96 κ= AA 47-68	Jarvinen *et al.* (2002)
	IgG epitopes (<20 kDa)	α_{S1}= AA93-98 AA161-175 β= AA121-140 κ= AA55-80	Spuergin *et al.* (1996) Cong *et al.* (2013) Cercedo *et al.* (2008) Chatchatee *et al.* (2001)
Alcalase	Cleavage site	Leu (L)	References
	Amino acid position	α_{S1}=3,4,6,14,26,35,36,110,113,114,124,135,164,171,182,184,213 α_{S2}=9,10,14,51,98,112,122,144,169,177,180,196,223 β=4,6,9,11,21,31,60,73,85,90,92,103,140,142,148,150,152,154,166,178,180,186,204,205,206 κ=7,12,14,16,19,53,61,71,77,95,100,116,124	

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Table 2.1: Continued

Expected peptides size	α_{S1}=AA3-213 α_{S2}=AA9-223 β=AA4-206 κ=AA7-124	
Molecular weight (<20 kDa)	α_{S1}= 211 residue sequence, 23.85 kDa α_{S2}= 215 residue sequence, 25.38 kDa β=202 residue sequence, 22.65 kDa κ=188 residue sequence, 13.63 kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA173-194 AA126-140 AA6-20 AA171-185 AA11-25 AA123-132 AA157-166 α_{S2}= AA171-180 β= AA149-164 AA167-178 AA173-184	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cerecedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

	κ= AA9-26 AA67-78 AA95-116 AA111-126	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA28-50 AA21-35 AA161-175	Cerecedo *et al.* (2001) Cong *et al.* (2013) Cerecedo *et al.* (2008)
	β= AA42-74 AA121-140 AA154-173	
	κ= AA15-24 AA55-80 AA105-116	

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Table 2.1: Continued

Alcalase	Cleavage sites	Lys (K)	References
	Amino acid position	α_{S1}=2,18,49,57,73,76,94,98,117,118,129,139,147,208 α_{S2}=2,16,18,37,40,48,50,86,92,96,107 129,151,152,165,166,168,174,181,182,197,204,207,213 β=2,43,44,47,63,112,114,120,122,128,147,184,191 κ=3,34,42,45,84,107,132,133,137	
	Expected peptides size	α_{S1}= 207 residue sequence, 23.43 kDa α_{S2}=212 residue sequence, 25.07 kDa β= 190 residue sequence, 21.3 kDa κ=135 residue sequence, 15.63 kDa	
	Molecular weight (<20 kDa)	α_{S1}= 207 residue sequence, 23.43 kDa α_{S2}=212 residue sequence, 25.07 kDa β= 190 residue sequence, 21.3 kDa κ=135 residue sequence, 15.63 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA 83-92 AA 135-144 AA 69-78 AA173-194 AA 126-140 AA 6- 20 AA 171-185 AA 11- 25 AA39-48 AA91-100	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cerecedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

		AA111-120 AA123-132 AA 157-166 α_{S2}= AA33-42 AA87-96 AA145-153 AA159-168 AA171-180 β= AA 149-164 AA 167-178 AA 173-184 κ= AA9-26 AA21-44 AA47-68 AA 9-26 AA 67-78 AA 95-116 AA 111- 126 AA 137-148	
	IgG epitopes (<20 kDa)	α_{S1}= AA 135-144 AA 183-208	Chatchatee *et al.* (2001).

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Table 2.1: Continued

	AA16-35 AA 28-50 AA73-92	Cerecedo *et al.* (2008) Spruengin *et al.* (1996) Cong *et al.* (2013)
	AA 19-30 AA93-98 AA141-150	Cerecedo *et al.* (2001)
	AA 21- 35 AA 56 -70 AA 161- 175	
	β= AA25-50 AA52-74 AA121-140 AA 154-173	
	AA25-50 AA52-74 AA121-140	
	κ= AA 15-24 AA 55-80 AA 105-116	

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Table 2.1: Continued

Papain	Cleavage site	Cys (C)	References
	Amino acid position	α_{S1}=8 α_{S2}=8,52,56 β=8 κ=31,32,109	
	Expected peptides size	α_{S1}=AA8 α_{S2}=AA8-56 β=AA8 κ=AA31-109	
	Molecular weight (<20 kDa)	α_{S1}=1 residue sequence, 0.12 kDa α_{S2}=49 residue sequence, 5.63 kDa β=1 residue sequence, 0.12 kDa κ=79 residue sequence, 9.28 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA 83-92 AA 135-144 AA 69-78 AA 126- 140 AA 6- 20 AA 11- 25 AA39-48 AA91-100 AA111-120 AA123-132	Chatchatee *et al.* (2001) Cong *et al.* (2013) Chatchatee *et al.* (2001) Chatchatee *et al.* (2021)

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Table 2.1: Continued

	IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA 28-50 AA73-92 AA 19-30 AA93-98 AA141-150 AA 21- 35 AA 56 -70 AA 161- 175 β= AA52-74 AA121-140 AA154-173 κ=AA 105-116	Cerecedo *et al.* (2008) Chatchatee *et al.* (2001)

Papain	Cleavage Site	His (H)	References
	Amino acid position	α_{S1}=19,23,136,145 α_{S2}=17,21,93,202 β=65,121,160 κ=119,121,123,154	

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Table 2.1: Continued

Expected peptides size	α_{S1}= AA19-145 α_{S2}= AA17-202 β= AA65-160 κ= AA119-154	
Molecular weight (<20 kDa)	α_{S1}=127 residue sequence, 14.42 kDa α_{S2}=186 residue sequence, 22.08 kD β=96 residue sequence, 10.85 kDa κ=36 residue sequence, 3.96 kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA 83-92 AA 135-144 AA 69-78 AA 126- 140 AA 6- 20 AA 11- 25 AA39-48 AA91-100 AA111-120 AA123-132 α_{S2}= AA 33-42 AA87-96	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cerecedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

	AA145-153 AA159-168 AA171-180 β=160 AA149-164 κ= AA111-126, AA 137-148 AA149-166	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA 28-50 AA73-92 AA 19-30 AA93-98 AA141-150 AA 21- 35 AA 56 -70 AA 161- 175 β= AA52-74 AA121-140 AA154-173 κ=AA 105-116	Spuergin *et al.* (1996) Cong *et al.* (2013) Cerecedo *et al.* (2008) Chatchatee *et al.* (2001)

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Table 2.1: Continued

Trypsin	Cleavage site	Arg (R)	References
	Expected peptides size	α_{S1}=AA16-166 α_{S2}=AA47-221 β=AA16-215 κ=AA37-118	
	Molecular weight (<20 kDa)	α_{S1}=152 residue sequence, 17.44 kDa α_{S2}=187 residue, 22.25 kDa β=132, residue sequence, 14.86 kDa κ=37 residue, 4.58 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA 69-78 AA83-92 AA135-144 AA6-20 AA11-25 AA126-140 AA39-48 AA91-100 AA111-120 AA123-132 AA 157-166	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002)

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Table 2.1: Continued

	α_{S2}= AA32-42 AA87-96 AA145-153 AA159-168 AA171-180 κ= AA21-44 AA67-78 AA95-116	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA28-50 AA73-92 AA135-144 AA19-30 AA93-98 AA141-150 AA 21- 35 AA 56 -70 AA 161- 175 β= AA25-50 AA52-74 AA121-140 AA 154-173	Cerecedo *et al.* (2008) Chatchatee (2001) Spuergin *et al.* (1996) Cong *et al.* (2013)

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Table 2.1: Continued

Trypsin	Cleavage sites	Lys (K)	References
	Amino acid position	α_{S1}=2,18,49,57,73,76,94,98,117,118,129,139,147,208 α_{S2}=2,16,18,37,40,48,50,86,92,96,107 129,151,152,165,166,168,174,181,182,197,204,207,213 β=2,43,44,47,63,112,114,120,122,128,147,184,191 κ=3,34,42,45,84,107,132,133,137	
	Expected peptides size	α_{S1}=AA2-208 α_{S2}=AA2-213 β=AA2-191 κ=AA3-137	
	Molecular weight (<20 kDa)	α_{S1}= 207 residue sequence, 23.43 kDa α_{S2}=212 residue sequence, 25.07 kDa β= 190 residue sequence, 21.3 kDa κ=135 residue sequence, 15.63 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA 83-92 AA 135-144 AA 69-78 AA173-194 AA 126-140 AA 6- 20 AA 171-185 AA 11- 25 AA39-48 AA91-100 AA111-120	Cerecedo *et al.* (2008) Chatchatee (2001) Spuergin *et al.* (1996) Cong *et al.* (2013) Cerecedo *et al.* (2008)

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Table 2.1: Continued

	AA123-132 AA 157-166	
	α_{S2}= AA33-42 AA87-96 AA145-153 AA159-168 AA171-180	
	β= AA 149-164 AA 167-178 AA 173-184	
	κ= AA9-26 AA21-44 AA47-68 AA 9-26 AA 67-78 AA 95-116 AA 111- 126 AA 137-148	
IgG epitopes (<20 kDa)	α_{S1}= AA 135-144 AA 183-208 AA16-35 AA 28-50 AA73-92 AA 19-30	Chatchatee *et al.* (2001) Cerecedo *et al.* (2008) Spruengin *et al.*(1996) Cong *et al.* (2013)

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Table 2.1: Continued

		AA93-98 AA141-150 AA 21- 35 AA 56 -70 AA 161- 175 β= AA25-50 AA52-74 AA121-140 AA 154-173 AA25-50 AA52-74 AA121-140 κ= AA 15-24 AA 55-80 AA 105-116	
Chymotrypsin	Cleavage sites	Phe (F)	References
	Amino acid position	α_{S1}=43,47,160,165,168,194 α_{S2}=3,4,6,104,108,135,163,179,190 β=67,77,102,126,134,172,203, 218 κ=5,6,18,38,39,76,126	

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Table 2.1: Continued

Expected peptides size	α_{S1}=AA43-194 α_{S2}=AA3-190 β=AA67-218 κ=AA5-126	
Molecular weight (<20 kDa)	α_{S1}=152 residue sequence, 17.54 kDa α_{S2}= 188 residue sequence, 22.15 kDa β=60 residue sequence, 6.67 kDa κ=122 residue sequence, 14.16 kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA 83-92 AA135-144 AA173-194 AA39-48 AA91-100 AA111-120 AA123-132 AA 157-166 AA126-140 AA171-185	Chatchatee *et al.* (2001) Jarvinen *et al.* (2002) Cercedo *et al.* (2008) Chatchatee *et al.* (2021)
	α_{S2}= AA 33-42 AA87-96 AA145-153 AA159-168 AA171-180	

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Table 2.1: Continued

	β= AA 149-164 AA 167-178 AA 173-184 AA167-178 κ= AA9-26 AA21-44 AA67-78 AA111-126 AA 137-148 AA 149-166	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA28-50 AA73-92 AA21-35 AA56-70 AA161-175 AA141-150 β= AA25-50 AA52-74 AA121-140 A154-173	Chatchatee *et al.* (2001) Jarvinen *et al.* (2002) Cong *et al.* (2013) Cercedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

		κ= AA15-24, AA55-80 AA105-116	
Chymotrypsin	Cleavage sites	Trp (W)	References
	Amino acid position	α_{S1}=179, 214 α_{S2}=125,192,209 β=158 k=97	
	Expected peptides size	α_{S1}=AA179-214 α_{S2}=AA125-209 β=AA158 k=AA97	
	Molecular weight (<20 kDa)	α_{S1}=36 residue sequence, 4.05 kDa α_{S2}= 85 residue sequence, 10.23 kDa β=1 residue sequence, 0.2 kDa κ=79 residue sequence, 9.28 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA173-194 AA171-185 α_{S2}= AA145-153 AA159-168 AA171-180	Chatchatee *et al.* (2001) Chong *et al.* (2013) Jarvinen *et al.* (2002)

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Table 2.1: Continued

		β= AA149-164 κ= AA95-117	
	IgG epitopes (<20 kDa)	α_{S1}= AA 183-208 AA141-150 β=AA 154-173	Spuergin *et al.* (1996) Cong *et al.* (2013) Cercedo *et al.* (2008) Chatchatee *et al.* (2001)
Chymotrypsin	Cleavage sites	Tyr (Y)	References
	Amino acid position	α_{S1}=95,106,109,119,158,161,169,174,180,181,188 α_{S2}=36,94,105,111,114,116,186,187,195,219,222 β=75,129,206 κ=46,51,56,59,63,64,79,81,82	
	Expected peptides size	α_{S1}=AA95-188 α_{S2}=AA36-222 β=AA75-206 κ=AA46-82	
	Molecular weight (<20 kDa)	α_{S1}=96 residue, 11.37 kDa α_{S2}=187 residue,22.25 kDa β=132, residue sequence, 14.86 kDa κ=37 residue, 4.58 kDa	

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Table 2.1: Continued

	IgE epitopes (<20 kDa)	α_{S1}= AA173-194 AA171-185 A91-100 AA111-120 AA123-132 AA 157-166 α_{S2}= AA 33-42 AA87-96 κ= AA 47-68	
	IgG epitopes (<20 kDa)	α_{S1}= AA93-98 AA161-175 β=AA121-140 κ=AA55-80	Chatchatee *et al.* (2001) Chong *et al.* (2013) Jarvinen *et al.* (2002)
Pepsin	Cleavage sites	Phe (F)	
	Amino acid position	α_{S1}=43,47,160,165,168,194 α_{S2}=3,4,6,104,108,135,163,179,190 β=67,77,102,126,134,172,203, 218 κ=5,6,18,38,39,76,126	

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Table 2.1: Continued

Expected peptides size	α_{S1}=AA43-194 α_{S2}=AA3-190 β=AA67-218 κ=AA5-126	
Molecular weight (<20 kDa)	α_{S1}=152 residue sequence, 17.54 kDa α_{S2}= 188 residue sequence, 22.15 kDa β=60 residue sequence, 6.67 kDa κ=122 residue sequence, 14.16 kDa	
IgE epitopes (<20 kDa)	α_{S1}= AA 83-92 AA135-144 AA173-194 AA39-48 AA91-100 AA111-120 AA123-132 AA 157-166 AA126-140 AA171-185 α_{S2}= AA 33-42 AA87-96 AA145-153 AA159-168 AA171-180 β= AA 149-164 AA 167-178 AA 173-184	Chatchatee *et al.* (2001) Jarvinen *et al.* (2002) Cong *et al.* (2013) Cercedo *et al.* (2008) Chatchatee *et al.* (2021)

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Table 2.1: Continued

	AA167-178	
	κ= AA9-26 AA21-44 AA67-78 AA111-126 AA 137-148 AA 149-166	
IgG epitopes (<20 kDa)	α_{S1}= AA16-35 AA28-50 AA73-92 AA21-35 AA56-70 AA161-175 AA141-150	Cerecedo *et al.* (2008) Cong *et al.* (2013) Spruengin *et al.* (1996) Chatchatee *et al.* (2001)
	β= AA25-50 AA52-74 AA121-140 A154-173	
	κ= AA15-24 AA55-80 AA105-116	

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Table 2.1: Continued

Pepsin	Cleavage site	Leu (L)	References
	Amino acid position	α_{S1}=3,4,6,14,26,35,36,110,113,114,124,135,164,171,182,184, 213 α_{S2}=9,10,14,51,98,112,122,144,169,177,180,196,223 β=4,6,9,11,21,31,60,73,85,90,92,103,140,142,148,150,152,154,166,178,180,186,204,205,206 κ=7,12,14,16,19,53,61,71,77,95,100,116,124	
	Expected peptides size	α_{S1}=AA3-213 α_{S2}=AA9-223 β=AA4-206 κ=AA7-124	
	Molecular weight (<20 kDa)	α_{S1}= 211 residue sequence, 23.85 kDa α_{S2}= 215 residue sequence, 25.38 kDa β=202 residue sequence, 22.65 kDa κ=188 residue sequence, 13.63 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA1-16 AA173-194 AA126-140 AA6-20 AA171-185 AA11-25 AA123-132 AA157-166	Chatchatee *et al.* (2001) Cong *et al.* (2013) Jarvinen *et al.* (2002) Cerecedo *et al.* (2008)

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Table 2.1: Continued

IgG epitopes (<20 kDa)	α_{S2}= AA171-180 β= AA149-164 AA167-178 AA173-184 κ= AA9-26 AA67-78 AA95-116 AA111-126 α_{S1}= AA16-35 AA28-50 AA21-35 AA161-175 β= AA42-74 AA121-140 AA154-173 κ= AA15-24 AA55-80 AA105-116	Cerecedo *et al.* (2001) Cong *et al.* (2013) Cerecedo *et al.* (2008)

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Table 2.1: Continued

Pepsin	Cleavage site	Tyr (Y)	References
	Amino acid position	α_{S1}=95,106,109,119,158,161,169,174,180,181,188 α_{S1}=36,94,105,111,114,116,186,187,195,219,222 β=75,129,206 κ=46,51,56,59,63,64,79,81,82	
	Expected peptides size	α_{S1}=AA95-188 α_{S2}=AA36-222 β=AA75-206 κ=AA46-82	
	Molecular weight (<20 kDa)	α_{S1}=96 residue, 11.37kda α_{S2}=187 residue,22.25 kDa β=132, residue sequence, 14.86 kDa κ=37 residue, 4.58 kDa	
	IgE epitopes (<20 kDa)	α_{S1}= AA173-194 AA171-185 AA91-100 AA111-120 AA123-132 AA 157-166	Chatchatee *et al.* (2001) Jarvinen *et al.* (2002) Cong *et al.* (2013)

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Table 2.1: Continued

IgG epitopes (<20 kDa)	α_{S2}= AA 33-42 AA87-96 κ= AA 47-68 α_{S1}= AA93-98 AA161-175 β= AA121-140 κ= AA55-80	Cerecedo *et al.* (2008) Spruengin *et al.* (1996) Chatchatee *et al.* (2001)

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2.2.4 Hydrolysis conditions

Research on various enzymes has shown significant differences in effectiveness and the conditions required for optimal hydrolysis. Optimal hydrolysis conditions for papain in cow's milk were determined to be a pH of 5.60, a temperature of 50 °C, an enzyme concentration of 2.5% (w/w), and a hydrolysis duration of 90 min (Liu et al., 2012). Under these conditions, papain facilitated the efficient degradation of casein proteins. Protease and trypsin in cow's milk demonstrated a significant increase in the degree of hydrolysis during the initial 20 min, attributed to elevated protein and enzyme concentrations. The plateaus exhibit a 45.2% increase after 45 min and remain stable during the hydrolysis process (Wang *et al.,* 2013).

The exploration of bromelain's potential for partial hydrolysis of casein, particularly at an enzyme-to-substrate ratio of 1:100, suggests its promise for developing hypoallergenic bovine milk formula (Medeiros *et al.,* 2014). This approach highlights the choice of enzyme and conditions to tailor the hydrolysis process for specific applications. The protein hydrolysis plays a crucial role in antigenicity reduction. Proteolytic enzymes, such as trypsin, α-chymotrypsin, and pepsin demonstrate varying levels of efficacy in reducing the antigenicity of α_{s1}-casein. Trypsin reduced antigenicity to 63% after 150 min, α-chymotrypsin to 60%, and pepsin to 38% (Ahmad *et al.,* 2015). This variation emphasises the significance of selecting appropriate enzyme to attain the intended reduction of allergenicity in milk proteins.

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Research on camel milk demonstrates that alcalase and papain exhibit effectiveness for 6 hours, whereas α-chymotrypsin is most effective at 4 h (Kumar *et al.,* 2016). Alcalase has been identified as the most effective enzyme within 150 min at 50 °C, pH 8.0, utilising a 2.0% enzyme concentration and a 4.0% substrate concentration (Shu *et al.,* 2016). Alcalase initially raises the degree of hydrolysis to 13.81% after 60 min; however, this value declines between 90 and 120 min (Liang *et al.,* 2020), demonstrating a time dependent effect on hydrolysis degree.

Optimal hydrolysis conditions for alcalase and neutrase in cow's milk involve an enzyme-to-substrate ratio of 6000 μ/g, a reaction temperature of 50 °C, a pH of 8.5, and a hydrolysis duration of 3 h. Under these conditions, the hydrolysates exhibited a 26.45% degree of hydrolysis and a 59.41% reduction in antigenicity (Wang *et al.,* 2023). The findings demonstrate that enzyme selection and hydrolysis conditions can be optimised to effectively decrease antigenicity and improve the functional properties of milk proteins. These studies demonstrate the complexity of the interplay between enzyme type, concentration, hydrolysis time and conditions in the optimisation of milk protein hydrolysis. The studies allow researchers to optimise the hydrolysis process to reduce food allergenicity risk according to specific requirements and applications. **Table 2.2** summarises the enzymatic hydrolysis conditions for ruminant milk types and enzyme.

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Table 2.2: Condition of enzymatic hydrolysis from different ruminant milk using various enzymes

Milk Type	Hydrolysis Conditions					References
	Enzyme	**pH**	**Temp (°C)**	**E:S**	**Time (min)**	
Cow's milk	Papain	6.8 6.4 6.0 5.6 5.2	60 55 50 45 40	3.0 % 2.5 % 2.0 % 1.5 % 1.0 %	40-140	Liu *et al.* (2012)
Cow's milk	Protease Trypsin	8.0	40	2500 U/g	10-80	Wang *et al.* (2013)
Cow's milk	Bromelain Rennet Pepsin Trypsin	6.5	30	1:50 1:200 1:75 1:65 1:100	-	Medeiros *et al.* (2014)
Buffalo milk	α-chymotrypsin Trypsin Pepsin	7.8 2.2 5.5	-	1:5000	0-150	Ahmad *et al.* (2015)
Camel Milk	Alcalase α-chymotrypsin Papain	8.0 8.0 6.5	55 37 55	1:100	0-360	Kumar *et al.* (2016).
Cow's milk	Alcalase	-	55 50 50	6000 u/g-10000 u/g	20	Liang *et al.* (2020)
Cow's milk	Alcalase and Neutrase	7.5, 8.0, 8.5, 9.0 and 9.5	40 45 50 55 60	2000 u/g-8000 u/g (2:1, 3:1, 1:1, 1:2, 1:3)	60-180	Wang *et al.,* (2023)

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Table 2.2: Continued

Goat's Milk	Alcalase	7.0 7.5 8.0 8.5 9.0	45 50 55 60 65	1.0 % 1.5 % 2.0 % 2.5 % 3.0 %	30 -240	Shu *et al.* (2016)

2.2.5 Bioactive peptide and structure-activity relationship

Bioactive peptides are bioactive specific protein fragments with health-promoting properties that have drawn considerable scientific attention. However, goat milk has emerged as a potential alternative in peptide synthesis because of its diverse nutritional composition and functionality among different milk sources. The enzymatic hydrolysis of the goat's milk proteins generates bioactive peptides with various biological activities, such as antihypertensive, antioxidant, hypoglycemic, anti-inflammatory, antimicrobial, and immunomodulatory (Ahmed *et al.,* 2015; Du *et al.,* 2022). The section discusses bioactive peptides from goat's milk concerning their amino acid sequences, structure-activity relationships, and their significance in functional activities.

The primary proteins in goat's milk, caseins, and whey proteins act as precursors for bioactive peptides. These peptides are released through enzymatic hydrolysis, facilitated by gastrointestinal or non-gastrointestinal enzymes. Both endogenous and exogenous enzymatic processes could contribute significantly to generating bioactive peptides, thereby influencing their diverse functional properties (Anusha & Bindhu, 2016).

Notably, hydrolysis of casein produces many bioactive peptides with well-documented functionalities (Punia *et al.,* 2020). The amino acids sequences

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of bioactive peptides largely determine their biological activities. Some peptides from goat milk proteins have already been identified to exhibit various biofunctional properties. Peptides with angiotensin-converting enzyme (ACE) inhibitory activity have shown beneficial effects in controlling blood pressure, and enzymes like subtilisin and trypsin hydrolyse these peptides to yield ones equally as potent as the antihypertensive drug captopril (Ibrahim *et al.,* 2017). Moreover, aromatic and sulfur-containing amino acid-rich peptides showed significant antioxidant activity. The N-terminal residues have been identified to be particularly important for radical scavenging and metal ion-binding interactions requiring the presence of tyrosine (i.e., in peptides such as ATVY (Vinderola *et al.,* 2019; Liu *et al.,* 2018). The mechanism of action of the peptides includes free radical scavenging and mitigation of oxidative stress (De Gobba *et al.,* 2014). Furthermore, whey protein-derived peptides, including beta-lactoglobulin, have been reported to have immunomodulatory activity by regulating cytokine secretions and immune cell functions (Pihlanto, 2006; Korhonen & Pihlanto, 2006). In silico studies suggest that goat milk peptides have been observed to interfere with interleukin-6 (IL-6), one of the significant inflammatory pathways (Rohmah *et al.,* 2015).

