Revealing the Potential of Bioactive Peptides from Sheep Milk Casein and Whey: An In-silico Exploration

Sathika.K*, *Priyadharshini*.M, *Harini*.B, *Puniethaa* Prabhu, *Rengesh* Balakrishnan

Department of Biotechnology, K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India.

Abstract. Sheep casein and whey proteins, α-lactalbumin, β-lactalbumin, and β-lactoglobulin 1/B, are good sources of bioactive peptides that have potential health benefits including antimicrobial, antioxidant, antihypertensive, and immunomodulatory activities. The following paper discusses the in-silico analysis of these proteins retrieved from the UniProtKB database, with emphasis on their amino acid compositions and the bioactive peptides formed after hydrolysis with pepsin and trypsin. Their physicochemical properties, bioactivity, and allergenicity were determined by the use of sophisticated bioinformatics tools. One of the interesting findings includes their inhibition activity against dipeptidyl peptidase IV, which might be of great importance in the management of type 2 diabetes. The allergenicity predictions where a mixed bag as some peptides had the potential to be flagged as allergens due to the similarities in their sequence with the proteins in bovine milk. Others were predicted to be nonallergenic and safer for further use. The results suggest that bioactive peptides from sheep could potentially be used in food and pharmaceutical applications, although the need for further experimental validation, especially on safety, is critical. In-silico approaches provide an efficient and cost-effective means for screening peptides.

Keywords: Sheep milk proteins, bioactivity, in-silico analysis, casein, whey, allergenicity, toxicity.

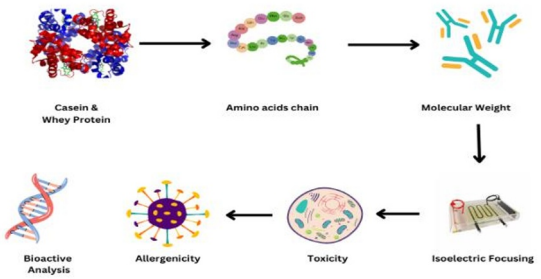

Fig. The overall process of In-silico Analysis of Ovein Protein.

* Corresponding author: sathikakannan@gmail.com

1 Introduction

Sheep milk contains high-quality proteins such as caseins (αs1, αs2, β, κ) and whey proteins, which serve as precursors for bioactive peptides with health benefits. Enzymes can hydrolyze caseins to produce bioactive peptides that may have therapeutic benefits. [2]. Other important constituents of the bioactive peptide profile of sheep milk are whey proteins such as β-lactoglobulin-1/B and α-lactalbumin. Among them, β-lactoglobulin is an important factor in producing peptides with antioxidant, antimicrobial, antihypertensive, and immunomodulatory activities. These proteins, upon enzymatic hydrolysis either by proteolytic enzymes or under conditions similar to gastrointestinal, have generated the generation of bioactive peptides promising high potential applications in functional