Aside from functional activity, bioactive peptides' biological activity–related structural properties are fundamentally essential. Peptide length, three-dimensional conformation, charge distribution, and hydrophobicity are primary determinants of bioactivity.

Short-chain peptides, generally consisting of 2 to 10 amino acids, exhibit enhanced bioavailability and functional potency (Sanchez & Vazquez, 2017).

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Research on circular dichroism indicates that bioactive peptides typically exhibit helical or beta-sheet secondary structures, which markedly enhance their binding affinity to biological targets (Kumar *et al.,* 2018). Peptide solubility and membrane permeability are affected by their net charge and hydrophobicity. Hydrophobic peptides demonstrate enhanced cellular uptake, which increases their bioactivity (Gangnon *et al.,* 2017). Although bioactive peptides possess considerable potential, challenges including elevated production costs and prolonged bioactivity evaluation impede their broad application. The utilisation of computational tools for peptide identification and potential activity prediction has increased to address these limitations.

Tools like BIOPEP and BLAST enable identifying and characterising peptide sequences with potential bioactivity (FitzGerald *et al.,* 2020). Quantitative structure-activity relationship (QSAR) models correlate peptide structure with functional properties, thereby optimising peptide selection and enzyme efficiency (Nongonierma *et al*., 2018). AI-driven systems like DeepAP and iBitter-SCM utilise advanced deep-learning methodologies to enhance peptide analysis by predicting bioactivity, optimising synthesis pathways, and identifying potential therapeutic targets. While cow's milk has traditionally been the main source of bioactive peptides, recent studies indicate the possible advantages of peptides obtained from goat's milk.

Peptides derived from goat's milk exhibit superior digestibility and bioavailability compared to cow's milk, making them more appropriate for human consumption (Roy *et al*., 2020; Sarker *et al.,* 2020). These peptides

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exhibit hypoglycemic effects by inhibiting dipeptidyl peptidase IV (DPP-IV), a crucial enzyme in glucose metabolism, indicating their potential role in diabetes management. Bioactive peptides derived from goat's milk demonstrate significant antimicrobial properties, showing enhanced antibacterial activity against pathogens such as *Escherichia coli* and *Listeria monocytogenes* (Esmaeilpour *et al.,* 2016).

Investigating bioactive peptides derived from goat milk presents opportunities for developing functional foods and therapeutic agents. The biological activity of these peptides is determined by their amino acid sequences and physicochemical properties. Future research should aim to clarify the specific mechanisms that govern peptide bioactivity, optimise hydrolysis processing conditions to enhance biofunctional properties and explore new applications in nutraceuticals and pharmacological studies. Predictive models enhanced by AI will facilitate identifying and utilising bioactive peptides. In addition to their therapeutic uses, peptides derived from goat milk significantly improve food preservation by reducing lipid oxidation and inhibiting microbial growth in various food systems (Roy *et al.,* 2020; Sarker *et al.,* 2020). Using computational tools and comparative studies allows for exploring goat's milk peptides' novel medical and food feasibility.

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2.3 ELISA assessment method in milk

An enzyme-linked immunosorbent assay (ELISA) is a preferred technique in the detection of various antigens (Heyrapetyan *et al.*, 2023; Nouri *et al.,* 2018; Lin, 2015) owing to its various advantages, including cost-effectiveness and high specificity, and rapidity (Holzhauser *et al.,* 2020). The source of antibodies and treatment can significantly affect the binding of these antibodies. Mohsin *et al.* (2020) reported that achieving high-quality outcomes of polyclonal antibodies depends on the criticality of antibody purification, specifically, Nter-IgG purification, which resulted in superior results compared to antiserum at comparable concentrations. The study found that purification impacted binding affinity significantly within the system; hence, to increase antigen-antibody binding, the antiserum is suggested to be purified. Moreover, in a study by Ramahan *et al.*, (2015), it was shown that ELISA required heating of β-lactoglobulin above 120 °C. In contrast, Mohsin *et al.,* (2023) advocated heating of α_{S1}-casein at 60°C as pre-treatment to alter its structure for antigen-antibody bindings. Differences in the molecular structures of samples cause this temperature variation. ELISA method offers a valuable supplementary method for food analysis, reducing the need for complex, costly, and time-consuming while preserving the sensitivity and dependability of this approach. **Table 2.3** shows different types of methods in ELISA to detect different milk proteins.

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Table 2.3: Development of ELISA for detection of different milk proteins reported in the literature

Format	Sample/ Antigen	Types of antibodies	Concentration of protein	Findings	Reference
Direct	Cow's milk (β-lactoglobulin)	1° Ab: anti β-lactoglobulin-HRP	NA	Minimal antigenicity was achieved by heating β-LG solutions to 120 °C at pH 5 with shearing (100–1,000 /s	Ramahan *et al.,* (2015)
Indirect	Cow's and buffalo's milk (κ-casein)	1° Ab: Mouse monoclonal antibody against bovine milk protein 2° Ab: κ-casein-HRP	1:100-1:1000 (v/v)	LOD: 0.1 to 2.5 µg/mL 0.05 µg/mL	Nermin Sajic & Essers (2015)
Indirect	Goat's milk (β-casein)	1° Ab: 0.1 µg/ml of the indicated purified mouse monoclonal antibody 2° Ab: 1:6,000 (v/v) goat anti-mouse IgG-HRP secondary antibody	10 µg/mL	6A12 monolconal antibodies observed a high sensitivity for goat's milk as compared to 1H3 mAb	Castillo & Cassola (2017)
Direct	Cow's milk (β-lactoglobulin)	1° Ab: anti β-lactoglobulin (β-LG) recombinant Immunoglobulin E (IgE) Secondary antibody: anti-human kappa-HRP	5 µg/mL		Orcajo *et al.* (2019)

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Table 2.3: Continued

Indirect	Goat's milk (α_{S1}-casein)	1° Ab: 1:1,000 Nter-IgG vs 1:1,000 antisera 1:10,000 2° Ab: Streptavidin-HRP	100 µL	N-ter IgG has higher binding affinity for peptide-BSA and goat α_{S1}-casein at a Kd value of 5.063 x 10^{-3} M compared to 9.046 x 10-3 for the C-ter IgG	Mohsin *et al.* (2020)
Sandwich	Cow's milk (β-lactoglobulin)	Capture Ab: 1:10,000 (v/v) polyclonal rabbit antiserum 1° Ab: 1:10,000 (v/v) polyclonal mice anti- β-LG serum 2° Ab: 1:6000 (v/v) HRP-labelled rabbit anti-mice IgG	1.0–0.01 µg/mL	LOD: 0.011 ppm	Khan *et al.,* (2022)
Sandwich	Goat milk (α_{S1}-casein)	Capture antibody: 1:100 Nter-IgG Primary antibody 1:100 Cter-IgG-biotin Secondary antibody 1:15,000 Streptavidin-HRP	16.7 µg/mL	LOD: 0.063 µg/mL LOQ: 0.21 µg/mL	Mohsin *et al.* (2023)

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Commercial ELISA kits are essential in milk protein analysis, offering reliable and efficient methods for detecting and quantifying specific milk proteins. These kits are developed to address diverse analytical requirements, providing high sensitivity and accuracy, critical for research and quality control applications. **Table 2.4** provides an overview of commercially available ELISA, each designed to target specific milk proteins. This table illustrates the versatility and applicability of these kits for various milk types and protein targets, emphasising their significance in maintaining product consistency, safety, and quality within the dairy industry. These commercial kits enable researchers and manufacturers to achieve accurate protein detection, thereby advancing milk protein research and product development. In addition to ELISA, various other methods are employed to evaluate the allergenic properties of milk. A notable technique is immunoblotting, commonly called Western blotting, which separates milk proteins based on size and identifies specific allergens via antibody binding. This method enables the precise detection and characterisation of allergenic proteins with notable specificity and sensitivity (Bureau *et al.,* 2018). Mass spectrometry is an effective method for identifying and quantifying allergenic peptides in milk proteins, yielding comprehensive information regarding their molecular structure and potential (Tian *et al.,* 2021). In vitro binding assays, including competitive inhibition assays, quantify the interaction between milk proteins and IgE antibodies, thereby aiding in evaluating their allergenic potential (Kumar *et al.,* 2019). These methods enhance ELISA by providing varied insights into milk allergenicity, thereby contributing to a more thorough understanding and management of milk allergies.

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Table 2.4: **Commercial kits of ELISA for the detection of different targeted milk proteins**

Target Protein	Commercial name of kits	Manufacturer (city, country)	Detection range (µg/mL)
α-casein	Casein	ELISA Systems™ (Darmstadt, Germany)	1.0 to 10
Non-fat dry milk (Casein)	Veratox Casein	Neogen (Michigan, USA)	2.5 to 15
Casein	RIDASCREEN Fast Casein	R-Biopharm (Darmstadt, Germany)	0.5 to 13.5
β-lactoglobulin	BLG	ELISA Systems™ (Darmstadt, Germany)	0.1 to 1
Whole milk powder (β-lactoglobulin)	BioKits™ BLG	Neogen (Michigan, USA)	2.5 to 40
β-lactoglobulin	RIDASCREEN Fast BLG	R-Biopharm (Darmstadt, Germany)	0.5 to 13.5
β-lactoglobulin	AgraQuant BLG	Romer Labs (Tulln, Austria)	0.01 to 0.4
Milk powder (β-casein)	RIDASCREEN FAST Casein	R-Biopharm (Darmstadt, Germany)	0.71 to 2.5

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CHAPTER 3

EFFECT OF HYDROLYSIS CONDITIONS USING GASTROINTESTINAL DIGESTIVE AND NON-DIGESTIVE ENZYMES

3.1 Introduction

The allergenicity of milk protein is a significant concern in cow's milk protein allergy (CMPA) individuals. Heat treatment, enzymatic hydrolysis, acid treatment, microbial fermentation, and high-pressure processing are examples of processing methods used to alleviate allergenicity (Maryniak *et al.,* 2022; Cruz-Casas *et al.,* 2021). Of these, enzymatic hydrolysis is the most efficient, as it is capable of selectively cleaving structural (linear and conformational) epitopes, thereby experiencing changes in the primary, secondary, tertiary, and quaternary structures of proteins (Nutten *et al.,* 2020).

The efficiency of enzymatic hydrolysis depends on several parameters that affect the degree of hydrolysis (DH) and peptide profile. DH is an indispensable measurement representing the ratio of cleaved peptide bonds to total available bonds (Baharudin *et al.,* 2016). During enzymatic hydrolysis, peptide bonds are cleaved and peptide fragments with varying sizes are generated, depending on the types of enzymes and DH (Bao *et al.,* 2017; Kaur *et al.,* 2023). Consequently, protein hydrolysates produced will be used in infant formula (Vandenplas *et al.,* 20142).

The most common enzymes for protein hydrolysis include gastrointestinal (GI) enzymes such as pepsin, trypsin, and chymotrypsin (Fu *et al.,* 2021), plant-

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derived (e.g., bromelain, papain) (Mohd Azmi *et al.*, 2023), and microbial (e.g., alcalase,) (Anh *et al.,* 2020) enzymes. These enzymes cleave with different specificities, producing different peptide profiles and immunoreactivities (Liang *et al.,* 2021). While enzymatic hydrolysis is a promising method of reducing allergenicity, they have varying enzyme specificity and inconsistent peptide yields.

Though all selected enzymes are digestive proteases, they can be distinguished in gastrointestinal (GI) (those working under physiological conditions such as pepsin or trypsin) and non-gastrointestinal (non-GI) enzymes (those with broader hydrolytic abilities such as bromelain or alcalase). Enzymatic hydrolysis is a promising approach for reducing allergenicity via enzymatic hydrolysis. However, further work is needed until hydrolysis can be optimised for different allergens since there is considerable variation in enzyme specificity and peptide yields are inconsistent. Due to the lack of knowledge of how different enzymes can affect the reduction of allergenicity through goat's milk casein hydrolysis, the aim of this work was designed to assess hydrolysis conditions of GI and non-GI enzymes and produce smaller, potentially less allergenic peptides. The results will provide information on the efficiency of each enzyme and favourable conditions for hydrolysis.

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3.2 Materials and Methods

3.2.1 Chemicals, reagent, and instrument

The proteolytic enzyme alcalase, was acquired from Novozymes (from *Bacillus licheniformis,* 2.4 U/mg Bagvaerd, Hovedstade, Denmark). Trypsin (from bovine pancreas, 225 U/mg) was procured from Fisher Scientific (Fair Lawn, New Jersey, USA). Pepsin (from porcine gastric mucosa, 0.5 U/mg) and bromelain (from pineapple stem, 2.4-3.0 U/mg) were purchased from Acros Organic (Geel, Antwerp, Belgium). Enzyme units (U) are a standardised measurement of enzyme activity. A unit (1U) is the enzyme required to convert 1 micromole of substrate into a product per minute under certain circumstances, including temperature, pH, and substrate concentration. This measure provides a standardised basis for assessing enzyme activity across experiments. Sodium phosphate dibasic heptahydrate (Merck KGaA, Burlington, MA), sodium phosphate, sodium phosphate monobasic monohydrate, Tris-base, potassium chloride, sodium acetate and acetic acid were purchased from R&M (Semenyih, Selangor). O-phataldialdehdyde (OPA), was purchased from Thermo Fisher Scientific (Waltham, MA). Instruments used were centrifuge (Sartorius, Gottingen, Germany), freeze drier (Labconco, USA), water bath (Memmert GmbH and Co. KG, Buchenbach, Germany), and microtiter plate reader (Millipore, Darmstadt, Germany). Deionised water was obtained from Milli-Q Water Purification System (Merck KGaA, Burlington, MA.

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3.2.2 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.

3.2.3 Casein hydrolysis

The procedure of casein hydrolysis was done according to Zarei *et al.,* (2015) with modifications. Before hydrolysis, lyophilised casein (0.65 g) was added to a 50 mM buffer at enzyme-specific pH and temperatures. A buffer concentration of 50 mM was chosen to preserve enzyme stability and activity while not disrupting the reaction process. pH regulation was achieved by precise buffer preparation, with fluctuations minimised by utilising a consistent batch of buffer solutions. The pH meter was calibrated before to each usage to guarantee accurate results. The temperature was controlled using a regulated water bath to provide uniform testing conditions.For the preparation of a 1 L of 50 mM phosphate buffer, 10.107 g of sodium phosphate dibasic heptahydrate (molecular weight: 268 g/mol) and 1.697 g of sodium phosphate monobasic monohydrate (molecular weight: 137.94 g/mol) were utilised. A 50 mM Tris-HCl buffer was prepared using 121.14 g of Tris base (molecular weight: 121.14 g/mol). To prepare 1 L of a 50 mM KCl-HCl buffer, 7.45 g of KCl-HCl (molecular weight: 74.55 g/mol) was dissolved. A 50 mM sodium acetate buffer was formulated with 3.678 g of sodium acetate (82.03 g/mol)

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and 295.5 µL of acetic acid (60.05 g/mol). The pH of all solutions was modified utilising 1M HCl or 1M NaOH. A casein solution was formulated by dissolving 0.65 g of casein in 35 mL of the corresponding buffer solution within a 50 mL Scotch bottle. The mixture was heated at the specified temperature for 20 min to guarantee the complete dissolving of the casein. At this juncture, enzymes were not included. After the heating procedure, enzymes were added to the casein-buffer solution (C+B) at designated enzyme-to-substrate (E:S) ratios. A 5 mL of the reaction mixture were allocated into separate tubes, and hydrolysis was conducted for periods varying from 0 to 6 h under regulated circumstances.

The enzymatic process was terminated by heating the tubes in boiling water for 20 min. The samples were centrifuged at 10,000 × *g* for 10 min at 30 °C. The resultant supernatant was collected and preserved in a refrigerated environment for further examination. To evaluate hydrolysis conditions, both gastrointestinal (GI) digestive enzymes (trypsin and pepsin) and non-GI digestive enzymes (bromelain and alcalase) were examined. The enzymatic reactions were performed at various E:S ratios: 3:1 (Control), 1:1, 1:10, 1:50, 1:75, 1:100, and 1:250. Temperature was utilised between 30 °C and 60 °C, whilst pH values spanned from 1.5 to 9.0. The length of hydrolysis was evaluated at time intervals ranging from 1 to 6 h. The experimental settings were chosen based on the findings of Shu *et al*. (2016), Kumar *et al.* (2016), Liang *et al.* (2021), Bahri *et al.* (2020), Kasuma *et al.* (2018), and Medeiros *et al.* (2013), as depicted in **Figure 3.0 (a)** and **(b).** The varied experimental conditions were created to assess the effectiveness of enzymatic hydrolysis

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for different applications in food enzymology, utilising known research to optimise hydrolysis parameters.

During hydrolysis, positive and non-enzymatic controls were included to validate the hydrolysis system. The positive control, using concentrated enzyme, achieved optimal efficiency, while the non-enzymatic (unhydrolysed) control served as a reference point. A control experiment employed a 3:1 enzyme-to-substrate ratio to assess the effectiveness of the optimised ratio. Another control used was a consistent enzyme to ensure consistent performance during hydrolysis.

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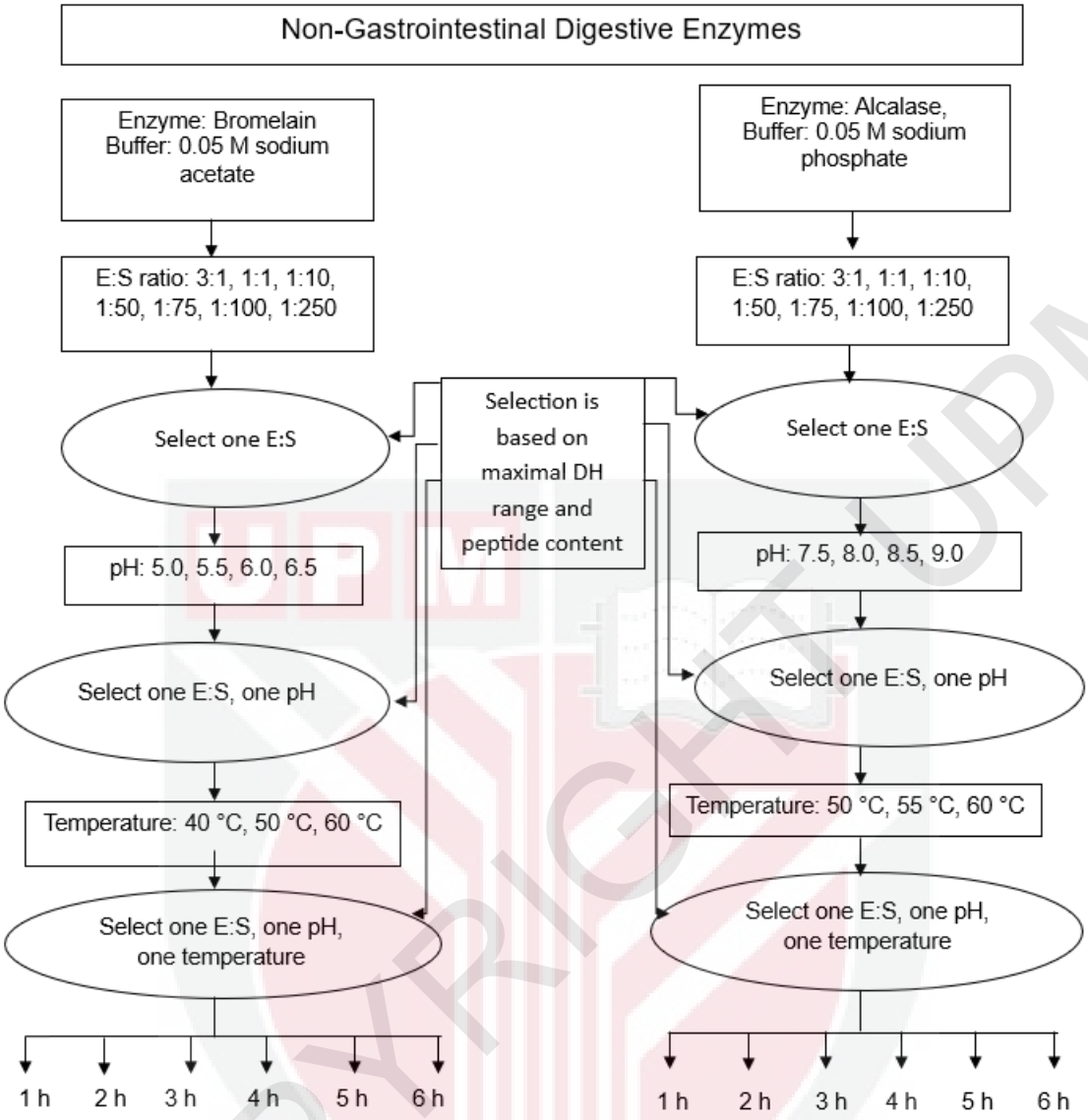

Figure 3.0(a): Non-gastrointestinal digestive enzymes and hydrolysis conditions used for hydrolysis of goat's milk casein.

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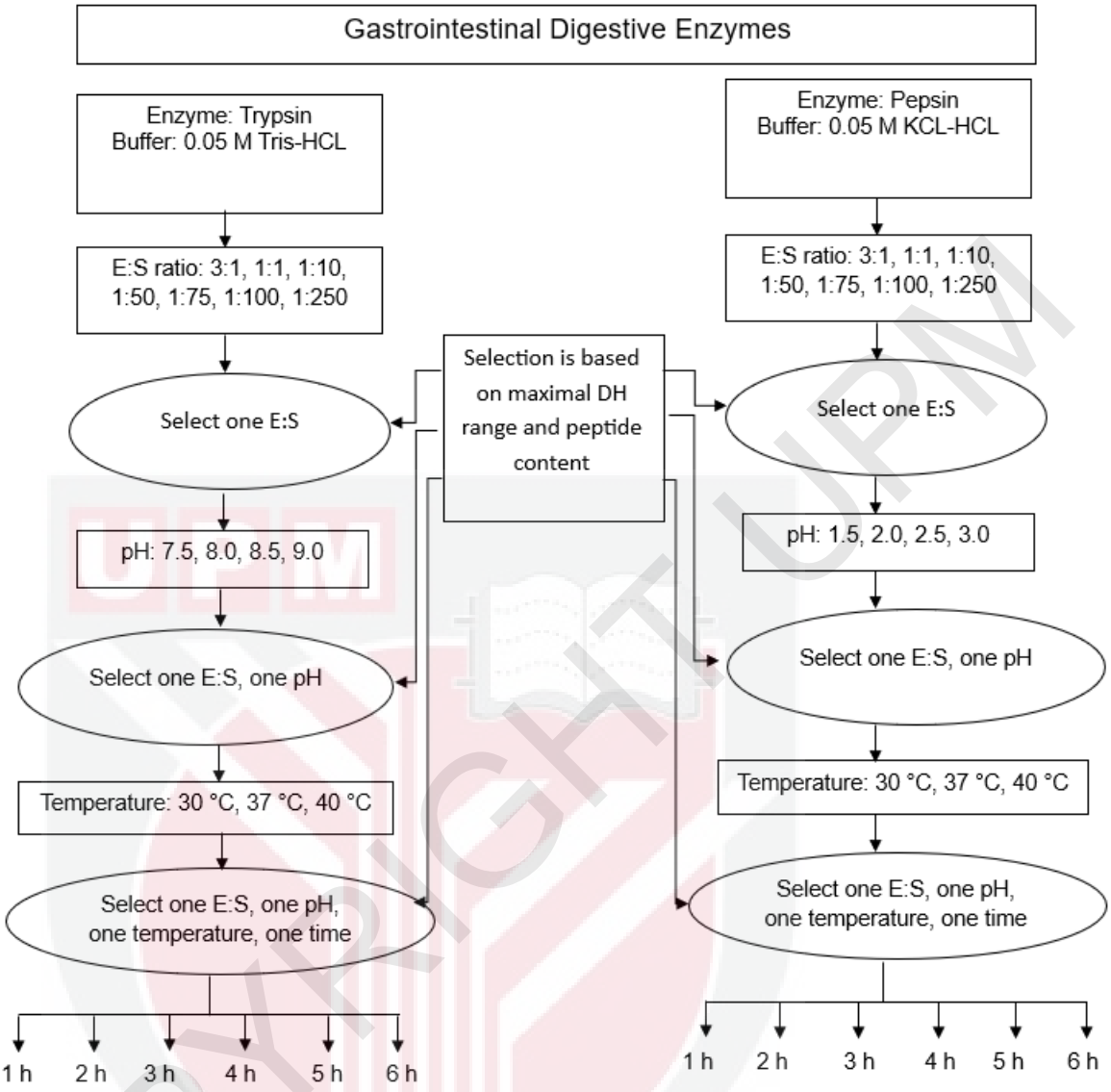

Figure 3.0(b): Gastrointestinal digestive enzymes and hydrolysis conditions used for hydrolysis of goat's milk casein.

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3.2.4 Determination of degree of hydrolysis (DH)

The OPA (ortho-phtalaldehyde) method is a precise methodology for assessing the degree of hydrolysis by measuring free amino groups liberated during protein hydrolysis. OPA interacts with primary amines in the presence of a thiol molecule, forming a quantifiable chromophore. This work used OPA to assess the enzymatic hydrolysis of goat's milk casein, providing excellent sensitivity, specificity, and quick analysis for precise measurement of free amino groups.

An OPA (ortho-phathalaldehyde) solution was prepared by combining solutions A1, A2, and A3 in the specified proportion as shown in **Table 3.0** according to Qoms *et al.,* (2023). In a Maxisorp 96-well plate (Nunc, Thermo Scientific, Waltham, USA), 36 µL of hydrolysed sample was mixed with 270 µL of OPA solution (A1, A2, A3). The solution was kept in the dark at room temperature for 2 min before measuring absorbance at 340 nm.

Table 3.0: A mixture of different types of solution to make ortho-phtalaldehyde solution

Type of solution	Ortho-phtalaldehyde
A1	Sodium dodecyl sulfate (SDS) (200 mg) and sodium tetrahydroborate (7.62 g) in deionised water (150 mL)
A2	OPA (160 mg) in 96% ethanol (4 mL)
A3	β-mercaptoethanol (400 µL) in 50 mL of deionised water

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The degree of hydrolysis was determined using the procedure Qoms of *et al.,* (2023). The hydrolysis extent was assessed using the following formula:

DH (%) = (Absorbance after hydrolysis-Absorbance before hydrolysis/total hydrolysis) *100**.***

Total hydrolysis was measured by ensuring a complete breakdown of casein to free amino acid. First, 0.2 g, 0.3 g, 0.4 g, and 0.5 g of casein were mixed with 15 mL 6N HCl and incubated at 100 °C for 24 h. After cooling at room temperature for 1 h, the samples were centrifuged at 10, 000 *x g* for 10 min to collect the supernatant. The absorbances were measured by mixing 36 µL of samples with 270 µL OPA (o-phataldehyde) solution in triplicates. Absorbance < 1.0 was selected as total hydrolysis value.

3.2.5 Determination of peptide content

The peptide content determination technique quantifies the overall peptide concentration, providing insight into the number of peptides generated, irrespective of size. It is often founded on colourimetric or UV absorbance tests. The two approaches are complementary: OPA measures hydrolysis efficiency, whereas peptide content estimates the total peptide produced. The peptide content was quantified using a colorimetric method that measures peptides by UV absorption. This approach guarantees precise peptide quantification and corresponds with the study's objective of evaluating enzymatic hydrolysis efficiency and overall peptide yield.

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To quantify the peptide content in hydrolysed casein, an L-serine standard curve was established from 2 mg/mL L-serine stock solution. This involved preparing a series of 12 dilutions to cover a broad range of concentrations. By measuring the absorbance of these dilutions and plotting the data, a standard curve was generated with the equation (y =13.549x-1E-05; R^2 = 0.9971). Here, y denotes the absorbance and x represents the peptide concentration. This well-defined standard curve provides a reliable method for quantifying the peptide content in hydrolysed casein, ensuring accurate and consistent results in the analysis.

3.2.6 Statistical analysis

All data was measured in triplicates (n=3). Absorbance data were presented as mean and standard deviation. GraphPad Prism (San Diego, California) statistical software version 8.0 was applied for two-way analysis of variance (ANOVA). A post-hoc Tukey test was then conducted to evaluate the pairs of mean from treatment levels. Significant differences were calculated at α=0.05 (95% confidence interval).

3.3 Results and Discussion

3.3.1 Evaluation of hydrolysis conditions

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3.3.1.1 Hydrolysis using alcalase

Enzyme specificity is crucial in enzymatic hydrolysis as it dictates the cleavage sites of peptides (Kilara & Chandan, 2011). Alcalase, recognised for its capacity to cleave at the carboxyl side of hydrophobic amino acids like glutamine (E), methionine (M), tyrosine (Y), leucine (L), and lysine (K), is proficient in hydrolysing casein (Jung *et al.,* 2016). Alcalase produces a mixture of peptides and free amino acids, augmenting diverse functional capabilities (Rao *et al.,* 2020). The experiment was performed at 340 nm, where nicotinamide adenine dinucleotide (NADH), a molecule involved in enzymatic activities, shows considerable light absorption, unlike its oxidised form, NAD^+. This allows for efficient observation of fluctuations in NADH levels throughout the process. Measuring absorbance at 340 nm enables precise monitoring of enzymatic reactions over time, as NADH is either produced or consumed during these processes, providing high sensitivity.

Table 3.1(a) demonstrates that the degree of hydrolysis (DH) with alcalase increases over time at higher enzyme-to-substrate (E:S) ratios, peaking at 1:10 and 1:50, with DH values between 40% and 60%. The 40–60% DH range was chosen for its efficacy in reducing protein allergenicity while maintaining the functioning of bioactive peptides. Research indicates that hydrolysis effectively destroys allergenic epitopes within this region, hence reducing immune recognition (Sun *et al*., 2024). Moreover, peptides produced at this DH level have preserving bioactivity. A 3:1 enzyme-to-substrate ratio was utilised, consistent with prior research on plant proteins, where this ratio significantly improved hydrolysis efficiency (Qoms *et al.,* 2023). A 1:50 ratio was used to

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enhance enzyme utilisation, attaining saturation and obtaining Vmax, when all active sites are occupied, hence assuring optimal reaction efficiency. This equilibrium improves hydrolysis yield while preserving cost-effectiveness. The hydrolysis within this DH range promotes the liberation of bioactive peptides possessing functional attributes, such as immunomodulatory and antioxidant capabilities (Sun *et al.,* 2024). These peptides facilitate the decrease of allergenicity, hence endorsing the selection of this DH range for the development of hypoallergenic protein hydrolysates.

Alcalase hydrolysed goat's milk casein most effectively at pH 8.0, as seen in **Table 3.1(b).** Elevating the pH to 8.5 significantly improved hydrolysis efficiency, presumably due to augmented negative charges on casein micelles, which enlarged and destabilised their structures, enhancing enzyme accessibility (Sinaga *et al.,* 2016). Trindade *et al.* (2015) assert that a protein's charge is determined by its isoelectric point (pI), the pH at which the protein has a zero net charge. The protein's acidic functional groups (e.g., carboxyl ($-COO^-$)) are deprotonated at pH above the isoelectric point, leading to a negatively charged protein. Conversely, beneath the isoelectric point, the protein possesses a net positive charge at sufficiently low pH due to the protonation of fundamental groups, such as amino ($-NH_3^+$). This explains why a protein with a pI of 8.5 would have a negative charge at pH values above 8.5 and a positive charge at far lower pH levels. Similar results were noted using cow's milk casein (Liu & Guo, 2008; Madadlou *et al.,* 2009); however, elevated pH levels (exceeding 9.5) may destabilise micelles and denature the enzyme, diminishing hydrolysis efficiency. While these findings align with cow's milk

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casein, more research is required to validate their relevance to goat's milk casein. Maintaining a regulated alkaline environment is essential for balancing micelle expansion and enzyme stability, guaranteeing effective hydrolysis.

Temperature substantially influences DH and peptide concentration. The optimal DH and peptide yield was attained at 55 °C (**Table 3.1(c)**), corroborating prior research (Shu *et al.,* 2016). Decreased temperatures diminish enzyme activity, hence impeding hydrolysis, whereas temperatures over 60 °C result in enzyme denaturation, reducing hydrolysis efficiency. Our findings correspond with the ideal temperature range for alcalase activity (50 °C-60 °C) (Shu *et al.,* 2016; Kumar *et al.,* 2016; Liang *et al.,* 2020), which inhibits enzyme denaturation while promoting casein degradation. Comparative investigations have shown differing optimum conditions for alcalase activity. Wang *et al.* (2023) determined that optimal conditions for the hydrolysis of cow's milk casein were 50 °C, pH 8.5, and a period of 3 h, resulting in a degree of hydrolysis (DH) of 26.45%. Kumar *et al.* (2016) similarly documented a 20% degree of hydrolysis using camel milk casein at 55 °C and pH 8.0 for 6 h. Although not directly comparable due to substrate variations, these investigations highlight alcalase's adaptability in protein hydrolysis across different milk types. Proper temperature settings are essential for maximising DH and peptide production while avoiding enzyme denaturation.

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Table 3.1(a): Degree of hydrolysis and peptide content at different hydrolysis times and substrate: enzyme (E:S) ratios using alcalase

Time (h)	Enzyme-to-substrate ratio						
	3:1 (Control)	1:1	1:10	1:50	1:75	1:100	1:250
	Degree of hydrolysis (%)						
1	75.53 ± 0.04^{Ae}	72.34 ± 0.06^{Bf}	56.59 ± 0.02^{Ce}	44.54 ± 0.03^{Df}	32.79 ± 0.012^{Ef}	32.77 ± 0.036^{Ef}	17.01± 0.04^{Ff}
2	79.00 ± 0.02^{Ab}	76.26 ± 0.06^{Be}	55.52 ± 0.05^{Cf}	48.56 ± 0.03^{Ce}	47.06 ± 0.000^{De}	39.81 ± 0.023^{Ee}	21.70± 0.02^{Fe}
3	77.54 ± 0.02^{Ac}	79.46 ± 0.06^{Bb}	55.64 ± 0.03^{Cd}	55.00 ± 0.02^{Dd}	44.46 ± 0.016^{Ed}	43.16 ± 0.022^{Fd}	23.30± 0.02^{Gd}
4	80.00± 0.010^{Aa}	80.00 ± 0.02^{Aa}	57.91 ± 0.05^{Cc}	60.13 ± 0.003^{Bc}	49.34 ± 0.03^{Dc}	43.72 ± 0.010^{Ec}	27.58± 0.01^{Fc}
5	76.00± 0.001^{Ad}	78.70 ± 0.00^{Bc}	60.91 ± 0.01^{Cb}	61.76 ± 0.07^{Db}	51.32 ± 0.00^{Eb}	49.16 ± 0.006^{Fb}	31.45± 0.00^{Gb}
6	80.00 ± 0.03^{Aa}	78.01 ± 0.00^{Bd}	61.63 ± 0.02^{Ca}	62.05 ± 0.00^{Da}	51.74 ± 0.00^{Ea}	50.60 ± 0.004^{Fa}	31.77± 0.03^{Ga}
	Peptide content (mg/mL)						
1	3.95 ± 0.04^{Ae}	3.13 ± 0.036^{Bc}	2.37 ± 0.012^{Cc}	1.87 ± 0.03^{De}	1.36 ± 0.02^{Ed}	1.36 ± 0.06^{Ee}	0.66 ± 0.04^{Fe}
2	3.84 ± 0.02^{Af}	3.30 ± 0.023^{Bb}	2.38 ± 0.000^{Cc}	2.06 ± 0.03^{Dd}	1.99 ± 0.05^{Dc}	1.67 ± 0.06^{Ed}	0.86± 0.02^{Fd}
3	4.19 ± 0.02^{Ad}	3.45 ± 0.022^{Bb}	2.48 ± 0.016^{Cb}	2.35 ± 0.02^{Dc}	1.97 ± 0.03^{Ec}	1.82 ± 0.06^{Fc}	0.94± 0.02^{Gc}
4	4.43 ± 0.01^{Ac}	3.53 ± 0.010^{Ba}	2.48 ± 0.03^{Dd}	2.58 ± 0.003^{Cb}	2.10 ± 0.05^{Eb}	1.84 ± 0.02^{Fc}	1.13± 0.01^{Gb}
5	4.65 ± 0.00^{Aa}	3.41 ± 0.006^{Ba}	2.61 ± 0.00^{Ca}	2.65 ± 0.07^{Ca}	2.19 ± 0.01^{Da}	2.09 ± 0.00^{Eb}	1.32± 0.001^{Fa}
6	4.58 ± 0.03^{Ab}	3.48 ± 0.004^{Ba}	2.65 ± 0.00^{Da}	2.67 ± 0.00^{Ca}	2.20 ± 0.02^{Ea}	2.16 ± 0.00^{Ea}	1.32± 0.03^{Fa}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different enzyme-substrate ratios. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.1(b): Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and pH using alcalase

Time (h)	pH value				
	Control	**7.0**	**7.5**	**8.0**	**8.5**
	Degree of hydrolysis (%)				
1	40.21 ± 0.001^{Af}	26.42 ± 0.04^{Ef}	34.17 ± 0.01^{Cf}	33.05 ± 0.01^{Df}	35.41± 0.000^{Bf}
2	47.68 ± 0.001^{Ab}	34.05 ± 0.02^{Ee}	41.85 ± 0.02^{De}	42.97 ± 0.02^{Ce}	43.37 ± 0.010^{Bd}
3	45.72 ± 0.003^{Be}	36.49 ± 0.002^{Ed}	43.45 ± 0.002^{Cd}	46.28 ± 0.02^{Ad}	42.41 ± 0.010^{De}
4	47.60 ± 0.018^{Ac}	39.45 ± 0.001^{Ec}	45.88 ± 0.003^{Cb}	46.56 ± 0.02^{Bc}	44.40 ± 0.002^{Dc}
5	46.32 ± 0.000^{Cd}	40.29 ± 0.03^{Db}	45.76 ± 0.054^{Cc}	47.68 ± 0.003^{Ab}	46.32± 0.003^{Ba}
6	48.98 ± 0.000^{Aa}	40.65 ± 0.02^{Ea}	47.60 ± 0.00^{Ca}	48.76 ± 0.054^{Ba}	46.16± 0.003^{Db}
	Peptide content (mg/mL)				
1	1.69 ± 0.001^{Ac}	1.26 ± 0.04^{De}	1.51 ± 0.01^{Bf}	1.37 ± 0.01^{Ce}	1.48 ± 0.000^{Bc}
2	2.03± 0.001^{Aa}	1.46 ± 0.02^{Dd}	1.63 ± 0.02^{Ce}	1.81 ± 0.02^{Bd}	2.03 ± 0.010^{Aa}
3	1.94 ± 0.003^{Ab}	1.50 ± 0.002^{Cd}	1.75 ± 0.002^{Bd}	1.96 ± 0.02^{Ac}	1.94 ± 0.010^{Ab}
4	2.02 ± 0.018^{Aa}	1.84 ± 0.001^{Cb}	1.80 ± 0.003^{Cc}	1.97 ± 0.02^{Bc}	2.02 ± 0.002^{Aa}
5	1.97 ± 0.000^{Ab}	1.79 ± 0.03^{Cc}	1.94 ± 0.054^{Bb}	2.03 ± 0.003^{Ab}	1.97 ± 0.003^{Ab}
6	2.08 ± 0.000^{Ba}	2.06 ± 0.02^{Ba}	2.16 ± 0.00^{Aa}	2.07 ± 0.054^{Ba}	2.08 ± 0.007^{Ba}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different pH. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.1(c): Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and temperatures using alcalase

Time (h)	Temperature			
	Control	**50 °C**	**55 °C**	**60 °C**
	Degree of hydrolysis (%)			
1	39.01 ± 0.02^{Af}	21.90 ± 0.00^{Df}	30.44 ± 0.01^{Bf}	27.82 ± 0.01^{Cf}
2	42.61 ± 0.02^{Ae}	27.00 ± 0.02^{Dc}	38.79 ± 0.01^{Ce}	39.00 ± 0.02^{Be}
3	43.35 ± 0.01^{Ad}	24.58 ± 0.00^{De}	43.00 ± 0.01^{Bd}	40.59 ± 0.03^{Cc}
4	44.60 ± 0.00^{Bc}	25.16 ± 0.01^{Dd}	47.68 ± 0.02^{Ab}	41.79 ± 0.03^{Cb}
5	48.24 ± 0.01^{Bb}	30.44 ± 0.02^{Db}	48.98 ± 0.01^{Aa}	39.75 ± 0.01^{Cd}
6	51.88 ± 0.02^{Aa}	41.23 ± 0.02^{Da}	46.34 ± 0.03^{Bc}	45.14 ± 0.02^{Ca}
	Peptide content (mg/mL)			
1	1.64 ± 0.02^{Ae}	0.88 ± 0.00^{Ce}	1.26 ± 0.01^{Bd}	1.64 ± 0.01^{Ae}
2	1.80 ± 0.02^{Ad}	1.14 ± 0.02^{Cc}	1.63 ± 0.01^{Bc}	1.80 ± 0.02^{Ad}
3	1.83 ± 0.01^{Ad}	1.00 ± 0.00^{Cd}	1.70 ± 0.01^{Bb}	1.83 ± 0.03^{Ad}
4	1.88 ± 0.00^{Ac}	1.02 ± 0.01^{Cd}	1.71 ± 0.02^{Bb}	1.88 ± 0.03^{Ac}
5	2.05 ± 0.01^{Ab}	1.26 ± 0.02^{Cb}	1.67 ± 0.01^{Bb}	2.05 ± 0.01^{Ab}
6	2.21 ± 0.02^{Aa}	1.74 ± 0.02^{Ca}	1.91 ± 0.03^{Ba}	2.21 ± 0.02^{Aa}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different temperature. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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3.3.1.2 Hydrolysis using bromelain

Bromelain is a proteolytic enzyme that hydrolyses peptide bonds at hydrophobic and charged/hydrophilic amino acids, including arginine, lysine, phenylalanine, and tyrosine (Ee *et al.,* 2019), further hydrolysing protein substrates into smaller peptide fragments. The enzyme-to-substrate (E:S) ratio significantly influences the degree of hydrolysis (DH) and peptide synthesis when bromelain is utilised as the enzyme. An optimal ratio of enzymes and substrates in the cleavage reaction will result in improved peptide bond cleavage, elevated peptide yields, and decreased peptide costs. The DH is observed to rise as anticipated with an increasing E:S ratio **(Table 3.2(a)),** and a ratio of 1:100 was determined to produce the optimal DH range of 40–60%. This ratio represents an optimal equilibrium between hydrolysis degree and cost-effectiveness. An excessively low ratio may lead to diminished enzyme efficiency, whereas an excessively high ratio might cause enzyme depletion. The accurate breakdown of proteins necessitates a modification of the ratio based on the substrate volume and the appropriate enzyme concentration.

The pH plays a critical role in bromelain's efficiency, functioning well at a somewhat acidic pH range of 5 to 8 (Medeiros *et al.,* 2014; Kumar *et al.,* 2023). **Table 3.2(b)** shows that the most significant degree of hydrolysis (60.35% ± 0.002) was recorded at pH 5.5 and a peptide concentration of 2.86 ± 0.002 mg/mL at 6 h. This signifies that bromelain reliably generates peptides at this pH, particularly as the process progresses. **Table 3.2(c)** reveals that the optimal temperature of 50 °C yields a maximum DH (60.47 ± 0.010%) and peptide concentration (2.91 ± 0.010 mg/mL), which were subsequently

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observed while maintaining the optimal pH of 5.5. Comparable results were documented by Purbanigrum (2019), which identified peak enzyme activity at 50 °C, exhibiting specific activities of 2 U/mg throughout the pH range of 5.5-6.0. Meanwhile, Lestari and Suyata (2019) determined that the optimal temperature for bromelain is 55 °C; however, the enzyme diminishes beyond this range due to thermal denaturation. Elevated temperatures undermine the enzyme's integrity, altering its secondary and tertiary structures, hence impairing its enzymatic function (Robinson, 2015).

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Table 3.2(a): Degree of hydrolysis and peptide content at different hydrolysis times and substrate: enzyme (E:S) ratios using bromelain

Time (h)	Enzyme-to-substrate ratio						
	3:1 (Control)	1:1	1:10	1:50	1:75	1:100	1:250
	Degree of hydrolysis (%)						
1	56.00 ± 0.00^{Bf}	58.15 ± 0.00^{Af}	7.35 ± 0.003^{Gc}	18.51 ± 0.009^{Ca}	8.31 ± 0.00^{Fe}	15.51 ± 0.002^{Dc}	11.83 ± 0.007^{Ea}
2	66.30 ± 0.014^{Ad}	60.03 ± 0.01^{Be}	6.36 ± 0.00^{Gd}	17.75 ± 0.00^{Cc}	7.35 ± 0.006^{Ff}	15.11 ± 0.001^{Dd}	8.79 ± 0.002^{Ec}
3	66.50 ± 0.006^{Bc}	71.42 ± 0.02^{Ac}	5.60 ± 0.003^{Ge}	18.39 ± 0.002^{Cb}	9.07 ± 0.001^{Fd}	14.55 ± 0.004^{De}	9.75 ± 0.006^{Eb}
4	63.51 ± 0.05^{Be}	68.46 ± 0.01^{Ad}	5.60 ± 0.002^{Ge}	16.71 ± 0.01^{Ce}	11.91 ± 0.003^{Ec}	13.90 ± 0.00^{Df}	8.19 ± 0.00^{Fd}
5	90.93 ± 0.02^{Ab}	90.73 ± 0.02^{Ab}	8.27 ± 0.006^{Eb}	16.87 ± 0.004^{Dd}	20.26 ± 0.001^{Cb}	27.98 ± 0.004^{Bb}	7.63 ± 0.002^{Ec}
6	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	16.26 ± 0.008^{Da}	14.51 ± 0.009^{Ef}	36.89 ± 0.01^{Ca}	42.77 ± 0.02^{Ba}	7.35 ± 0.002^{Ff}
	Peptide content (mg/mL)						
1	2.40 ± 0.00^{Be}	2.50 ± 0.00^{Af}	0.22 ± 0.003^{Fc}	0.72 ± 0.009^{Ca}	0.27 ± 0.00^{Ee}	0.59 ± 0.002^{Dc}	0.22 ± 0.007^{Ff}
2	2.85 ± 0.014^{Ac}	2.58 ± 0.01^{Be}	0.17 ± 0.00^{Fd}	0.69 ± 0.00^{Cb}	0.22 ± 0.006^{Ef}	0.57 ± 0.001^{Dc}	0.24 ± 0.002^{Ee}
3	2.87 ± 0.006^{Bc}	3.09 ± 0.02^{Ac}	0.15 ± 0.003^{Gd}	0.72 ± 0.002^{Ca}	0.30 ± 0.001^{Ed}	0.55 ± 0.004^{Dc}	0.26 ± 0.006^{Fd}
4	2.73 ± 0.05^{Bd}	2.95 ± 0.01^{Ad}	0.15 ± 0.002^{Gd}	0.64 ± 0.01^{Cc}	0.43 ± 0.003^{Ec}	0.52 ± 0.00^{Dd}	0.33 ± 0.00^{Fb}
5	3.96 ± 0.02^{Ab}	3.94 ± 0.02^{Ab}	0.27 ± 0.006^{Eb}	0.65 ± 0.004^{Dc}	0.80 ± 0.001^{Cb}	1.15 ± 0.004^{Bb}	0.29 ± 0.002^{Ec}
6	4.98 ± 0.00^{Aa}	4.72 ± 0.00^{Ba}	0.62 ± 0.008^{Ea}	0.54 ± 0.009^{Fd}	1.54 ± 0.01^{Da}	1.81 ± 0.02^{Ca}	0.42 ± 0.002^{Ga}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different enzyme-substrate-ratio. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.2(b): **Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and pH using bromelain**

Time (h)	pH value				
	Control	**5.0**	**5.5**	**6.0**	**6.5**
	Degree of hydrolysis (%)				
1	40.12 ± 0.000^{Af}	3.60 ± 0.0005^{Ef}	9.79 ± 0.006^{De}	18.98 ± 0.001^{Bf}	14.23 ± 0.006^{Cf}
2	47.68 ± 0.0007^{Ab}	10.63 ± 0.0005^{De}	6.43 ± 0.003^{Ef}	22.38 ± 0.017^{Ce}	22.50 ± 0.003^{Be}
3	45.72 ± 0.007^{Ae}	12.71 ± 0.0005^{Ed}	16.11 ± 0.004^{Dd}	25.38 ± 0.025^{Cc}	27.98 ± 0.002^{Bd}
4	47.60 ± 0.0003^{Ac}	13.10 ± 0.000^{Ec}	37.13 ± 0.004^{Bc}	23.02 ± 0.000^{Dd}	36.39 ± 0.000^{Cb}
5	46.32 ± 0.010^{Ba}	21.86 ± 0.008^{Db}	47.52 ± 0.011^{Ab}	32.13 ± 0.007^{Cb}	32.13 ± 0.000^{Cc}
6	48.98 ± 0.010^{Ca}	25.22 ± 0.000^{Ea}	60.35 ± 0.002^{Aa}	60.15 ± 0.007^{Ba}	42.69 ± 0.008^{Da}
	Peptide content (mg/mL)				
1	1.70 ± 0.000^{Ad}	0.06 ± 0.0005^{Ee}	0.33 ± 0.006^{De}	0.74 ± 0.001^{Bf}	0.53 ± 0.006^{Cc}
2	2.03 ± 0.0007^{Aa}	0.37 ± 0.0005^{Cd}	0.18 ± 0.003^{Df}	0.90 ± 0.017^{Be}	2.03 ± 0.003^{Aa}
3	1.94 ± 0.007^{Ac}	0.46 ± 0.0005^{Dc}	1.03 ± 0.025^{Bc}	1.03 ± 0.025^{Bc}	1.94 ± 0.002^{Ab}
4	2.02 ± 0.0003^{Aa}	0.48 ± 0.000^{Dc}	1.55 ± 0.004^{Bc}	0.92 ± 0.000^{Cd}	2.02 ± 0.000^{Aa}
5	1.97 ± 0.010^{Bb}	0.87 ± 0.008^{Db}	2.02 ± 0.011^{Ab}	1.49 ± 0.007^{Cb}	1.97 ± 0.007^{Bb}
6	2.08 ± 0.000^{Ca}	1.02 ± 0.000^{Da}	2.86 ± 0.002^{Aa}	2.58 ± 0.007^{Ba}	2.08 ± 0.008^{Ca}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different pH. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.2(c): Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and temperatures using bromelain

Time (h)	Temperature			
	Control	40 °C	50 °C	60 °C
	Degree of hydrolysis (%)			
1	29.50 ± 0.009^{Af}	9.95 ± 0.003^{Bf}	5.52 ± 0.001^{Cf}	3.60 ± 0.001^{Dd}
2	37.41 ± 0.016^{Ae}	15.19 ± 0.010^{Be}	9.83 ± 0.004^{Ce}	2.52 ± 0.007^{Df}
3	43.53 ± 0.023^{Ac}	21.30 ± 0.02^{Bd}	10.27 ± 0.003^{Cd}	2.96 ± 0.003^{De}
4	42.21 ± 0.0007^{Ad}	26.34 ± 0.02^{Bc}	25.14 ± 0.004^{Cc}	6.75 ± 0.002^{Dc}
5	45.16 ± 0.013^{Ab}	28.38 ± 0.01^{Cb}	39.09 ± 0.002^{Bb}	21.26 ± 0.004^{Db}
6	47.18 ± 0.013^{Ba}	39.53 ± 0.01^{Ca}	60.00 ± 0.010^{Aa}	22.06 ± 0.012^{Da}
	Peptide content (mg/mL)			
1	1.21 ± 0.009^{Af}	0.34 ± 0.003^{Bf}	0.14 ± 0.001^{Cf}	0.06 ± 0.001^{Dd}
2	1.57 ± 0.016^{Ae}	0.57 ± 0.010^{Be}	0.33 ± 0.004^{Ce}	0.01 ± 0.007^{Df}
3	1.84 ± 0.023^{Ac}	0.85 ± 0.02^{Bd}	0.35 ± 0.003^{Cd}	0.03 ± 0.003^{De}
4	1.78 ± 0.0007^{Ad}	1.07 ± 0.02^{Bc}	1.02 ± 0.004^{Cc}	0.20 ± 0.002^{Dc}
5	1.91 ± 0.013^{Ab}	1.16 ± 0.01^{Cb}	1.64 ± 0.002^{Bb}	0.84 ± 0.004^{Db}
6	2.00 ± 0.013^{Ba}	1.66 ± 0.01^{Ca}	2.91 ± 0.010^{Aa}	0.88 ± 0.012^{Da}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different temperature. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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3.3.1.3 Hydrolysis using trypsin

To evaluate protein hydrolysis with trypsin, the enzyme-to-substrate (E:S) ratio, pH, and temperature must be controlled since they greatly influence the degree of hydrolysis (DH) and peptide yield. The data in **Table 3.3(a)** reveals that the optimal enzyme-substrate (E:S) ratio was established at 1:10, consistently yielding a degree of hydrolysis (DH) between 40% and 60%. This ratio equilibrates the hydrolysis efficiency and cost-effectiveness, mitigating excessive enzyme loss. An inadequate E:S ratio may yield reduced enzyme efficacy, whereas an excessively high E:S ratio might result in unnecessary enzyme utilisation and jeopardise cost-effectiveness. Hence, the E:S ratio must be adjusted based on substrate availability to maintain the appropriate concentration of active enzymes for effective protein breakdown. Maximum peptide production occurred at 6 h of hydrolysis, yielding 2.45 ± 0.00 mg/mL and 2.41 ± 0.00 mg/mL, respectively. This indicates that the hydrolysis process progresses nearly to an ideal level within these periods, suggesting that 6 h is sufficient for substantial peptide production among the assessed parameters. The prolonged period of the experiment, up to 6 h, is essential to achieve the entire or near-complete hydrolysis of casein, as enzymatic processes often need a longer time to get the appropriate level of hydrolysis. Maintaining a steady temperature is crucial for preserving enzymes' stability and effectiveness during the reaction, preventing thermal denaturation and ensuring the hydrolysis process progresses effectively.

The ideal pH for hydrolysis was determined to be 9, yielding a maximum degree of hydrolysis (DH) of 54.64 ± 0.02% and a peptide concentration of

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2.34 ± 0.02 mg/mL in tissue after 6 h (**Table 3.3 (b)**). These findings align with the results reported by Custodio *et al.* (2005), who identified pH 9 as the optimal environment for the actions of trypsin on cheese whey proteins. Nonetheless, our results do not align with those of Jung *et al.* (2014), which indicated that trypsin attains its optimal activity at pH 7-8. Similarly, Lim *et al.* (2019) conducted trypsin hydrolysis of bovine milk proteins at pH 7 and 45 °C, resulting in only 15% degree of hydrolysis (DH). These discrepancies arise from differing experimental settings, including substrate type, enzyme purity, and reaction length. Our findings indicate that pH 9 is optimal for trypsin action on the proteins examined in this study.

The temperature findings indicated that the optimal temperature for trypsin was 37 °C, yielding a DH of 46.52 ± 0.00% and a peptide concentration of 1.97 ± 0.00 mg/mL in 6 h of hydrolysis (**Table 3.3(c)**). This discovery aligns with Cheison *et al.* (2011) and Alkhalidy *et al.* (2011), who found that the optimal temperature for trypsin is 37 °C. However, increasing the temperature to 45 °C, resulted in a noticeable decrease, suggesting that the enzyme may begin to denature at these temperatures. Elevated temperatures can lead to enzyme denaturation, compromising the secondary and tertiary structures of the enzyme, hence diminishing catalytic activity and restricting peptide synthesis (Galvão *et al.,* 2001). Any deviations from this temperature would significantly reduce hydrolysis, reaffirming the essential temperature for enzymatic function in hydrolysis. The study conducted by Rostammiry *et al.* (2017) further supports the need for optimal temperature control in trypsin-mediated hydrolysis. Their research employed Response Surface Methodology (RSM)

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to investigate the effects of temperature and time on soy protein hydrolysate. It concluded that the optimal temperature was 41.85 °C with a hydrolysis duration of 1 h. This could be due to variations in the enzyme-substrate ratios, types of substrates used, or differences in experimental design.

The hydrolysis of proteins by trypsin was maximised and ideal at a 1:10 enzyme-to-substrate ratio, a pH of 9, and a temperature of 37 °C. This will provide the most considerable DH and peptide output, as demonstrated by the findings of this study. Any variation from ideal circumstances, such as higher temperature or pH, results in diminished enzyme function and thus reduced production of peptides. These findings reinforce the understanding of the necessary conditions for enhancing the effectiveness of trypsin-mediated hydrolysis and establish a foundation for further efforts to optimise protein hydrolysis procedures for diverse applications.

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Table 3.3(a): Degree of hydrolysis of goat's milk casein hydrolysate at different hydrolysis time and substrate: enzyme (E:S) ratio using trypsin

Time (h)	Enzyme-to-substrate ratio						
	3:1 (Control)	**1:1**	**1:10**	**1:50**	**1:75**	**1:100**	**1:250**
	Degree of hydrolysis (%)						
1	67.35 ± 0.05^{Ae}	53.84 ± 0.02^{Bd}	42.17 ± 0.02^{Ce}	19.03 ± 0.00^{De}	27.14 ± 0.02^{Ef}	26.26 ± 0.06^{Fe}	18.47 ± 0.02^{Gf}
2	72.06 ± 0.00^{Aa}	51.84 ± 0.05^{Be}	44.21 ± 0.05^{Cc}	19.82 ± 0.03^{Fd}	30.86 ± 0.05^{De}	26.33 ± 0.06^{Ee}	19.25 ± 0.01^{Ge}
3	70.26 ± 0.03^{Ac}	50.70 ± 0.03^{Bf}	42.89 ± 0.03^{Cd}	18.91± 0.01^{Gf}	31.30 ± 0.03^{Dd}	28.94 ± 0.057^{Ed}	23.03 ± 0.04^{Fd}
4	67.55 ± 0.02^{Ad}	54.49 ± 0.01^{Bc}	47.48 ± 0.01^{Cb}	20.14 ± 0.01^{Gc}	33.65 ± 0.05^{Db}	30.01 ± 0.016^{Ec}	23.15 ± 0.03^{Fc}
5	71.28 ± 0.06^{Ab}	57.1 ± 0.00^{Bb}	57.19 ± 0.00^{Ba}	28.30 ± 0.01^{Eb}	33.45 ± 0.01^{Cc}	33.33± 0.004^{Db}	27.18 ± 0.02^{Fb}
6	70.46 ± 0.02^{Ab}	57.10 ± 0.00^{Ba}	57.10 ± 0.00^{Ba}	30.58 ± 0.00^{Ea}	40.65 ± 0.02^{Ca}	35.57 ± 0.005^{Da}	30.22 ± 0.007^{Fa}
	Peptide content (mg/mL)						
1	2.90 ± 0.05^{Ac}	2.30 ± 0.02^{Bb}	1.78 ± 0.02^{Cc}	0.75 ± 0.00^{Dc}	1.11 ± 0.02^{Ed}	1.07 ± 0.06^{Ed}	0.72 ± 0.02^{Dd}
2	3.12 ± 0.00^{Aa}	2.21 ± 0.05^{Bc}	1.87 ± 0.05^{Cc}	0.78 ± 0.03^{Fc}	1.27 ± 0.05^{Dc}	1.07 ± 0.06^{Ed}	0.75 ± 0.01^{Fd}
3	3.04 ± 0.03^{Ab}	2.31 ± 0.03^{Bb}	1.81 ± 0.03^{Cc}	0.74± 0.01^{Gc}	1.30 ± 0.03^{Dc}	1.19 ± 0.057^{Ec}	0.92 ± 0.04^{Fc}
4	2.91± 0.02^{Ac}	2.33 ± 0.01^{Bb}	2.02 ± 0.01^{Cb}	0.80 ± 0.01^{Gc}	1.40 ± 0.05^{Db}	1.24 ± 0.016^{Ec}	0.93 ± 0.03^{Fc}
5	3.08 ± 0.06^{Ab}	2.27 ± 0.00^{Cb}	2.45 ± 0.00^{Ba}	1.16 ± 0.01^{Eb}	1.40 ± 0.01^{Db}	1.39± 0.004^{Db}	1.11 ± 0.02^{Eb}
6	3.04± 0.02^{Ab}	2.42 ± 0.00^{Ba}	2.41 ± 0.00^{Ba}	1.26 ± 0.00^{Ea}	1.71 ± 0.02^{Ca}	1.49 ± 0.005^{Da}	1.25 ± 0.007^{Ea}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different enzyme-to-substrate ratio. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.3(b): Degree of hydrolysis of goat's milk casein hydrolysate at different hydrolysis time and pH using trypsin

Time (h)	pH value				
	Control	**7.5**	**8.0**	**8.5**	**9.0**
	Degree of hydrolysis (%)				
1	30.90 ± 0.01^{Df}	26.86 ± 0.03^{Ef}	38.37 ± 0.07^{Bf}	37.37 ± 0.03^{Cf}	38.81 ± 0.01^{Af}
2	36.57 ± 0.02^{De}	28.94 ± 0.04^{Ee}	39.09 ± 0.02^{Ce}	45.52 ± 0.01^{Be}	47.40 ± 0.02^{Ae}
3	43.17 ± 0.01^{Da}	32.69 ± 0.02^{Eb}	44.68 ± 0.02^{Cd}	46.16 ± 0.06^{Bd}	48.20 ± 0.00^{Ad}
4	42.39 ± 0.02^{Dc}	31.93 ± 0.02^{Ec}	45.04 ± 0.04^{Cc}	47.28 ± 0.03^{Bc}	49.92 ± 0.00^{Ac}
5	42.61 ± 0.03^{Db}	35.17 ± 0.01^{Ea}	52.00 ± 0.02^{Ba}	51.92 ± 0.02^{Cb}	53.40 ± 0.00^{Ab}
6	39.05 ± 0.01^{Dd}	31.18 ± 0.01^{Ed}	46.92 ± 0.01^{Cb}	52.98 ± 0.01^{Ba}	54.64 ± 0.00^{Aa}
	Peptide content (mg/mL)				
1	1.28 ± 0.01^{Dd}	1.10 ± 0.03^{Ed}	1.61 ± 0.07^{Bd}	1.57 ± 0.03^{Cc}	1.63 ± 0.01^{Ad}
2	1.53 ± 0.02^{Dc}	1.19 ± 0.04^{Ec}	1.59 ± 0.02^{Cd}	1.93 ± 0.01^{Bb}	2.01 ± 0.02^{Ac}
3	1.82 ± 0.01^{Da}	1.36 ± 0.02^{Eb}	1.89 ± 0.02^{Cc}	1.96 ± 0.06^{Bb}	2.05 ± 0.00^{Ac}
4	1.79 ± 0.02^{Da}	1.32 ± 0.02^{Eb}	1.91 ± 0.04^{Cc}	2.01 ± 0.03^{Bb}	2.13 ± 0.00^{Ab}
5	1.80 ± 0.03^{Ca}	1.47 ± 0.01^{Da}	2.22 ± 0.02^{Ba}	2.21 ± 0.02^{Ba}	2.28 ± 0.00^{Aa}
6	1.69 ± 0.01^{Db}	1.29 ± 0.01^{Eb}	2.00 ± 0.01^{Cb}	2.24 ± 0.01^{Ba}	2.34 ± 0.02^{Aa}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different pH. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.3(c): Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and temperatures using trypsin

Time (h)	Temperature			
	Control	**30 °C**	**37 °C**	**45 °C**
	Degree of hydrolysis (%)			
1	35.57 ± 0.07^{Bf}	32.78 ± 0.07^{Cf}	38.25 ± 0.01^{Ac}	38.24 ± 0.02^{Ac}
2	43.49 ± 0.01^{Ae}	33.00 ± 0.01^{De}	34.00 ± 0.03^{Cf}	34.69 ± 0.01^{Be}
3	47.12 ± 0.07^{Ac}	34.45 ± 0.07^{Dc}	36.00 ± 0.03^{Cd}	46.32 ± 0.07^{Ba}
4	46.76 ± 0.02^{Ad}	44.32 ± 0.02^{Bb}	35.53 ± 0.03^{Ce}	32.65 ± b 0.02^{Df}
5	51.44 ± 0.03^{Aa}	34.13 ± 0.03^{Dd}	43.99 ± 0.04^{Bb}	37.89 ± 0.03^{Cd}
6	49.72 ± 0.05^{Ab}	43.89 ± 0.05^{Ca}	46.52 ± 0.00^{Ba}	41.69 ± 0.05^{D}
	Peptide content (mg/mL)			
1	1.26 ± 0.07^{Bd}	1.36 ± 0.07^{Ab}	1.38 ± 0.01^{Ad}	1.26 ± 0.02^{Bc}
2	1.70 ± 0.01^{Ab}	1.40 ± 0.01^{Bb}	1.32 ± 0.03^{Bc}	1.70 ± 0.01^{Ab}
3	1.71 ± 0.07^{Ab}	1.44 ± 0.07^{Cb}	1.53 ± 0.03^{Bb}	1.71 ± 0.07^{Ab}
4	1.76 ± 0.02^{Bb}	1.87 ± 0.02^{Aa}	1.48 ± 0.03^{Cb}	1.76 ± 0.02^{Bb}
5	1.47 ± 0.03^{Cc}	1.42 ± 0.03^{Cb}	1.89 ± 0.04^{Aa}	1.67 ± 0.03^{Bb}
6	1.91 ± 0.05^{Aa}	1.90 ± 0.05^{Aa}	1.97 ± 0.00^{Aa}	1.91 ± 0.05^{Aa}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different temperature. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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3.3.1.4 Hydrolysis using pepsin

This section delineates the composition of peptides of varying lengths and the corresponding degree of hydrolysis (DH) for pepsin under varied experimental settings. The maximal for both DH and peptide content (**Table 3.4(a)**) were seen at E:S ratios of 1:1 and 1:10, respectively. An E:S ratio of 1:10 was used for subsequent investigation as it exemplified a balance between hydrolytic efficiency and economic expenditure. Excessive or insufficient enzyme concentration can result in suboptimal performance since the enzyme may either become saturated with substrate or enzyme depletion. **Table 3.4(b)** indicates that pH significantly influenced pepsin function, with the enzyme attaining a DH of 59.76 ± 0.02% after 5 h at pH 1.5 and 59.43 ± 0.03% after 6 h at pH 2.0, so demonstrating pepsin's functions under very acidic circumstances, which aligns with its role in stomach digesting.

Pepsinogen undergoes autocleavage in the presence of HCL, resulting in its activation (Heda *et al.*, 2023). However, beyond a pH of 2.0, both DH and peptide content diminished, perhaps due to the altered substrate binding, resulting in reduced enzymatic stability. Pepsin, in contrast to trypsin, remains active and stable at acidic pH levels.

Temperature also plays a crucial role in pepsin function, as shown in **Table 3.4include,** exhibiting the peak DH (59.84 ± 0.01%) and peptide (2.89 ± 0.02mg/m) concentrations at 37 °C after 6 h, aligning with the documented range of 40-60 °C for pepsin (Sholeh *et al.,* 2020). However, at 45 °C, both DH and peptide concentrations declined, perhaps due to partial enzyme

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denaturation at elevated temperature or loss of stability. A notable limitation is that pepsin hydrolysis has a limited number of previous studies, hence constraining a broader understanding and limited comparison of pepsin's efficacy under varying situations. Subsequent research should explore a wider array of hydrolysis parameters to enhance the optimised ability of these findings.

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Table 3.4(a): Degree of hydrolysis of goat's milk casein hydrolysate at different hydrolysis times and substrate: enzyme (E:S) ratios using pepsin

Time (h)	Enzyme-to-substrate ratio						
	3:1 (Control)	1:1	1:10	1:50	1:75	1:100	1:250
	Degree of hydrolysis (%)						
1	47.96± 0.05^{Ae}	52.60± 0.02^{Ba}	18.82 ± 0.02^{Cf}	10.35 ± 0.00^{Df}	7.67 ± 0.02^{Ge}	12.11 ± 0.06^{Fd}	16.70 ± 0.02^{Eg}
2	57.15± 0.00^{Ad}	46.53 ± 0.05^{Bb}	19.10 ± 0.05^{De}	11.19 ± 0.03^{Ee}	6.08 ± 0.05^{Gf}	8.67 ± 0.06^{Ff}	20.94 ± 0.01^{Cf}
3	71.86± 0.03^{Ac}	46.04 ± 0.03^{Bc}	39.65 ± 0.03^{Ca}	16.15 ± 0.01^{Fd}	17.35 ± 0.03^{Ed}	11.83 ± 0.057^{Ge}	23.86 ± 0.04^{De}
4	78.22± 0.02^{Ab}	42.45 ± 0.01^{Be}	39.65 ± 0.016^{Cb}	21.34 ± 0.01^{Ec}	21.34 ± 0.05^{Fc}	15.48 ± 0.016^{Gc}	21.62 ± 0.03^{Dd}
5	100.00± 0.06^{Aa}	43.41 ± 0.00^{Bd}	35.57 ± 0.01^{Cc}	21.74 ± 0.01^{Fb}	22.86 ± 0.01^{Eb}	15.74± 0.004^{Gb}	25.22 ± 0.02^{Db}
6	100.00± 0.02^{Aa}	36.42 ± 0.00^{Bf}	34.57 ± 0.00^{Cd}	31.18 ± 0.00^{Da}	23.06 ± 0.02^{Fa}	19.46 ± 0.005^{Ga}	24.58 ± 0.007^{Ea}
	Peptide content (mg/mL)						
1	2.40 ± 0.00^{Be}	2.50 ± 0.00^{Af}	0.22 ± 0.003^{Fc}	0.72 ± 0.009^{Ca}	0.27 ± 0.00^{Ee}	0.59 ± 0.002^{Dc}	0.22 ± 0.007^{Ff}
2	2.85± 0.014^{Ac}	2.58 ± 0.01^{Be}	0.17 ± 0.00^{Fd}	0.69 ± 0.00^{Cb}	0.22 ± 0.006^{Ef}	0.57 ± 0.001^{Dc}	0.24 ± 0.002^{Ee}
3	2.87 ± 0.006^{Bc}	3.09 ± 0.02^{Ac}	0.15 ± 0.003^{Gd}	0.72 ± 0.002^{Ca}	0.30 ± 0.001^{Ed}	0.55 ± 0.004^{Dc}	0.26 ± 0.006^{Fd}
4	2.73 ± 0.05^{Bd}	2.95 ± 0.01^{Ad}	0.15 ± 0.002^{Gd}	0.64 ± 0.01^{Cc}	0.43 ± 0.003^{Ec}	0.52 ± 0.00^{Dd}	0.33 ± 0.00^{Fb}
5	3.96 ± 0.02^{Ab}	3.94 ± 0.02^{Ab}	0.27 ± 0.006^{Eb}	0.65 ± 0.004^{Dc}	0.80 ± 0.001^{Cb}	1.15 ± 0.004^{Bb}	0.29 ± 0.002^{Ec}
6	4.98 ± 0.00^{Aa}	4.72 ± 0.00^{aB}	0.62 ± 0.008^{aE}	0.54 ± 0.009^{Fd}	1.54 ± 0.01^{Da}	1.81 ± 0.02^{Ca}	0.42 ± 0.002^{Ga}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different enzyme-to-subtrate ratio. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.4(b): Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis times and pH using pepsin

Time (h)	pH value				
	Control	**1.5**	**2.0**	**2.5**	**3.0**
	Degree of hydrolysis (%)				
1	35.00 ± 0.002^{Af}	8.078 ± 0.004^{Bf}	8.00 ± 0.007^{Ce}	7.03 ± 0.001^{De}	1.32 ± 0.000^{Ef}
2	47.80 ± 0.020^{Ae}	11.55 ± 0.002^{Ce}	6.43 ± 0.000^{Df}	15.00 ± 0.012^{Bd}	2.76 ± 0.020^{Ee}
3	49.24 ± 0.014^{Ad}	30.14 ± 0.007^{Bd}	14.39 ± 0.002^{Dc}	16.67 ± 0.001^{Cb}	6.12± 0.0014^{Ed}
4	52.00 ± 0.001^{Aa}	40.00 ± 0.000^{Bb}	13.39 ± 0.003^{Dd}	15.00 ± 0.004^{Cd}	9.71 ± 0.001^{Ec}
5	51.72 ± 0.001^{Bb}	57.07 ± 0.018^{Aa}	24.42 ± 0.01^{Cb}	20.58 ± 0.007^{Da}	18.51 ± 0.001^{Ea}
6	50.28 ± 0.03^{Bc}	31.25 ± 0.012^{Cc}	56.47 ± 0.007^{Aa}	16.55 ± 0.002^{Dc}	10.91 ± 0.003^{Eb}
	Peptide content (mg/mL)				
1	0.97 ± 0.002^{Ad}	0.26 ± 0.004^{Cf}	0.27 ± 0.007^{Be}	0.21 ± 0.001^{Dd}	0.00 ± 0.000^{Ee}
2	2.03 ± 0.02^{Ac}	0.41 ± 0.002^{Ce}	0.18 ± 0.00^{Df}	0.82 ± 0.012^{Bb}	0.02 ± 0.020^{Ee}
3	2.10 ± 0.014^{Ab}	1.24 ± 0.007^{Bd}	0.54 ± 0.002^{Dc}	0.64 ± 0.001^{Cc}	0.17± 0.0014^{Ed}
4	2.22 ± 0.001^{Aa}	1.84 ± 0.000^{Bb}	0.49 ± 0.003^{Dd}	0.56 ± 0.004^{Cc}	0.33 ± 0.001^{Ec}
5	2.21 ± 0.001^{Ba}	2.45 ± 0.018^{Aa}	0.99 ± 0.01^{Cb}	0.82 ± 0.007^{Da}	0.72 ± 0.001^{Ea}
6	2.14 ± 0.030^{Bb}	1.29 ± 0.012^{Cc}	2.42 ± 0.007^{Aa}	0.63 ± 0.002^{Dc}	0.38 ± 0.003^{Eb}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different pH. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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Table 3.4©: Degree of hydrolysis and peptide content of goat's milk casein hydrolysate at different hydrolysis time and temperatures using pepsin

Time (h)	Temperature			
	Control	**30 °C**	**37 °C**	**45 °C**
	Degree of hydrolysis (%)			
1	39.45 ± 0.02^{Af}	7.91 ± 0.003^{Df}	11.67 ± 0.009^{Cf}	2.36 ± 0.000^{Bf}
2	40.01 ± 0.00^{Ce}	15.11 ± 0.000^{Ad}	14.55 ± 0.007^{Be}	4.48 ± 0.000^{De}
3	46.00 ± 0.00^{Ad}	14.87 ± 0.001^{Ce}	17.51 ± 0.001^{Bd}	6.51 ± 0.001^{Dd}
4	46.53 ± 0.020^{Ac}	18.00 ± 0.004^{Cc}	27.78 ± 0.004^{Bc}	14.98 ± 0.02^{Dc}
5	52.76 ± 0.00^{Aa}	29.62 ± 0.016^{Cb}	39.53 ± 0.019^{Bb}	24.74 ± 0.00^{Db}
6	50.60 ± 0.00^{Bb}	30.14 ± 0.021^{Da}	55.16 ± 0.00^{Aa}	39.00 ± 0.004^{Ca}
	Peptide content (mg/mL)			
1	1.66 ± 0.02^{Ac}	0.25 ± 0.003^{Cc}	0.15 ± 0.009^{Df}	0.99 ± 0.000^{Bd}
2	1.69 ± 0.00^{Ac}	0.57 ± 0.000^{Cb}	0.55 ± 0.007^{Ce}	1.08 ± 0.000^{Bd}
3	1.99 ± 0.001^{Ab}	0.56 ± 0.001^{Db}	0.68 ± 0.001^{Cd}	1.04 ± 0.001^{Bd}
4	1.98± 0.020^{Ab}	0.49 ± 0.004^{Db}	1.14 ± 0.004^{Cc}	1.81 ± 0.02^{Bc}
5	2.26 ± 0.00^{Aa}	1.21 ± 0.016^{Da}	1.66 ± 0.019^{Cb}	1.99 ± 0.00^{Bb}
6	2.16 ± 0.004^{Ca}	1.24 ± 0.021^{Da}	2.36 ± 0.000^{Ba}	2.64 ± 0.004^{Aa}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts, are significantly different ($p < 0.05$) within the same group, while different uppercase superscripts signify significant differences ($p < 0.05$) among different pH. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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3.3.1.5 Comparison between various enzymes

Each enzyme requires an optimised enzyme-to-substrate (E:S) ratio, pH, and incubation time, which are determined based on the degree of hydrolysis (DH) and peptide concentration. This study employed the E:S ratio that yielded the highest DH and peptide concentration and was subsequently used for further evaluation of other parameters. To determine the impact of duration on hydrolysis efficiency, the time was set between 1 to 6 h. The process and hydrolysis conditions determined from experimental data for DH and peptide content were depicted in **Figures 3.1 (a)** and **(b).** The hydrolytic performance data of several enzymes is presented in **Table 3.4.** Bromelain exhibited the greatest level of hydrolysis and peptide concentration among all enzymes studied, underscoring its exceptional efficacy in casein hydrolysis. Its wide cleavage specificity facilitates widespread casein hydrolysis, hence affirming its efficacy. These findings indicate that bromelain is especially effective for applications necessitating significant protein degradation, such as decreasing antigenicity or allergenicity in dairy products. Moreover, its extensive availability, affordability, and accessibility render it a pragmatic option for industrial and research applications.

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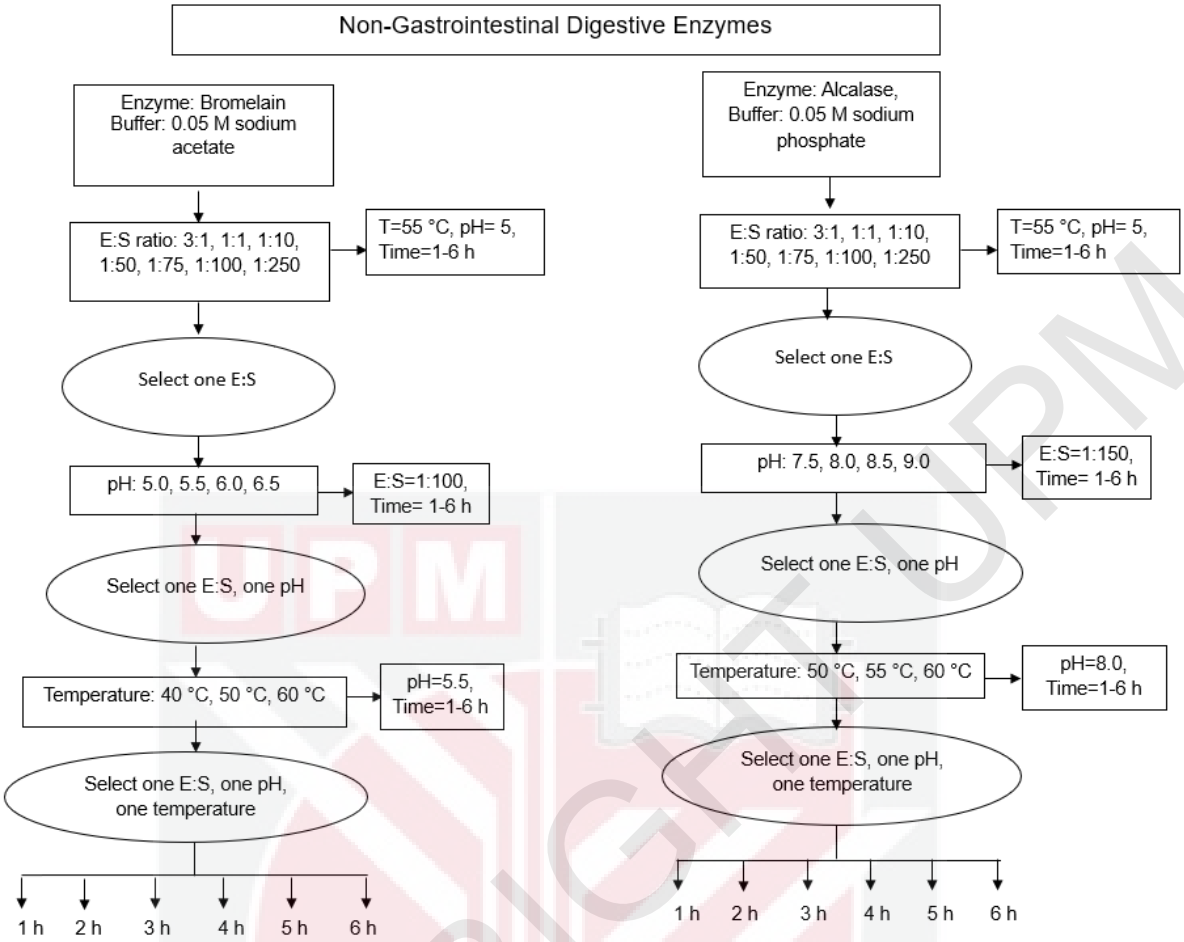

Figure 3.1(a): A comprehensive workflow of non-gastrointestinal digestive enzymes after selection of hydrolysis conditions used for hydrolysis of goat's milk casein

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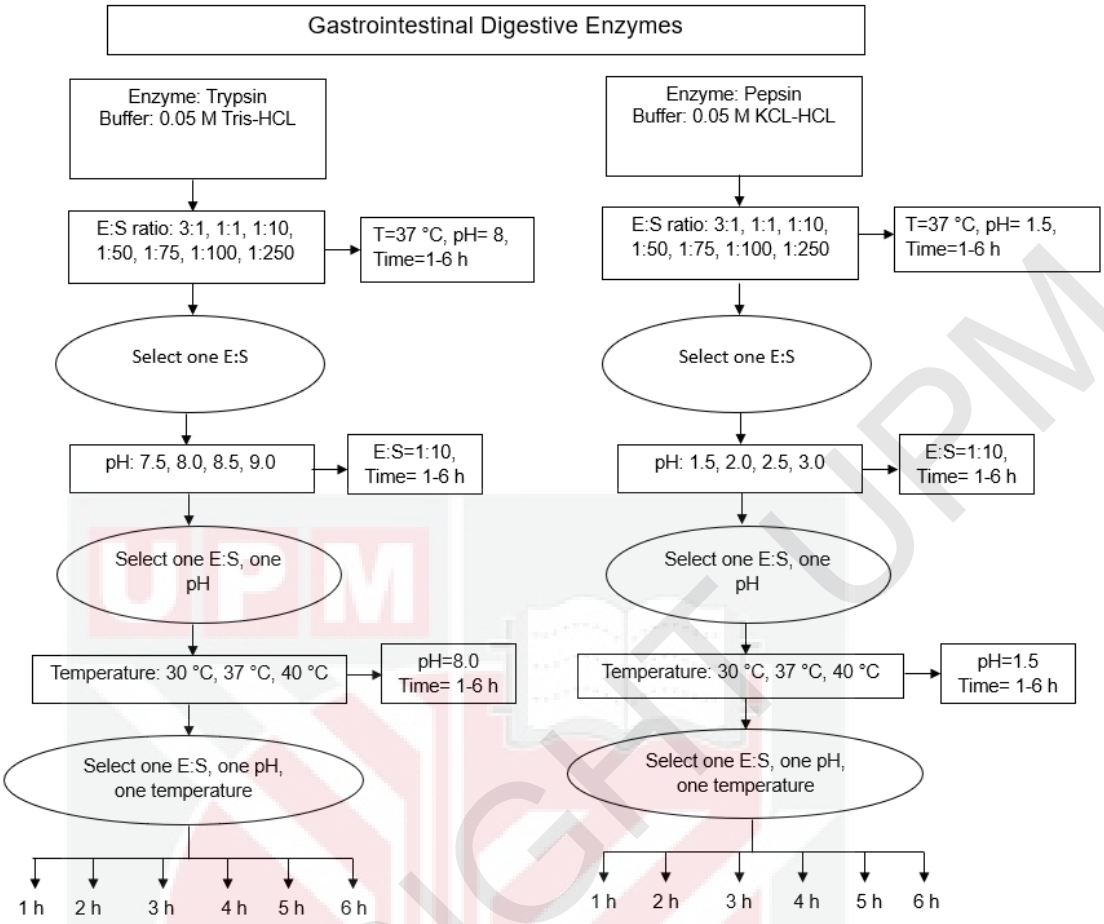

Figure 3.1(b): A comprehensive workflow of gastrointestinal digestive enzymes after selection of hydrolysis conditions used for hydrolysis of goat's milk casein

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Table 3.5: Comparison of degree of hydrolysis and peptide content using different types of enzymes

Enzymes	Condition				Degree of hydrolysis (%)	Peptide Content (mg/mL)
	E:S	pH	Temp	Time		
Alcalase	1:50	8.0	55	6	46.34 ± 0.03^{d}	1.91 ± 0.03^{d}
Bromelain	1:100	5.5	50	6	60.00 ± 0.010^{a}	2.91 ± 0.010^{a}
Trypsin	1:10	5.5	50	6	46.52 ± 0.00^{c}	1.97 ± 0.00^{c}
Pepsin	1:10	1.5	37	6	55.16 ± 0.00^{b}	2.36 ± 0.000^{b}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$), among different enzymes. The absorbance of the well of goat's milk casein hydrolysate was detected at 340 nm.

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3.4 Conclusion

One Factor at a Time (OFAT) approach was used to ensure that no critical elements were overlooked when evaluating hydrolysis conditions. Response Surface Methodology (RSM) provides a methodical framework for a 3×3 factorial design at a time. In contrast, One Factor at a Time (OFAT) adopts a more flexible strategy by sequentially assessing the impact of individual components to achieve optimal circumstances. Consequently, the methodology was employed to thoroughly assess the key parameters, including enzyme-to-substrate ratio, pH, duration, and temperature, without missing potential interactions that may influence hydrolysis efficiency. Additionally, all hydrolysis data were presented in tabular format to offer a systematic overview of observed values and enable precise reference and comparison across various situations. The table format was prioritised in this study to ensure transparency and thoroughness when presenting the experimental outcomes.

The study has effectively examined the hydrolysis efficiency of gastrointestinal and non-gastrointestinal digestive enzymes. Four parameters (enzyme-to-substrate (E:S) ratio, pH, temperature, and incubation time) were evaluated using the One-Factor-at-a-Time (OFAT) methodology to attain a maximum degree of hydrolysis (DH) of 40-60% and a peptide yield of approximately 2 mg/mL, with peptide concentration. Among the evaluated enzymes, gastrointestinal non-digestive enzymes, namely bromelain, showed superior efficacy to gastrointestinal digestive enzymes in hydrolysis. Under 111ptimized circumstances (E:S 1:100, pH 5.5, 50 °C), bromelain attained the maximum

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degree of hydrolysis (DH, around 60%). Alcalase exhibited optimal performance at an enzyme-to-substrate ratio of 1:50, pH 8.0, and 55 °C. In contrast, the gastrointestinal digestive enzymes trypsin (E:S 1:10, pH 9.0, 37 °C) and pepsin (E:S 1:10, pH 1.5, 37 °C) demonstrated effective albeit significantly lower hydrolysis efficiency. While DH and peptide concentrations give early insights on hydrolysis efficiency, they lack antigenicity reduction indices. Further studies must include antigenicity analyses to prove a direct relationship between enzymatic hydrolysis and decreased allergenicity. Characterisation of hydrolysed peptides structurally will also aid our understanding of how enzymatic modifications of protein substrates affect allergenicity. Additional methodological studies will be required to exploit enzyme-mediated hydrolysis to generate functional hypoallergenic dairy products fully.

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CHAPTER 4

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

4.1 Introduction

Milk protein allergens have remained a major focus in food safety research. Analytical techniques including high-performance liquid chromatography (HPLC) (Costa *et al.,* 2015), liquid chromatography-spectrometry (LC-MS) (Holzhauser & Roder, 2015; Villa *et al.,* 2022), and DNA-based methods (Lu *et al.,* 2023) have been employed to detect milk proteins. However, these methods have significant limitations, such as high cost and excessive use of organic solvents. In contrast, enzyme-linked immunosorbent assay (ELISA) provides a low-cost, user-friendly, highly specific, and rapid approach to protein detection (Holzhauser *et al.,* 2020). (Lin, 2015; Nouri *et al.,* 2018; Zhu *et al.,* 2022; Heyrapetyan *et al.,* 2023). Despite ELISA being a common detection method, few studies exist on this method to determine the goat's milk casein composition, likely due to the disparity observed in commercial antibody availability for goat's casein. To address this issue, the study aimed to evaluate the binding ability of goat's milk casein to antibodies derived from caprine α_{s1}-casein peptides and anti-bovine casein polyclonal antibodies. ELISA detection methods, include direct, indirect, and sandwich assays, have different sensitivities and specificities. However, the optimal format for detecting goat's milk casein remain uncertain.

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Moreover, antibody specificity is critical for the performance of ELISA, as highlighted by He *et al.* (2023). The presence of other critical parameters including the concentration of antibodies used, type of blocking reagents and pre-treatments also have a significant impact on the sensitivity and reliability of ELISA (Minic et al., 2021).

In contrast to earlier studies aimed at determining specific goat's milk casein subtypes as α_{s1}-casein (Mohsin *et al.,* 2023), this study intended to increase the applicability of different ELISA formats (**Figure 4 (a–c)**) for determination of goat's milk total casein composition. Due to the lack of antibodies raised against goat's milk casein are commercially available, polyclonal antibodies against bovine casein and antibodies raised against caprine α_{s1}-casein peptides (N-terminal IgG-biotin conjugates, and C-terminal IgG-biotin conjugates) were employed. Given the 88% similarity of the amino acid sequence of bovine and goat's casein (Prosser, 2021), bovine-specific antibodies can aid in determining this approach. This chapter discusses optimisation of ELISA for the detection of goat's milk casein. Systematic evaluation of the key parameters (e.g., ELISA formats, antibody selection, blocking reagents and pre-treatment conditions) led to the development of a highly sensitive and specific detection method.

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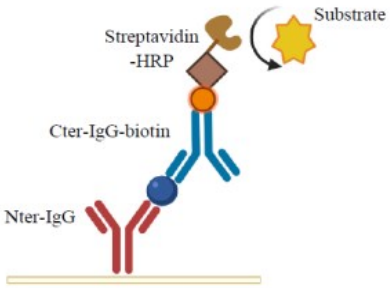
a)



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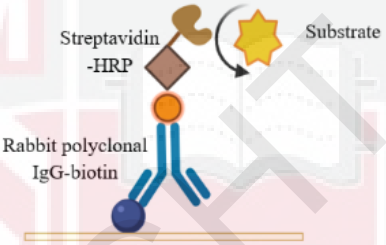

c)

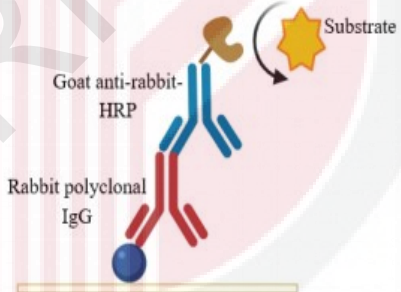

Figure 4.0: Different formats of ELISA; (a) sandwich (b) direct (c) indirect

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4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Chemicals used for SDS-PAGE were HCl, glycerol, TEMED, ammonium persulfate and β-mercaptoethanol, Coomassie Blue R-250 were purchased from Merck (Kenilworth, NJ). Precision plus proteinTM dual colour standards, pure Tris's base (~99.8%), 30% acrylamide/bis solution (37:5:1) and sodium dodecyl sulfate (SDS), ammonium persulfate, dithiothreitol (DTT) were obtained from Bio-Rad (Hercules, CA).

Antibodies used for sandwich ELISA include 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 containing 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4.2H_2O$, and 18 mM KH_2PO_4. 1X PBS was used for all dilutions and solutions. All chemicals for PBS were obtained from R&M (Semenyih, Selangor).

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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. Bovine serum albumin (BSA) was obtained from Avantor Lifescience (Missouri, TX), Pierce™ Protein-Free (PBS) (1X concentration), Superblock™, and ELISA substrate (1-Step Ultra TMB), as well as Maxisorp 96-well plate, were acquired from ThermoFisher Scientific (Waltham, MA). Deionised water was obtained from Milli-Q Water Purification System (Merck KGaA, Burlington, MA).

4.2.2 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.

4.2.3 Protein determination (Kjeldahl)

Nitrogen content and crude protein were assessed in accordance with AOAC 991.20 (1991). This study quantified nitrogen derived from proteins, enabling the calculation of crude protein content. In the Kjeldahl technique, 0.15 g of the dried material was weighed in triplicate. Due to the sample being a liquid (milk), a dropper was employed to acquire an accurate measurement. Subsequently, 0.8 g of a mixed catalyst was used to enhance the digestion process, followed by the incorporation of concentrated sulphuric acid (100%).

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The tube was gently agitated to achieve thorough mixing. The digesting procedure involved incrementally heating the tube on a heating coil beneath a fume hood until the solution became clear and exhibited a blue-green, signifying the completion of digestion. This procedure typically requires thirty minutes. The solution evolved from black and hazy throughout digestion to dark brown, clear orange, bright orange, dark green, and ultimately blue-green. The blank was utilised to assess the uniformity of the sample colouration. After digestion, heating was discontinued, and the tube was let to pre-cool for 10 min, verifying the bottom of the tube to confirm adequate cooling. Cooling for over 15 min may lead to the crystallisation and freezing of the sample. If crystallisation occurred, 10 mL of deionised water was added immediately to the tube. The solution was thereafter spun gently to ensure proper mixing, as inadequate mixing might result in hazardous circumstances for the subsequent phase. If there is adequate phase separation and the acid and water were not well mixed prior to the addition of sodium hydroxide, splashing may occur due to the phase separation. This indicates thorough mixing was essential to dilute the concentrated acid before neutralisation.

Subsequently, a 45% NaOH solution was incrementally introduced to a distinct phase. The distillation apparatus was securely attached to the condenser at the start of the distillation process. The operation was executed by operating the system for two minutes. The distillate was collected in a conical flask containing 10 mL of 2% boric acid and two drops of an indicator solution. This combination was covered with aluminium foil to safeguard it against contamination. The conical flask was positioned on the distillation platform to

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ensure the tip of the distillation tube was fully immersed in the acid solution. The distilled ammonia solution was gathered in the conical flask until the total reached 120 mL. The contents were combined by swirling, and the unreacted boric acid was titrated with 0.05 N sulphuric acid (H_2SO_4) to achieve a neutral endpoint. To guarantee precision, identical procedures were executed on a blank sample. The nitrogen and protein content were determined using the following equations:

Calculation *Vs*=tritation sample, *Vb*=titration blank, Normality (H_2SO_4) =0.05 N, *W*=weight of sample

Percentage of nitrogen (%) = $\frac{(Vs-Vb) \times 0.05\ N \times 1.4}{W} \times 100$
Percentage of protein (%) = percentage nitrogen × conversion factor.

4.2.4 Casein Isolation

Isolation of casein from goat's milk was conducted as described by Hodgkinson *et al.* (2012) with some modifications on washing precipitate. Goat's milk was first thawed at 36 ˚C in a water bath for 30 min and cooled at ambient temperature (24 ˚C). At 36 ˚C, the temperature is mild enough to minimise the thermal denaturation of proteins, thus preventing protein aggregation or precipitation (Zhao *et al.,* 2019). A controlled water bath promotes equal heat transfer with homogeneous conditions for thawing, protecting the physical properties of the goat's milk. Then, 800 mL of milk was transferred into a 1 L centrifuge bottle (Sartorius, Gottingen, Germany) at 5,232 × g for 30 min while maintaining a temperature of 4 ˚C . Subsequently, the skimmed milk was poured into a new container, and the fat layer was removed from the fat layer in a centrifuge tube by using a spatula. The pH of

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the solution was attuned to 4.2 by adding 1 M HCl to induce precipitation of the casein. The skimmed milk was stirred using a magnetic stirrer for 30 min, followed by subsequent centrifugation at 5,232 × *g* for 15 min at 20 ˚C to retrieve the casein precipitate. Then, the casein residue was subjected to washing by suspending it in purified water at ten times the initial volume of milk (5.0 L dH_20 in a 5 L conical flask: 70 g casein) (v/v). The suspension was then stirred at ambient temperature (24 ˚C) for 1 h before 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 analysis.

4.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The lyophilised casein was analysed using SDS-PAGE as described by Laemmli (1970). Resolving gel (12%) was prepared by mixing before stacking gel (5%). Sample buffer was prepared by a mixture 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. A total volume of 20 µL sample and sample buffer were heated at 90-100 ˚C for 5 min before loaded on the gel (10 µL) while the protein marker was added at a volume of 5 µL. The electrophoresis was connected to the power supply with 200 V running conditions for 40 min.

The image of the gel was stained using 0.05% (w/v) Coomassie Brilliant Blue G 250, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid overnight. Then, the gel was destained with a destaining solution until the blue background disappeared so the band could be seen clearly. The gel was then rinsed with distilled water and stored in the solution. The casein bands were identified

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using Precision Plus Protein™ dual colour standards (Bio-rad Laboratories Inc, Hercules, CA, USA).

4.2.6 Sandwich ELISA

Immunoassay was conducted using a method established by Mohsin *et al.* (2023). N-terminal and C-terminal-biotin polyclonal antibodies were produced in rabbits by both N-terminal and C-terminal peptides of goat a_{s1}-casein. 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 in 1X 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, ELISA plate was positioned in the dark, and the development of a blue colour was monitored. The reaction was halted by incorporating 100 µL of 0.1 M H_2SO_4. Finally, absorbance values were

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acquired at a wavelength of 450 nm via Multiscan FC (ThermoFisher Scientific, Waltham, MA). The signal-to-noise was determined by (Absorbance of samples/Absorbance of background noise). A 450 nm is the standard wavelength for TMB (3, 3', 5,5'-tetramethybenzidine reaction products in the ELISA experiment. It produces yellow colour changes before absorbance measurement.

4.2.6.1 Antibody concentration of caprine alpha-s1 casein peptides

Nter-IgG at 1:1,000 (v/v) and Cter-biotin-IgG was used at (1:1,000, 1;2,500) (v/v) and streptavidin-horseradish peroxidase (Streptavidin-HRP) was applied at ratios of (1:7,500, 1:10,000; 1:15,000) (v/v) in phosphate-buffered saline (PBS). All reagents were prepared in 1X PBS, pH 7.4.

4.2.6.2 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) (1X) (Pierce™), and Superblock™ (Pierce) blocking buffer performed directly to each well.

4.2.6.3 Casein Pre-treatment

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

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4.2.7 Bovine casein polyclonal antibodies

4.2.7.1 Direct ELISA

Direct ELISA was done as described by Liang *et al.* (2021) 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 executed at 100 µL by including various treatments at a concentration of 10 µg/mL (casein samples) diluted with 1:500 and 1:1,000 (v/v) in PBS (pH 7.4). Then, the plate underwent three washes using PBS. The plate was then 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 1:1,000, 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 adding streptavidin-HRP 1:7,500 (v/v) in PBS and incubated at the same condition. After the washing step, a 1-step ultra TMB-ELISA substrate solution (50 µL) was administered and subsequently incubated for 20 min at ambient temperature (24 ˚C) without light. The reaction was halted by adding 0.1 M H_2SO_4 (50 µ). The absorbance value was measured at 450 nm.

4.2.7.2 Indirect ELISA

The indirect ELISA was done according to Liang *et al.* (2021), but with modifications. Firstly, a Maxisorp 96-well plate was subjected to an overnight

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coating process at 4 °C for 16 h. The coating was executed by including various treatments at a concentration of 10 µg/mL (casein samples) diluted with 1:500 and 1:1,000 (v/v) in PBS (pH 7.4). The casein samples were treated in two ways, an untreated control (raw) and a denatured sample, which was prepared using 0.1% sodium dodecyl sulphate (SDS) applied in combination with the following heating—either at 80 °C or 90 °C for 2 min in a water bath. Subsequently, the plate was washed three times with PBS. For blocking, 2.5% BSA solutions were used for 2 h at room temperature (24 °C) in an orbital shaker at 150 rpm. The plate was washed three times with PBST and incubated with rabbit anti-casein biotin conjugate in carbonate buffer (pH 9.6) for 1 h at room temperature. After three washes, 100 µL of goat anti-rabbit HRP conjugate (dilutions of 1:7,500, 1:10,000, or 1:15,000 (v/v) was introduced to the plate and incubated under identical conditions, followed by an additional wash. Subsequently, Streptavidin-HRP was introduced at dilutions of 1:7,500, 1:10,000, or 1:15,000 (v/v), and the plate was incubated for 2 h at ambient temperature (24 °C) on an orbital shaker set to 150 rpm. 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) without light. The reaction was halted by adding 0.1 M H_2SO_4 (50 µ). The absorbance value was measured at 450 nm.

4.2.8 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 to analyse variance (ANOVA). A

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post-hoc Tukey test was then conducted to compare the pairs of mean from treatment levels. Significant differences were calculated at α=0.05 (95% confidence interval).

4.3 Results and Discussion

4.3.1 Isolation of goat's milk casein

The isolation of defatted goat's milk casein obtained was 545.96 g/L, and the total soluble protein was 1.4 mg/mL using Bradford assay. Bradford assay is quick and straightforward, cost-effective, compatible with buffer compared to time-consuming and expensive reagents, and a complex protocol using Lowry and Bicinchoninic acid assay (Lu & Fu, 2020). The total nitrogen content of goat's milk was 0.68, and the total protein content was calculated to be 4.35 ± 1.36%. This is comparably reduced compared to the previous study by Mohsin *et al.* (2019), which reported a value of 4.83 ± 0.73%. There is a possibility that the variations in milk composition are attributable to factors such as breed, age, body weight, udder size, diet, lactation stage, season, dry period length, environment, and temperature (Park, 2010). Besides, a protein recovery was measured considering the mass, density, and volume. Given that the density of milk was 1.026 g/cm^3, thus with a total volume of milk used of 1 L (ca. 1000 cm^3), a recovery of the protein readout was calculated at 53.2% (g casein/g of crude protein). This value is slightly reduced than the 72% reported by Mansor *et al.* (2020), possibly linked to protein dispersion upon acid precipitation and variation in the experimental technique used, including differences in acidification conditions and separation methods. In this acid precipitation

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process, casein is primarily extracted, but lactose and whey proteins are left in the liquid phase trailing to lower the apparent total protein recovery.

Results from **Figure 4.1** showed protein bands of casein from goat's milk at 25-32 kDa. The estimation of molecular weight of casein subunits was similar to that reported by Wang *et al. (2013),* which states α_{s1}-=24.3 kDa, α_{s2}-=26.4 kDa, and β-=24.0 kDa. Detection of κ-casein by SDS-PAGE appeared to be challenging in this study. The high hydrophilicity and glycosylation of κ-casein may prevent its interaction with the SDS as well as its migration in the denaturing gel and thus reduce its detection (Lucey & Horne, 2018). Also, κ-casein forms macromolecular complexes with other fractions of casein, which are not easy to resolve during electrophoresis, making it challenging to detect using SDS-PAGE analysis (Jovanic *et al.,* 2007).

This SDS-PAGE was to verify the casein isolation was successful. Lane 1-2 represents skimmed milk from two different batches of goat's milk. Meanwhile, the substantial smear on the SDS gel is likely attributable to the elevated content of skim milk as no prior quantification of protein was made which may induce overloading and diffuse bands. Optimisation of sample concentration and gel loading is therefore necessary to obtain more representable bands in SDS PAGE.

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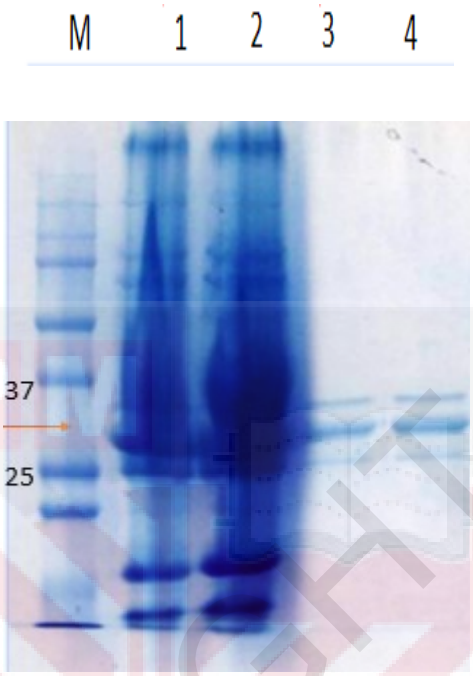

Figure 4.1: Separation of goat's milk protein based on size through SDS-PAGE M: Protein marker. Lanes 3-4: Casein bands at 25-32 kDa. Lane 1-2: Skimmed milk.

4.3.2 Sandwich ELISA

4.3.2.1 Background signal

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 4.0** shows the absorbance value of the background reading of HRP-conjugated streptavidin in a sandwich ELISA. The data shows comparable results ($p > 0.05$) between the streptavidin-HRP tested (1:7500, 1:10,000, and 1:15,000 v/v), as well as the plate reading.

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The absorbance values of streptavidin-HRP remained below 0.09, suggesting limited plate surface binding and background signal. This consistency confirms the stability of the system and establishes a <0.09 threshold for minimal background binding. Minimal non-specific-binding is vital in ELISA to provide a high signal. Therefore, 1:7,500 to 1:15,000 v/v is confirmed as the ideal range. Relative standard deviation (RSD) is shown in **Table 4.0** with RSD = (standard deviation/mean of sample) × 100. It is one of the most critical measures of precision, where values ≤10% are commonly accepted to ensure repeatability and robustness. Such a low RSD indicates that there is minimal range of variability in repeated measurements confirming the consistency of the data quality. Large RSD values would indicate variation due to sample preparation, or instrument sensitivity. RSD between readings indicates variations in the assay, and keeping it low enhances reproducibility and builds confidence in experimental results.

Besides, incorporating additional incubation and washing processes resulted 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, 2016). Due to the absence of any interaction between streptavidin-HRP and biotinylated molecules, the absorbance reading in the immunoassay is significantly reduced.

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Table 4.0: Background reading of streptavidin-HRP in phosphate-buffered saline (PBS) at different dilutions

Day	Streptavidin-HRP dilutions (v/v)			
	Plate background	1:7,500	1:10,000	1:15,000
1	0.052±0.004	0.089±0.009^{Aa}	0.093±0.007^{Aa}	0.078±0.006^{Ab}
2	0.056±0.0036	0.071±0.003^{Ba}	0.077±0.003^{Ba}	0.067±0.007^{Aa}
3	0.051±0.0023	0.073±0.006^{Ba}	0.067±0.002^{Ba}	0.060±0.002^{Aa}
4	0.052±0.0015	0.051±0.002^{Ca}	0.051±0.002^{Ca}	0.050±0.002^{Ba}
Average Relative	3.81	6.62	4.58	6.37

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. The absorbance of the well without goat's milk casein was detected at 450 nm. Relative standard deviation is obtained by (standard deviation /mean of data set × 100).

Following that, background reading of Cter-biotin IgG with streptavidin-HRP was evaluated at two different dilution ratios, (1:1,000; 1:2,500) (v/v), as shown in **Table 4.1.** The increase in absorbance after the addition of Cter-biotin IgG indicates that IgG plays a role in non-specific binding Güven *et al.* (2013). This phenomenon can be attributed to Cter-biotin IgG being a polyclonal antibody, which increases attachment within the streptavidin-biotin system and promotes immobilisation on the plate surface such that these findings are consistent with Lin *et al.* (2008) reported that streptavidin-horseradish peroxidase can bind four biotin molecules, whether conjugated to haptens, antigens or HRP, resulting in signal amplification in immunoassays. It is critical to optimise IgG

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concentration and blocking conditions to reduce non-specific interaction, and thus improves the specificity of the assay.

Table 4.1: 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
1:1,000	1:15,000	0.607 ± 0.075^{bA}	0.527 ± 0.050^{aA}	0.494 ± 0.081^{cB}
1:2,500	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.

Neto *et al.,* (2023) mention that 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 increased background signals. Nevertheless, this excessive binding can be minimised by increasing the frequency of washing and including blocking measures. In contrast, a study by Samarasinghe *et al*., (2017) observed a low signal due to biotin interference in sandwich ELISA. The antigen-antibody reactions have been extensively applied to immunoassays through the biotin-streptavidin system (Guo *et al.,* 2016; Cheah & Yamada, 2017; Yang *et al.,* 2021).

To further evaluate potential background interference, background readings of a complete sandwich ELISA in different solutions using Cter-biotin IgG at ratios of 1:2,500 (v/v) and 1:1,000 (v/v) were evaluated in **Table 4.2**. Results

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indicated that negligible background value was observed regardless of different dilution rations of Cter-biotin IgG. PBS was chosen for all dilutions of goat's milk casein and antibodies due to its lowest absorbance reading. Additionally, 2.5% BSA was identified as a suitable blocking reagent to reduce non-specific binding, while PBST can be used for regular washing steps. These choices were made since they improved specificities, with less background noise.

Table 4.2: 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 450nm
1:1,000	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}
1:2,500	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 1:1,000 (v/v) and streptavidin-HRP 1:15, 000 (v/v) The absorbance was detected at 450 nm.

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4.3.2.2 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 with 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 **Figure 3.2**, 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 h 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 study shows a lower signal-to-noise ratio of 2.29, as illustrated in **Figure 4.2**.

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This difference could be due to the variation in the type of casein used. The previous research utilised purified goat α_{s1}-casein, with antibodies targeting both terminals of α_{s1}-casein, resulting in a stronger binding affinity and, therefore, a higher signal-to-noise ratio. In contrast, our study did not use purified α_{s1}-casein, as this approach has already been explored in earlier studies and is limited by its high cost and low availability

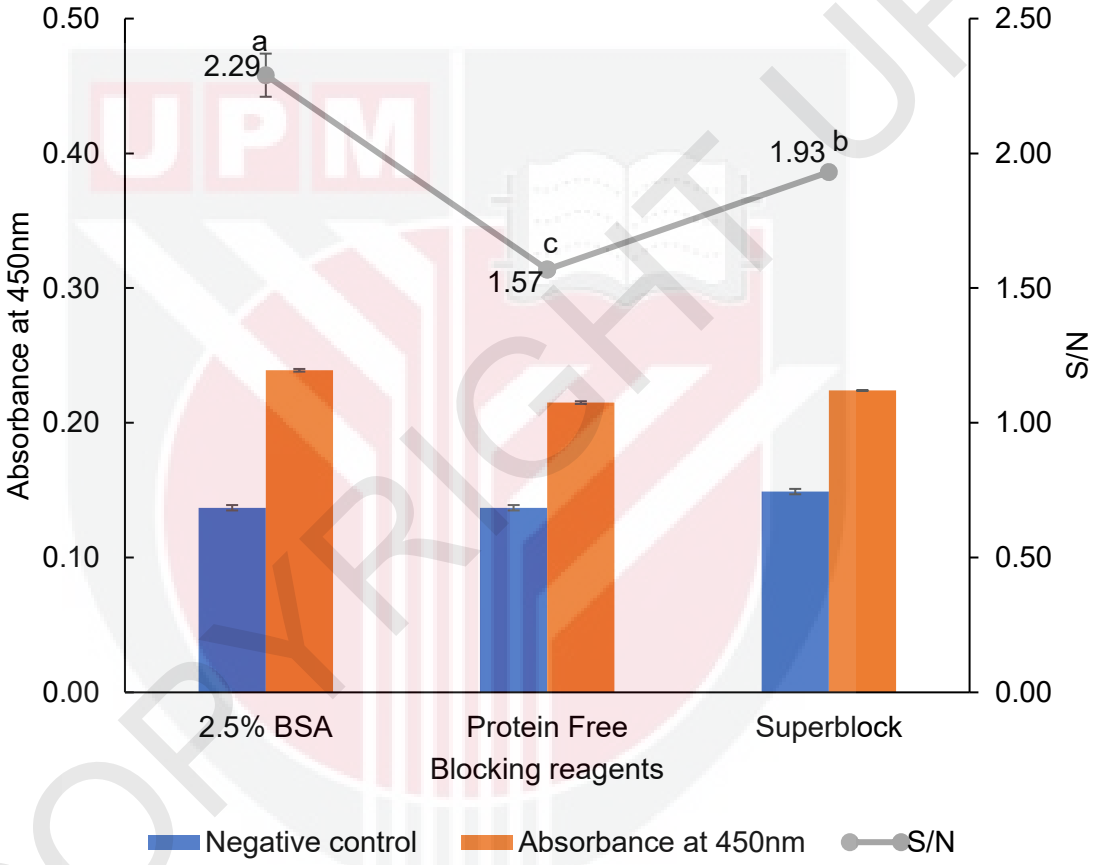

Figure 4.2: Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA using different types of blocking reagents.

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4.3.2.3 Antibody concentration and protein pre-treatment

A complete ELISA system was performed using Cter-biotin IgG (1:1,000; 1:2,500) (v/v) to detect goat's milk casein. Based on **Figure 4.3** 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 a 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 1:1,000 (v/v) of Nter-IgG, 1:1,000 (v/v) Cter- biotin IgG, and 1:15,000 (v/v) of streptavidin-HRP. However, a lower signal-to-noise ratio when employing a more dilute antibody concentration, 1:1,000 (v/v) Nter-IgG, 1:2,500 (v/v) Cter-biotin IgG, and 1:15,000 (v/v) streptavidin-HRP as shown in **Figure 4.4.**

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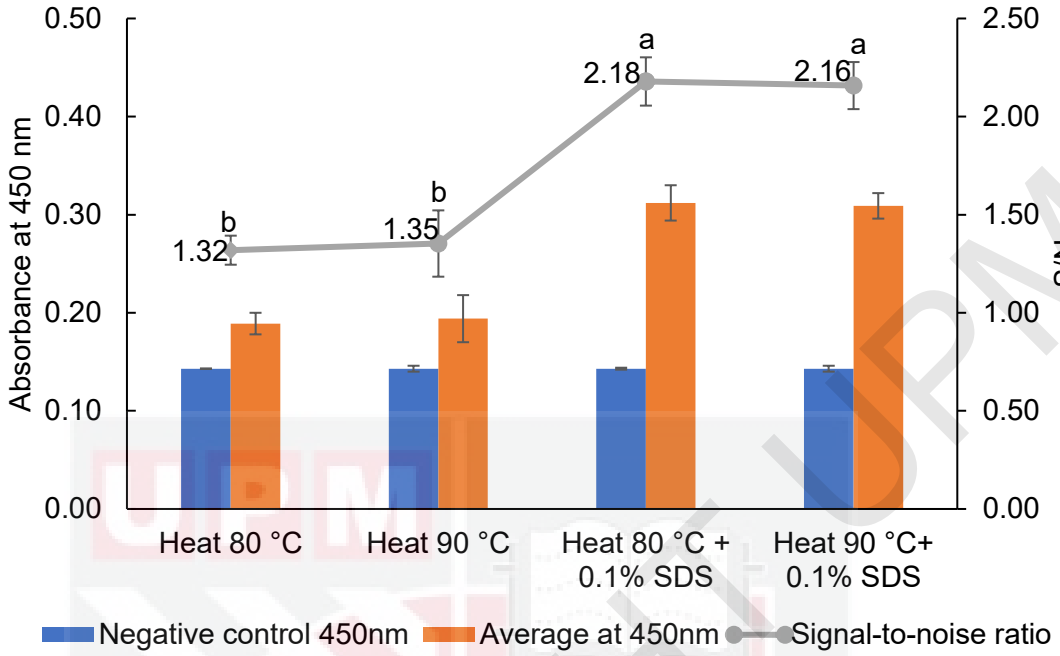

Figure 4.3: Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein using sandwich ELISA after heating and addition of denaturing agent 0.1% SDS. Dilution used in this experiment were 1:1,000 (v/v), 1:1,000 (v/v) Cter biotin-IgG, and 1:15,000 (v/v) streptavidin-HRP.

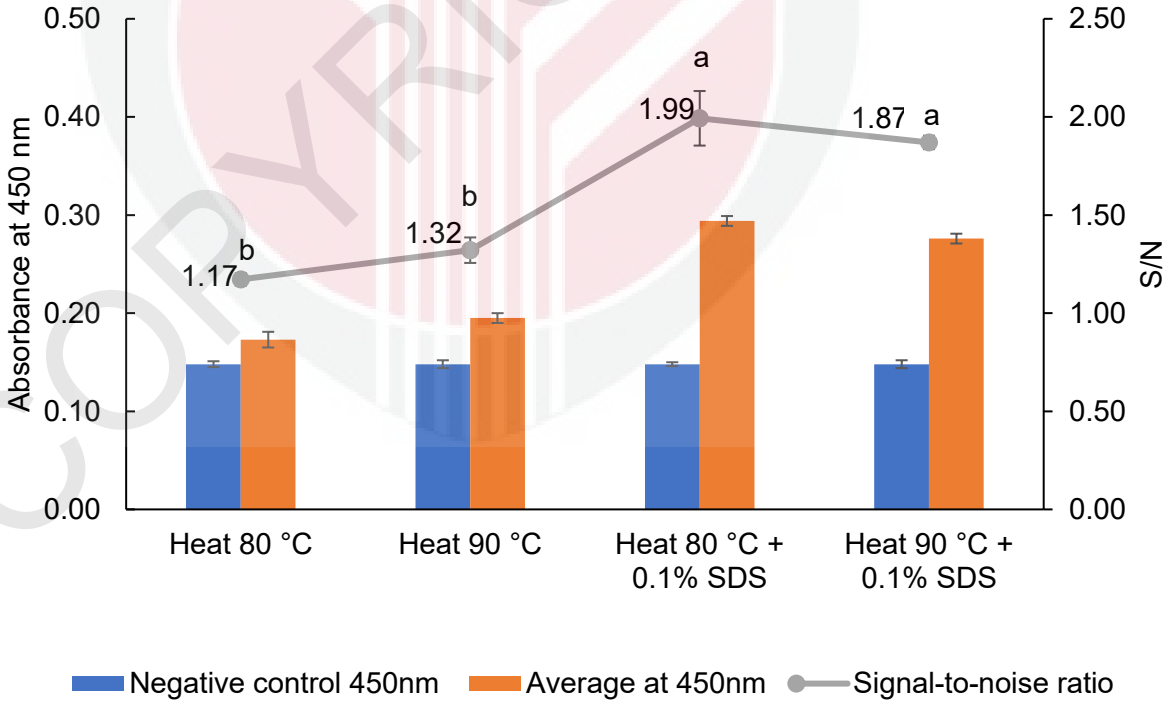

Figure 4.4: Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein in sandwich ELISA after heating and addition of 0.1% SDS. Dilution used in this experiment were 1:1,2500 (v/v) 1°Ab, 1:1,000 (v/v) 2°Ab, and 1:15,000 (v/v) streptavidin-HRP.

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A pre-treatment was performed on goat's milk casein in advance to improve the specificity of the antibodies. The antibodies used were specifically designed to detect goat's casein peptides, necessitating pre-treatment to ensure proper antigen exposure. Bovine-derived α-casein and phosphate-buffered saline (PBS, pH 7.4) were used as positive and negative controls, respectively, to confirm the specificity of the assay and to avoid false positive results. It is well-established that caseins undergo noteworthy structural changes in relation to heat treatment, with past research (Corredig *et al.,* 2019) demonstrating that temperatures over 60 °C have the potential to trigger protein unfolding, denaturation, and aggregate formation, ultimately resulting in a loss of its native structure. These structural changes mainly affect secondary and tertiary structures that are vital to antibody recognition (Pi *et al.,* 2023). Similar findings have been described by Sun *et al.* (2017) and Azdad *et al*. (2018); heating changes the secondary structure and conformational epitopes of casein, affecting its immunological properties. Hence, it was necessary to pre-treat goat's milk casein to ensure that antibodies only detected casein when it was in denatured form, enabling its detection in the native conformation.

The improved signal-to-noise ratio noted in the sandwich ELISA was probably due to interactions with α_{s1}-casein instead of aggregated casein. The enhancement was achieved using denaturant-heat treatment, which revealed concealed epitopes, hence improving antibody binding efficacy. In this study, whole casein was utilised instead of purified α_{s1}-casein in the presence of the

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antibody, which was originally designed to target a particular variation rather than the complete casein protein.

In addition, SDS treatment improved the ELISA signal as compared to heat treatment alone. Jelińska *et al.* (2017) have reported that SDS causes denaturation and aggregation of α_{s1}-casein, changing its structural integrity. Similarly, Winogradoff et al. (2020) and Yang *et al.* (2020) reported that SDS and heat treatment help in the unfolding of proteins by breaking the non-covalent interactions that maintain the conformation of proteins. This progress allows antibodies to more effectively recognise linear epitopes on ELISA assays.

Interestingly, though, the combined treatment with heat and the SDS denaturation gave a lower signal-to-noise ratio for total casein detection during this study. Nter-IgG and Cter-IgG recognise only goat α_{s1}-casein and not the complete casein fraction (Mohsin *et al.,* 2023), which could explain this reduction.

While the SDS treatment adequately exposes antigenic sites, the antigenicity was determined by the target of the antibody. Since it was impossible to obtain the described antibody specific to the goat's casein, we therefore explored alternative detection methods (direct and indirect ELISA) for casein detection. After searching worldwide, no commercial antibody specific to goat casein was found. Thus, we used a rabbit polyclonal antibody against bovine casein based on the 88% identical protein sequence between bovine and goat casein proteins (Prosser, 2021).

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Both direct and indirect ELISA methods were investigated to obtain the most reliable method for the detection of goat's casein and used for verification. Direct ELISA has a relatively simple detection format, which shortens assay time and decreases the risk of non-specific binding; however, indirect ELISA offers greater specificity due to the addition of a secondary antibody to amplify the signal. These results highlight the challenges in establishing a specific immunoassay for goat's casein and the need for different approaches to overcome the lack of antibodies. Further studies should also look into the development of custom antibodies or alternative detection/specificity methods, such as mass-spectrometry-based proteomics approaches.

4.3.3 Direct ELISA

Direct ELISA is a widely used immunoassay technique designed to detect and quantify specific antigens in a sample. In this method, the antigen is directly immobilised on a solid surface, such as a microplate, and then incubated with a primary antibody that is conjugated with a detectable label, often an enzyme like horseradish peroxidase (HRP). Following the incubation, a substrate is added that reacts with the enzyme to produce a measurable signal, typically a colorimetric change, indicating the presence and quantity of the antigen. This streamlined approach allows for straightforward and sensitive detection of antigens, making it an effective tool in various applications, including protein analysis and diagnostic assays.

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A rabbit polyclonal with biotin-conjugate at different dilutions 1:500 (v/v) **Figure 4.5**, 1:1,000 (v/v) **Figure 4.6** together with streptavidin-HRP was used in a direct ELISA system. Bovine-derived α-casein and phosphate-buffered saline (PBS, pH 7.4) were used as positive and negative controls, respectively, to confirm the specificity of the assay and to avoid false positive results. 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.

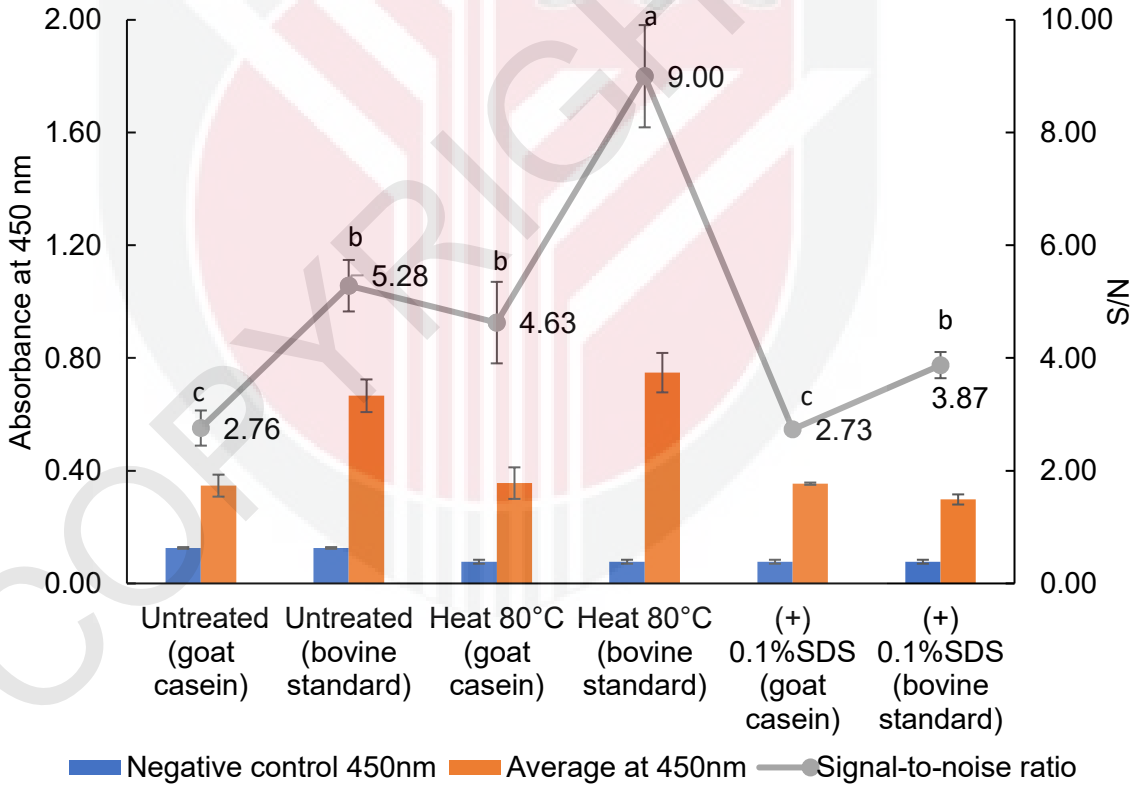

Figure 4.5: Absorbance and signal-to-noise ratio of goat's milk casein in direct ELISA using capture antibody and streptavidin-HRP, with 1:500 and 1:7,500 (v/v), respectively, after heating and addition of 0.1% SDS.

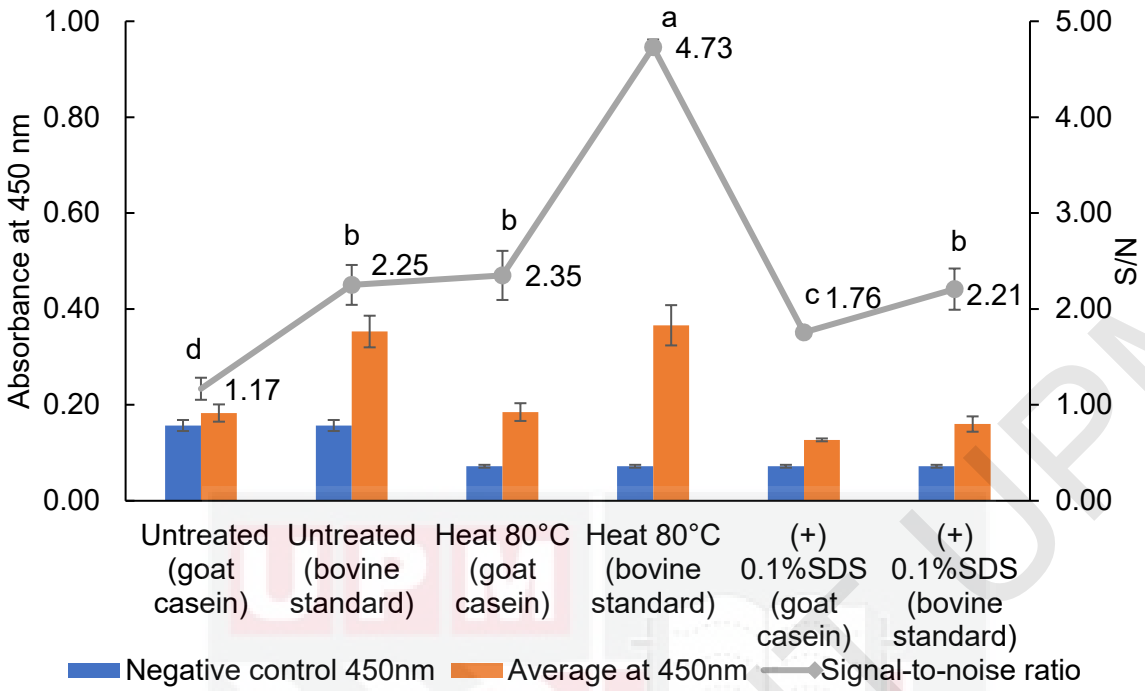

Figure 4.6: Signal-to-noise ratio and average absorbance of goat's milk casein in direct ELISA after heating and addition of 0.1% SDS, using a 1:1,000 (v/v) capture antibody and a 1:7,500 dilution of streptavidin-HRP.

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 **Figures 4.3** and **4.4** which showed a significant increase in the S/N ratio following both treatments. Heat treatment and SDS treatment both denature proteins, although they differ in their methods, influencing antibody interactions in ELISA. Heat treatment causes thermal unfolding, revealing concealed epitopes by destroying non-covalent interactions, but preserving certain protein structures and antigenicity. This facilitates enhanced antibody binding.

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SDS treatment chemically denatures proteins by binding to hydrophobic areas, fully unfolding and obscuring epitopes. This frequently diminishes antibody specificity. According to Anema *et al.,* (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 research, which suggested that the inclusion of SDS causes the separation of casein micelles and milk gel within a specific range of SDS concentrations (Nakano, 2017). 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.

4.3.4 Indirect ELISA

In this ELISA system, the results presented in **Figure 4.7** demonstrate that the indirect ELISA had the highest signal-to-noise ratio for goat casein (9.80) in the absence of any heat treatment as compared to **Figure 4.8.** 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 1:7,500 (v/v).

The observed effect may be attributed to the elevated specificity, sensitivity, and binding affinity levels. Indirect ELISA has been employed to detect

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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.

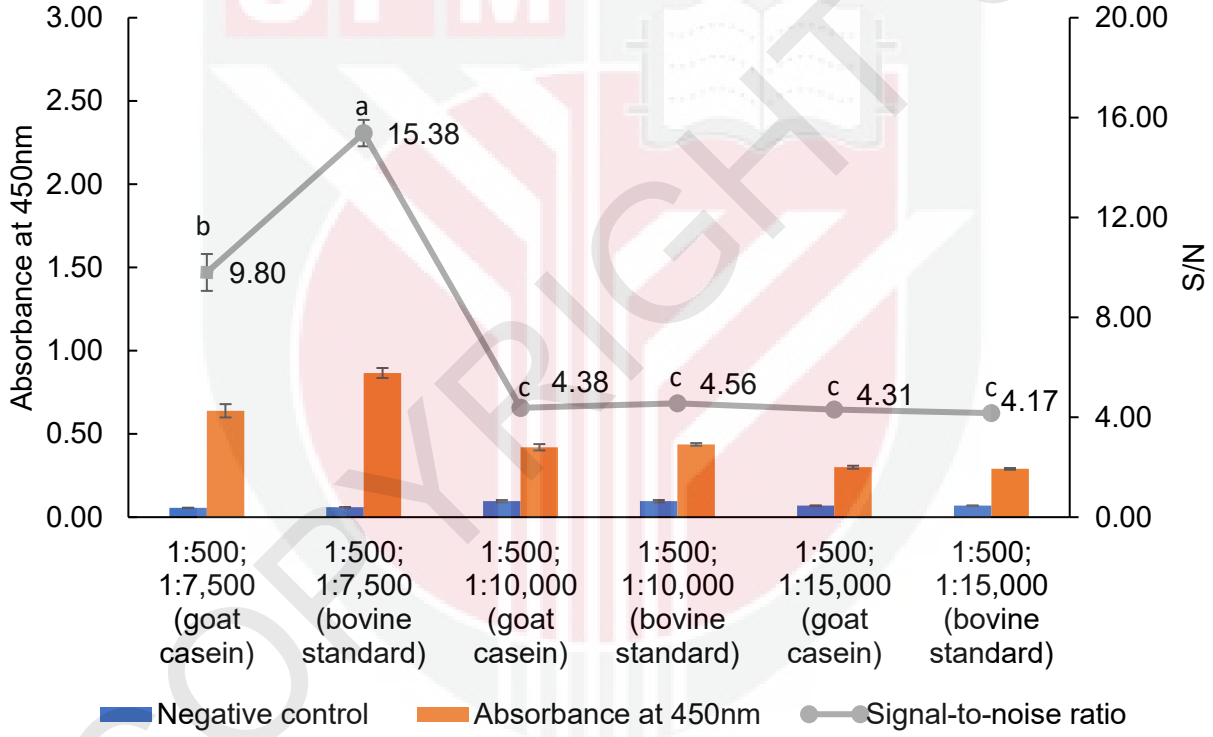

Figure 4.7: Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein and bovine standard using different concentrations of primary and secondary antibody in indirect ELISA.

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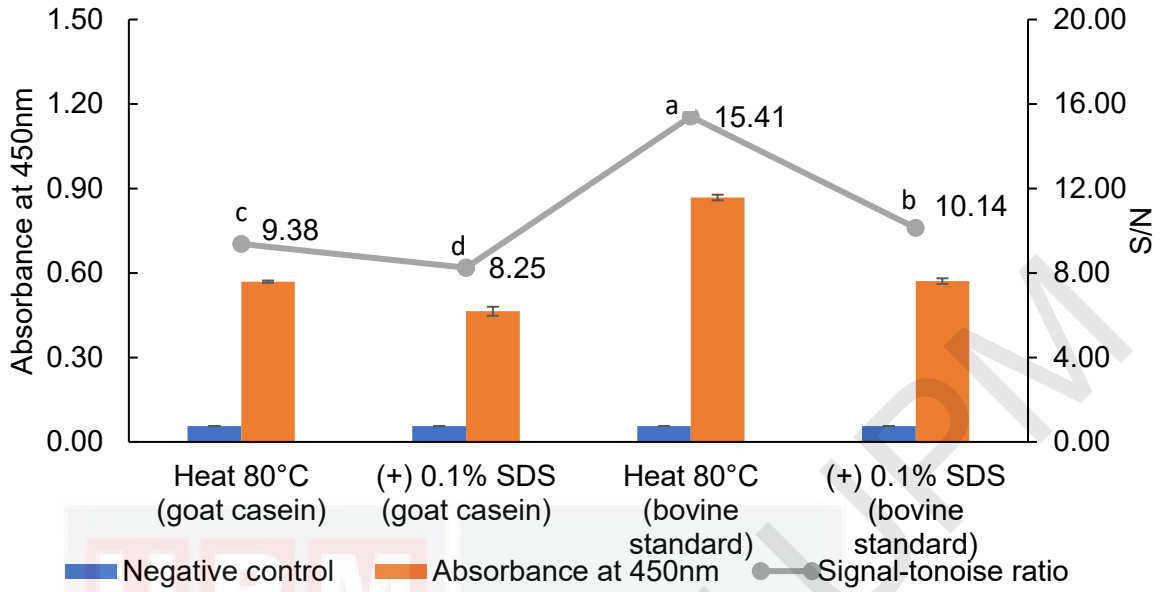

Figure 4.8: Signal-to-noise ratio and average absorbance at 450 nm of goat's milk casein and bovine standard in indirect ELISA after heating and addition of 0.1% SDS, using 1:500 (v/v) primary antibody and 1:7,500 (v/v) of secondary antibody.

The choice of ELISA format significantly influences the sensitivity and efficiency of casein detection. In this study, direct, indirect, and sandwich ELISA methods were evaluated to determine which provided the highest signal to detect total casein instead of casein subtypes. Sandwich ELISA offered the highest specificity, particularly for α_{s1}-casein, though it was less suitable for whole casein detection due to antibody specificity. Direct ELISA detected total casein but exhibited lower sensitivity due to single antibody binding. Indirect ELISA, on the other hand, demonstrated improved sensitivity through secondary antibody amplification. Since the objective was to identify the method yielding the strongest detection signal for total goat's milk casein, sensitivity variations across formats were expected. One notable limitation the high consumption of antibodies and buffers and multiple washing steps contributed to resource inefficiency. Selecting an ELISA format that balances sensitivity and efficiency is crucial in ensuring accurate casein detection. In

this study, indirect ELISA appeared to be the most efficient for detecting total casein with high sensitivity and specificity.

4.4 Conclusion

The present work successfully depicted the application of ELISA for detecting goat's milk casein with different levels of sensitivity and specificity based on assay type. Direct and indirect ELISA was able to recognise total casein (αs-, β- and κ-caseins were all recognised), and sandwich ELISA was used to detect α_{s1}-casein, demonstrating the significance of antibody specificity in the development of immunoassay. With 1 mg/mL pure casein as the optimal background system, the use of Nter-IgG, Cter-IgG-biotin, and streptavidin-HRP, sandwich ELISA system provides high signal-to-noise ratio due to the specificity of detection, heat and, additionally, SDS treatment. Indirect ELISA exhibited superior signal-to-noise performance without any heat treatment compared to direct and sandwich ELISA, even at reduced concentration of goat's casein, (10 µg/mL), indicating its potential as a more efficient detection method under certain conditions. Overall, the findings show that ELISA is highly specific to goat's milk casein, making it a valuable tool for food surveillance, dairy product authentication and allergen detection. These findings enhance the quality control mechanisms in the dairy sectors by enabling the accurate identification of milk proteins in processed products. Further developments in assay sensitivity and specificity through custom antibodies and other detection strategies are worth exploring in future studies.

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CHAPTER 5

ANTIGENICITY OF GOAT'S MILK CASEIN FROM GI DIGESTIVE AND NON-DIGESTIVE ENZYME HYDROLYSATE AND PEPTIDE SIZE

5.1 Introduction

Casein is a major protein in cow's milk and is known for its potential to induce allergic reactions in sensitive individuals. The allergenic potential of casein is largely attributed to its structure, which contains specific epitopes that trigger immune responses in those with cow's milk allergy (CMA) (Lerner *et al*., 2016). To reduce allergenicity, casein is enzymatically hydrolysed into smaller peptides and amino acids. The degree of hydrolysis critically influences the allergenicity of the resulting peptides. Partially hydrolysed formulas (pHF) contain peptides with molecular weights ranging from 3 to 10 kDa, which, while smaller than intact casein, may still retain allergenic epitopes that can trigger immune responses in sensitive individuals (Bode, 2009). This moderate hydrolysis provides some reduction in allergenicity but may not be sufficient for severe CMA cases. In contrast, extensively hydrolysed formulas (eHF) achieve a more thorough breakdown of casein, resulting in peptides typically smaller than 1.5 kDa and free amino acids (Jelinska & Kaczmarek, 2017). This extensive hydrolysis ensures the removal of most allergenic epitopes, significantly reducing allergenicity and making eHF a preferred option for managing severe CMA (Corredig & Delgleish, 2019).

The reduced size of peptides in eHF, combined with the absence of larger allergenic fragments, effectively minimizes the risk of allergic reactions. By

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understanding and manipulating peptide size through enzymatic hydrolysis, researchers can better address the varying needs of individuals with milk protein sensitivity, providing safer and more effective dietary solutions.

As for the management of cow's milk protein allergy, the most effective way is to avoid consuming any milk derived from cows. In addition, a hydrolysed cow's milk protein infant formula was introduced with reduced antigenicity and can serve as a replacement for breastfed milk. Nevertheless, there is a scarcity of research on hydrolysed goat's milk, thus this study aims to evaluate the decrease in antigenicity between hydrolysed and non-hydrolysed casein using different enzymes with optimal hydrolysis conditions through optimised ELISA. This study provides a theoretical foundation for the development of a hypoallergenic goat's milk formula.

5.2 Materials and Methods

5.2.1 Chemicals, reagents, and instruments

The proteolytic enzyme utilised was bromelain (1:100, pH 5.5, 50 °C, 6 h), alcalase (1:50, pH 8.0, 55 °C, 6 h), trypsin (1:10, pH 8.0, 37 °C, 6 h) and pepsin (1:10, pH 1.5, 37 °C, 6 h). The proteolytic enzyme alcalase, was acquired from Novozymes (from *Bacillus licheniformis,* 2.4 U/mg Bagvaerd, Hovedstade, Denmark). Trypsin (from bovine pancreas, 225 U/mg) was procured from Fisher Scientific (Fair Lawn, New Jersey, USA). Pepsin (from porcine gastric mucosa, 0.5 U/mg) and bromelain (from pineapple stem, 2.4-3.0 U/mg) were purchased from Acros Organic (Geel, Antwerp, Belgium).

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Sodium phosphate dibasic heptahydrate (Merck KGaA, Burlington, MA), sodium phosphate, sodium phosphate monobasic monohydrate, Tris-base, potassium chloride, sodium acetate and acetic acid were purchased from R&M (Semenyih, Selangor). Protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin was obtained from Thermo Fisher Scientific (Waltham, MA).

The chemicals employed for SDS-PAGE are detailed in section 4.2.5, and the antibodies used are outlined earlier in section 4.2.7.2 regarding Indirect ELISA.

5.2.2 Bradford assay

Bradford protein assay (microplate technique) was performed following the methodology provided by Bio-Rad Laboratories to measure the amount of total soluble protein in the milk sample, hydrolysed and unhydrolysed casein. To achieve precise findings, a standard curve was constructed by plotting the absorbance values of bovine serum albumin standards (2 mg/mL) against their known concentrations. Sample concentrations are determined by interpolation. If the sample absorbance exceeds this range, the samples are diluted and re-evaluated.

The absorbance of the blank was deducted from all measurements to compensate for background noise. Samples were prepared at 1:5, 1:10, 1:30 and 1:50 dilutions in phosphate buffer saline. Each standard sample solution was pipetted to 10 µL into individual wells. Bradford reagent of 200 µL was added into all wells containing standard and sample. Conducting measurements in triplicate guarantees precision. Absorbance values were

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measured at 595 nm. Total soluble proteins of samples were quantified using linear regression based on a calibration curve generated with BSA.

5.2.3 Indirect ELISA

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 hydrolysed and unhydrolysed casein at 1000 µg/mL in 1X PBS. Then, the plate underwent three washes using PBS. The plate was then 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 (250 µL) thrice and was thereafter incubated with anti-casein rabbit polyclonal antibody (100 µL; diluted at 1:500 (v/v) in PBS, pH 9.6 for 1 h at ambient temperature (24 ˚C). The plate underwent three additional washes before adding goat anti-rabbit HRP at 1:5,000 (v/v) in PBS and incubated at the same condition. After the washing step, a 1-Step ultra TMB-ELISA substrate solution (50 µL) was administered and subsequently incubated for 20 min at ambient temperature (24 ˚C) without light. The reaction was halted by incorporating 50 µL of 0.1 M H_2SO_4. The absorbance value was measured at 450 nm. The calculation for signal-to-noise ratio and antigenicity reduction were as follows:

- S/N=Mean Signal (Sample)/ Mean Background (Blank)

- Antigenicity reduction (%) = (Absorbance of unhydrolysed-hydrolysed)/ Absorbance of unhydrolysed) × 100

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5.2.4 Fractionation of goat's milk casein hydrolysate using viva spin column

The fractionation of casein hydrolysate from goat's milk, which was enzymatically hydrolysed using bromelain, alcalase, pepsin, and trypsin, was conducted utilising Sartorius VivaSpin columns with molecular weight cut-offs of 2 kDa, 5 kDa, and 10 kDa (ThermoFisher Scientific, Waltham, MA, USA). The hydrolysate samples were loaded into the columns and centrifuged at 8,000 *x g* for 10 min at 4 °C. This process allowed for the separation of the hydrolysate into distinct fractions based on molecular weight, with the larger molecules being retained in the respective columns and the smaller peptides passing through. The resulting fractions were collected and stored at -20 °C.

5.2.5 Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE)

The lyophilised casein was analysed using SDS-PAGE as described by Laemmli (1970). Resolving gel (15%) was prepared by mixing before stacking gel (5%). Sample buffer was prepared by a mixture of 3.55 mL deionized 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. A total volume of 20 µL sample (hydrolsate collected from optimal enzymes conditions) and sample buffer were heated at 90-100 ˚C for 5 min before loaded on the gel (10 uL) while the protein marker was added at a volume of 5 µL. The electrophoresis was connected to the power supply with 200 V running conditions for a duration of 40 min. The image of the gel was stained using 0.05% (w/v) Coomassie Brilliant Blue G 250, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid overnight. Then, the gel was destained with a destaining solution until the blue background disappeared for

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the band to be seen clearly. The gel was then rinsed with distilled water and stored in the storage solution. The casein bands were identified by referring to Precision Plus Protein™ dual colour standards (Bio-rad Laboratories Inc, Hercules, CA, USA).

5.2.6 Statistical analysis

All data was measured in triplicates (n=3). Absorbance data were analysed using mean and standard deviation. GraphPad Prism (San Diego, California) statistical software version 8.0.2 was applied for two-way analysis of variance (ANOVA). Post-hoc Tukey test was then made for evaluation between pairs of mean from treatment levels. Significant differences were calculated at a=0.05 (95% confidence interval).

5.3 Results and Discussion

5.3.1 Protein concentration

The buffer system and pH highly influence the solubility of casein and other proteins, which can significantly affect protein quantification results in both unhydrolysed and hydrolysed samples. Casein is an amphiphilic phosphoprotein whose solubility relies on buffer composition and ionic strength (Mehringer *et al.,* 2021). Additional buffers, such as sodium phosphate (for alcalase hydrolysis) and Tris-HCl (for trypsin hydrolysis), are crucial for regulating protein solubilisation (El-Shibiny & Shanab, 2015). These varying circumstances influence protein recovery and result in discrepancies in protein concentrations that must be meticulously considered when assessing

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hydrolysis outcomes.

PH levels significantly influence the solubility of casein. At pH values significantly far from its isoelectric point (pI = 4.6), casein demonstrates increased solubility owing to intensified electrostatic repulsion among molecules. At or close to its isoelectric point, casein molecules possess a negligible net charge, resulting in less electrostatic repulsion, enhanced aggregation, and eventual precipitation (Huppertz, 2016). Such a notion is crucial in hydrolysate processing, as pH variations affect solubility and can reduce undesirable precipitation.

In hydrolysed samples containing smaller peptides and free amino acids, quantifying protein concentration becomes more intricate. The sensitivity, speed, and compatibility with peptide-rich materials prompted the selection of the Bradford test. Coomassie Brilliant Blue dye binds to aromatic and basic residues, making it optimal for identifying casein fragments produced by this enzymatic process. It provides a less variable and more accurate assessment of soluble protein concentration compared to the Lowry test, which may be influenced by free amino acids and non-protein nitrogen (Sengupta & Chattopadhyay, 1993). Nonetheless, it may exhibit reduced sensitivity for short peptides, especially for diminutive peptides and those lacking foldable aromatic residues, leading to underestimating the overall protein content in severely hydrolysed materials. The 0h represent unhydrolysed casein samples (refer to **Table 5.0**) providing a benchmark for evaluating the hydrolysate produced during enzymatic hydrolysis. The conditions used for each enzyme

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were based on the optimised enzyme-to-substrate (E:S) ratio, pH, temperature, and time conditions established in Chapter 4.

Table 5.0: Total soluble protein of unhydrolysed and hydrolysed casein

Enzyme concentration, buffer, pH, temperature, time	Total protein concentration 0 h, unhydrolysed (µg/mL)	Hydrolysed casein (µg/mL)
Bromelain (2.4 U/mg) 0.05 M sodium acetate 1:100, pH 5.5, T=50 °C, time= 6 h	7.15 ± 0.05^{Ad}	3.39± 0.01^{Ba}
Alcalase (2.4 U/µg), 0.05 M sodium phosphate 1:50, pH 8.0, T=55 °C time= 6 h	166.65± 0.05^{Ab}	2.13± 0.02^{Bc}
Trypsin (225 U/mg), 0.05 M Tris-HCL 1:10, pH 9.0, T=37 °C time= 6 h	192.75± 0.05^{Aa}	1.00± 0.00^{Bd}
Pepsin (0.5 U/mg), 0.05 M KCl-HCl 1:10, pH 1.5, T=37 °C time=6 h	12.89± 0.04^{Ac}	2.60± 0.02^{Bb}

Values are represented by mean ± SD values of three absorbance readings (*n*=3). with different lowercase superscripts with similar columns and uppercase superscripts with similar rows, are significantly different ($p < 0.05$); the absorbance of the well of goat's milk casein hydrolysate was detected at 450 nm.

The increased results presumably signify the improved solubility of casein in certain buffers, as previously demonstrated. As hydrolysis advances, the breakdown of each casein affects its structural properties, modifying solubility and reactivity with the Bradford reagent. Since the assay primarily detects intact proteins with sufficient aromatic and basic residues, the increasing presence of short peptides with fewer dye-binding sites results in lower measured protein concentrations (Yao, 2021). Short peptides lack the

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structural components required for robust dye binding, possibly resulting in reduced quantification results (Song, 2019).

The differences in protein quantification between unhydrolysed and hydrolysed casein can be attributed to peptides demonstrating solubility following enzymatic cleavage. Certain hydrolysed peptides demonstrate solubility while remaining undetectable by the Bradford assay. Larger hydrolysed fragments may aggregate or precipitate, resulting in considerable material losses and underestimating protein concentration. The Bradford assay, a widely used reference method for quantifying total protein content in solution, may be insufficient for analysing hydrolysed protein samples due to its limited sensitivity to low peptide concentrations. The decrease in protein concentration in hydrolysed casein results from structural modifications and the assay's preference for intact proteins. Complementary analytical methods, such as spectrophotometry, chromatography, and mass spectrometry, are essential for accurately analysing hydrolysed protein fractions.

5.3.2 Antigenicity reduction using various enzyme

Enzymatic hydrolysis is a significant technique for reducing the antigenicity of milk proteins by cleaving them into smaller peptide fragments, thereby diminishing their potential to trigger immune responses. This study employed bromelain and assessed its efficacy in diminishing antigenicity compared to alcalase, trypsin, and pepsin in their ideal circumstances. The ideal circumstances for bromelain, as delineated in Chapter 4, served as a benchmark for comparison with the other enzymes. ELISA was employed to

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assess the efficacy of antigenicity reduction. **Figure 5.0** indicates that bromelain exhibited the highest reduction in antigenicity at 33%, followed by alcalase, trypsin, and pepsin.

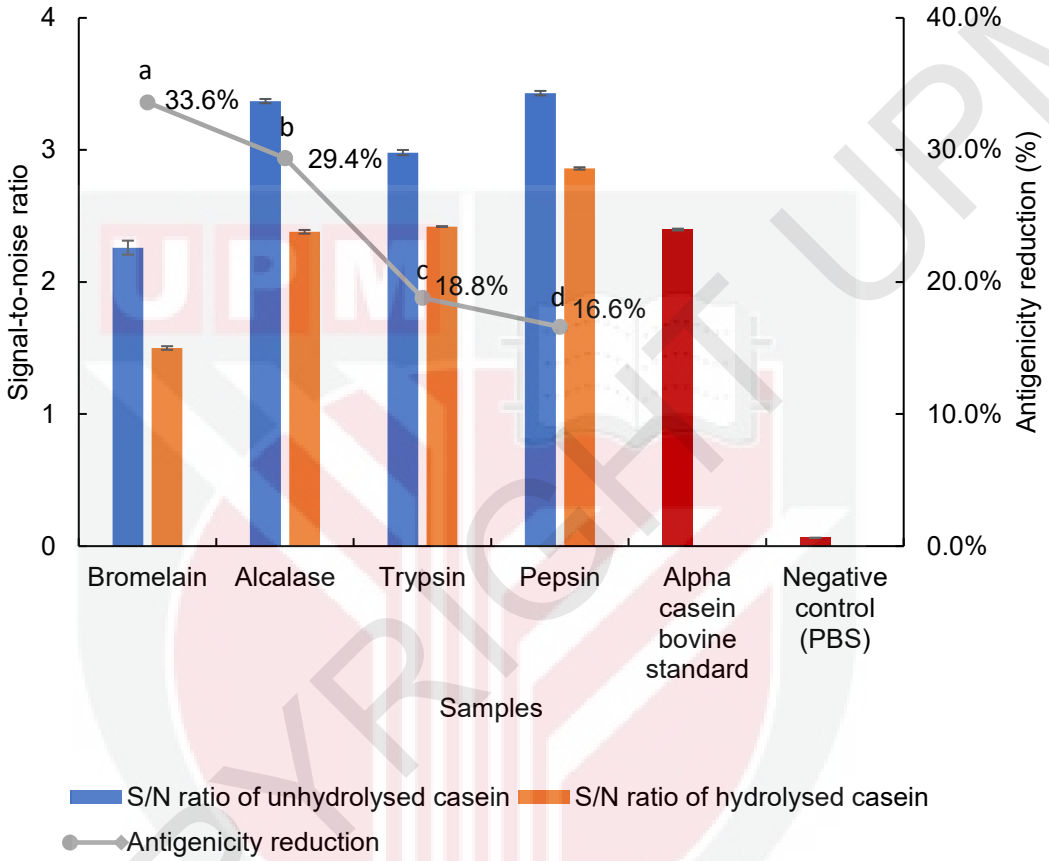

Figure 5.0: Antigenicity reduction by various enzymes. Values are represented by mean ± SD values of three absorbance readings (*n*=3). with different lowercase superscripts, are significantly different (p < 0.05), The absorbance of the well of goat's milk casein hydrolysate was detected at 450 nm.

Bromelain cleaves specific epitopes, sites that can trigger an immune response, and produces peptides with modified functional properties due to the cleavage of IgE-binding epitopes, which reduces immunological recognition (Villas-Boas *et al.,* 2015). Alternative enzymes may also hydrolyse peptide bonds; however, they do not specifically target many epitope sites, leading to a diminished decrease in antigenicity reduction. Alpha-casein

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served as both the standard and positive control to assess the binding efficiency of the antigen to the antibody and to eliminate false-positive results. PBS, with pH 7.4, functioned as a negative control to eliminate the possibility of cross-contamination.

The relationship between the signal-to-noise (S/N) ratio and antigenicity reduction is significant. A high signal-to-noise ratio signifies strong antigen-antibody interaction, whereas a low ratio suggests epitope disruption and diminished antigenicity. Hydrolysis disrupts the allergenic sequence, diminishing the ELISA signal and validating decreased antigenicity. This association demonstrates the efficacy of enzymes that hydrolyse peptides to evade detection by the immune system (Yu *et al.,* 2021).

5.3.3 Effect of peptide size on antigenicity

In assessing the impact of peptide size on antigenicity, it is crucial to determine the molecular weight of peptides generated during hydrolysis. Smaller peptide sizes are often associated with reduced antigenicity, as they are less likely to trigger immune responses. This study examined the size of hydrolysed peptides, utilising techniques such as SDS-PAGE and specific molecular cut-off filtration to effectively separate and quantify peptides, further exploring their relevance in reducing allergenic potential. The peptide size was <10 kDa on the normal SDS-PAGE as shown in **Figure 5.1.** To further separate the proteins based on their size, viva spin columns with specific sizes were used. The results showed the size of peptides with 10 kDa. This aligns with a study by Milessi *et al.,* (2022) who observed all the peptide sizes of cow's milk protein

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were lower than 12 kDa after hydrolysis. This suggests that the enzymatic hydrolysis was successfully done to reduce its size. Additionally, an antigenicity test was further tested to determine the effect of peptide size on antigenicity.

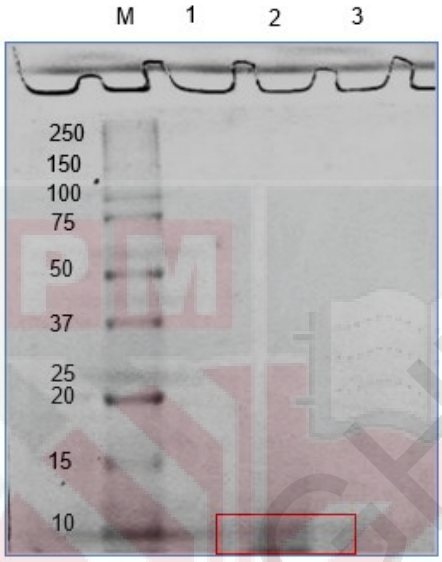

Figure 5.1: Protein separation of goat's milk casein hydrolysate using bromelain on SDS PAGE at Lane 2.

Antigenicity reduction is commonly assessed using normalised absorbance values in ELISA. Since **Figure 5.2** presents normalised absorbance, it inherently reflects antigenicity reduction, even if not explicitly mentioned. The absorbance values correlate with antigen-antibody interactions, meaning lower absorbance signifies reduced antigenicity. Based on the results of **Figure 5.2**, the peptide sequence with fewer amino acids (<2 kDa) had the lowest signal-to-noise ratio, indicating the highest antigenicity reduction. Meanwhile, the highest signal-to-noise ratio occurs in peptides with size >10 kDa. This aligns with a study by Hochwallner *et al. (2014),* who stated that activity of the immune system is reported to occur for proteins with greater than

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14 kDa. Certain peptide sequences exhibit a reduced likelihood of eliciting immune responses. Peptides shorter than 10 amino acids are typically insufficient in size to function as IgE epitopes (Lee *et al.,* 2016).

The findings have significant implications for minimising allergenicity in food products. The research indicates substantial efforts employing various enzymes under diverse conditions, highlighting the complexity of antigenicity reduction, providing deeper analysis. Enhanced execution, a comprehensive approach, and thorough analysis are essential for improving reproducibility and understanding. Future research should focus on peptide sequence, characterisations of peptides, and identifying allergenic epitopes and examining advanced methods to effectively reduce allergenicity.

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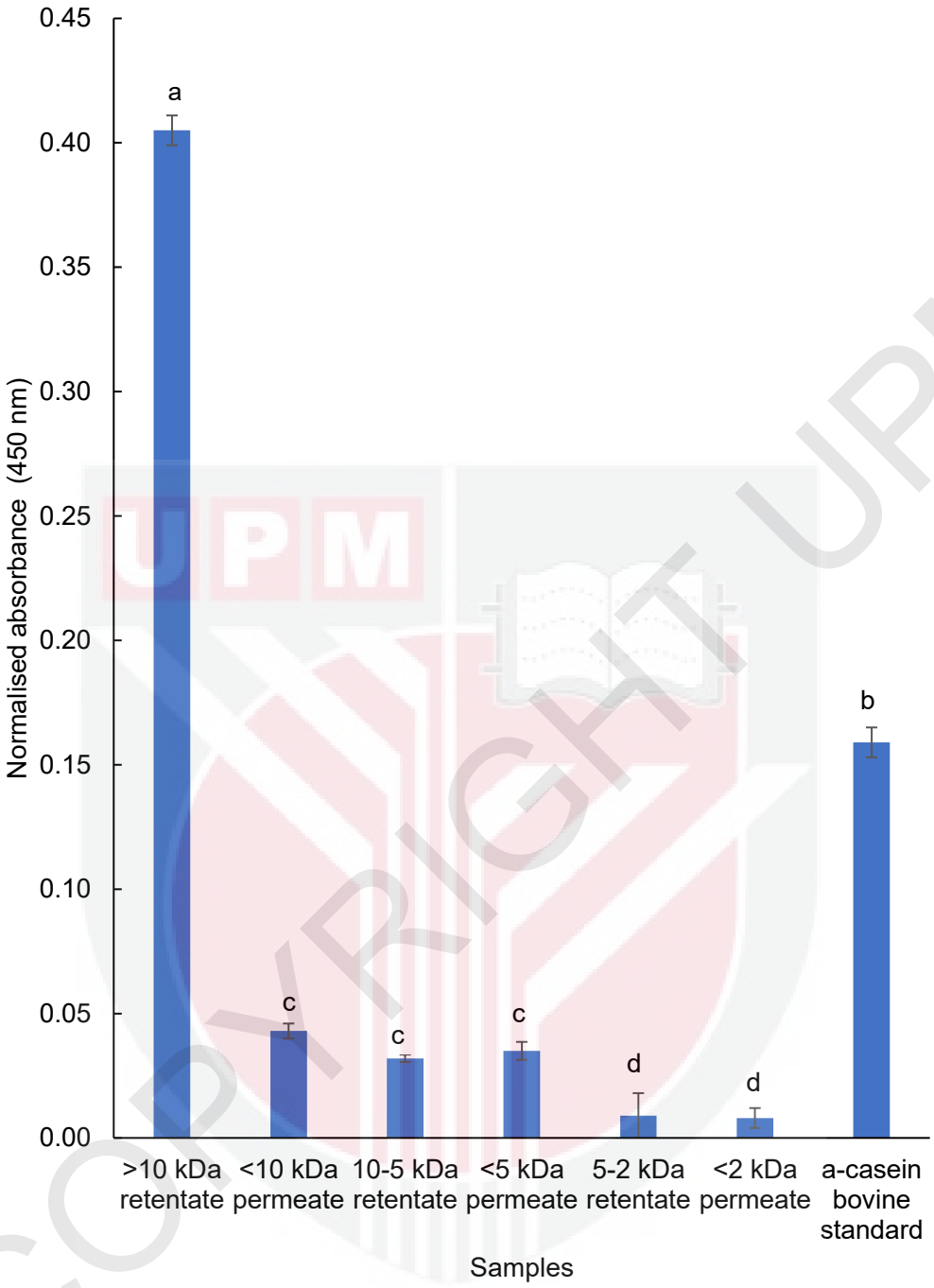

Figure 5.2: Effect of peptide size on the antigenicity of hydrolysed goat's milk casein after hydrolysis at 1:100 enzyme-to-substrate ratio, 50 °C, pH 5.5 at 6 h. Values are represented by mean ± SD values of three absorbance readings (*n*=3). Values with different lowercase superscripts are significantly different ($p < 0.05$), The absorbance of the well of goat's milk casein hydrolysate was detected at 450 nm.

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Bromelain-catalysed hydrolysis and further fractionation based on size-significantly reduced the allergenic potential in goat's milk casein. The peptides with molecular weights below 2 kDa exhibited maximum reduction in antigenicity, emphasising the role of optimum control over hydrolysis parameters for achieving maximum hypoallergenicity. Among the proteases used, bromelain resulted in the most pronounced effect and showed that the smaller the size of peptides, the lesser the allergenicity. These results suggests that bromelain is particularly effective at in the hydrolysis of casein into peptides, which are less likely to induce any immune response, making it a valuable tool in developing dairy products that are both hypoallergenic and nutritionally beneficial for individuals with milk protein sensitivities.

Antigenicity reduction is influenced not only by peptide size but also by specific peptide sequences, hydrophobicity, and sequence motifs. Amino acid residues can modify IgE binding, and hydrophobicity influences peptide solubility and epitope accessibility. Changes in sequence motifs may also impair antibody recognition. This study evaluated antigenicity through ELISA, emphasising antigen-antibody interactions. The study did not directly analyse factors such as hydrophobicity and sequence motifs, necessitating additional analytical techniques beyond its scope.

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5.4 Conclusion

This study successfully employed enzymatic hydrolysis using various enzymes to evaluate the conditions for hydrolysing goat's milk proteins and utilised ELISA to assess the decrease in the antigenicity of the hydrolysed protein preparations. In conclusion, the findings indicated that bromelain achieved the highest reduction in antigenicity at 33%, followed by alcalase, trypsin, and pepsin. The research addressed the issue statement with an optimised hydrolysis technique to diminish the biological product's antigenicity efficiently. Nonetheless, while ELISA measured the reduction in antigenicity, it could not identify particular differences in peptide sequences, variances in hydrophobicity, or changes in epitope conformations. Consequently, more research is necessary to enhance the comprehensive understanding of the structural and immunological properties of the hydrolysates. Nevertheless, the study offers significant insights into the feasibility of producing hypoallergenic dairy products, particularly in enhancing enzymatic hydrolysis procedures. These findings establish a foundation for improving food safety and allergy control in goat's milk products.

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CHAPTER 6

SUMMARY, CONCLUSION, FUTURE RECOMMENDATION

6.1 Summary

In summary, this study aimed to evaluate hydrolysis conditions to reduce the antigenicity of goat's milk. It comprised of three primary objectives: The first objective, presented in Chapter 3, sought to evaluate the hydrolysis conditions for goat's milk casein using gastrointestinal and non-gastrointestinal enzymes. Key parameters such as enzyme-to-substrate ratio, pH, time, and temperature were fine-tuned to establish optimal conditions for producing casein hydrolysate. The second objective, detailed in Chapter 4, focused on evaluating and optimising various ELISA approaches for detecting goat's milk casein. This involved optimising several parameters, including different ELISA formats, pre-treatment methods, blocking reagents, and antibody concentrations. Finally, the third objective, outlined in Chapter 5, involved assessing the antigenicity of goat's milk casein hydrolysate based on the use of different enzymes and varying peptide sizes. This structured approach ensured a comprehensive investigation into the antigenicity reduction of goat's milk through enzyme hydrolysis. The results demonstrate that bromelain is effective throughout the 6 h hydrolysis duration, positioning it as a viable option for regulated protein hydrolysis. Key performance indicators, such as degree of hydrolysis, peptide yield, and antigenicity reduction, validate bromelain as an efficient enzyme for generating hydrolysates with lower allergenic properties.

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6.2 Conclusion

This research effectively optimised the conditions for enzymatic hydrolysis and utilised ELISA to evaluate the decrease of antigenicity in hydrolysed goat milk proteins. The findings indicated that bromelain produced the most significant reduction in antigenicity (33%), succeeded by alcalase, trypsin, and pepsin. The findings fulfil the research purpose by emphasising the capability of enzymatic hydrolysis to diminish milk protein antigenicity while maintaining peptide integrity. The study also examines the underexplored casein of goat's milk, which has garnered less attention than cow's milk. Although goat's milk cannot serve as a direct substitute for cow's milk for those with allergies because of antigenic proteins, hydrolysed goat's milk offers a viable option. This study's enzymatic hydrolysis method showed the capability to alter antigenic characteristics, rendering goat's milk a suitable candidate for hypoallergenic compositions. The research addressed the initial problem by providing optimal hydrolysis conditions to diminish antigenicity. Nonetheless, while ELISA proficiently measured the decrease of antigenicity, it did not differentiate particular peptide sequence alterations, variations in hydrophobicity, or alterations in epitope conformations. Consequently, more research is required to enhance the understanding of the structural and immunological characteristics of the hydrolysates. Notwithstanding these constraints, the study offers significant insights for formulating safe and palatable goat’s milk-based products via regulated enzymatic hydrolysis. These findings establish a basis for enhancing dairy products' food safety and allergy control.

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6.3 Recommendation for future research

Additional characterisation of the resultant peptides warrants future investigation to potentially develop a hypoallergenic goat's milk formula. Proteins must be examined for their bioactive functions and evaluated for their properties and allergenic potential. To achieve a thorough knowledge, the biological activities of bromelain hydrolysates and biopeptides must be elucidated by specialised molecular docking and bioinformatics methods. The interaction between the peptide and antibody via molecular docking must be investigated to clarify the mechanisms underlying antigenicity and allergenicity and uncover possible linear epitopes. Identifying allergenic epitopes is crucial for evaluating food safety, particularly for those with allergies. Response Surface Methodology (RSM) may also be employed for the optimisation of diverse parameters in forthcoming experiments, such as pH, temperature, enzyme-to-substrate ratio, and reaction duration. This would enhance the efficiency of enzymatic hydrolysis and improve repeatability on a bigger scale. This research establishes a foundation for growing hydrolysed goat's milk as a feasible alternative for cow's milk protein allergy.

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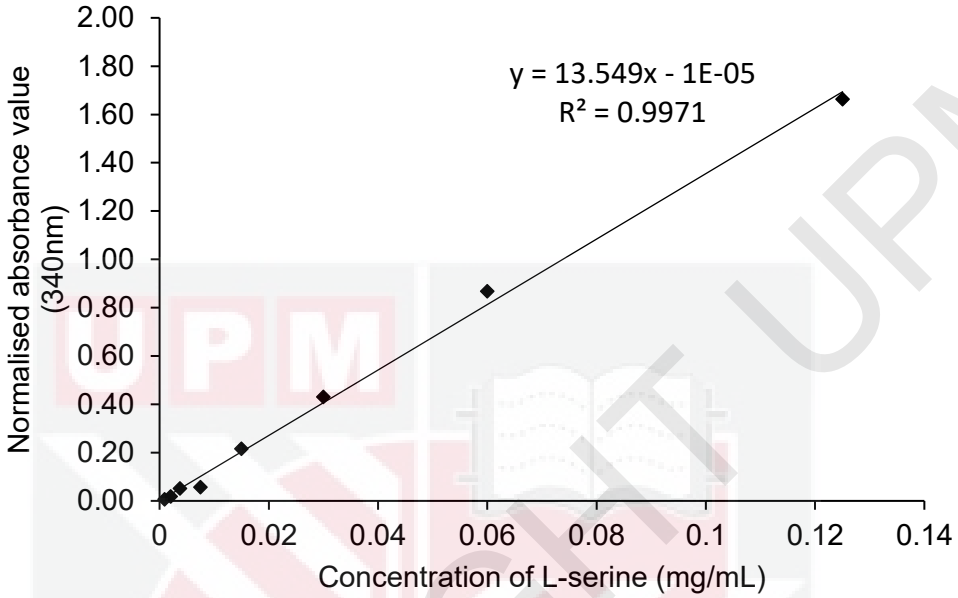
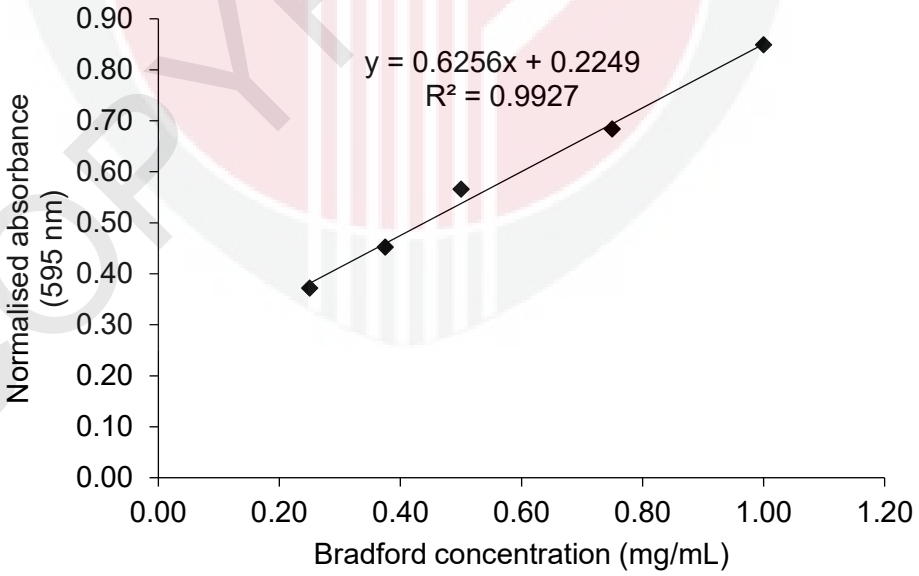
APPENDICES



Appendix A: L-serine standard curve for calculation of peptide content of hydrolysed casein.



Appendix B: Bradford assay standard curve

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Appendix C: Degree of hydrolysis, peptide concentration of goat's milk casein at a specific time of hydrolysis using different enzymes

Enzyme	Condition				Degree of hydrolysis (%)	Peptide Content (mg/mL)	Antigenicity Reduction
	E:S (ratio)	pH	Temp (°C)	Time (h)			
Bromelain	1:100	5.5	50	6	60	2.86	33.60± 0.014[a]
Alcalase	1:50	8.0	55	6	48	2.07	29.38 ± 0.014[b]
Trypsin	1:10	5.5	50	6	51	2.20	18.80 ± 0.001[c]
Pepsin	1:10	1.5	37	6	49	2.20	16.61 ± 0.009[d]

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BIODATA OF STUDENT

Nurul Izza Nabilah Binti Mohd Samsudin was born on 12th October 1999 at Ipoh Specialist Centre. She is the second child in the family. She received her primary education at Sekolah Kebangsaan Tarcisian Convent in Ipoh, Perak. Then, she completed her secondary education at Sekolah Menengah Kebangsaan Tarcisian Convent in Ipoh, Perak, until 2016. She continued her study at pre-university, Foundation in Science, at Universiti Putra Malaysia for one year after finishing high school. Later, in 2018, she pursued her study in Bachelor of Food Science and Technology at Universiti Putra Malaysia. In 2022, she further her study in Master of Science (Food Safety) by research. She aspires to achieve her goals and become successful in the future.

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LIST OF PUBLICATION

Samsudin, N. I. N. M., Sukor, R., Mohsin, A. Z., & Saari, N. (2024). 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. *International Journal of Food Science & Technology*, 59. 5181-5191. https://doi.org/10.1111/ijfs.17260

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Samsudin, N. I. N. M., Ying, C., Sukor, R., Mohsin, A. Z., & Saari, N. (2024). Isolation and purification of alpha-s1 casein from goat's milk using Hitrap Sepharose Fast Flow Cation Exchange Chromatography (IEC). Abstract presented in International Innovation Conference (INNOFEST-24). Politeknik Sandakan. (31st April). Oral. (Silver Award).

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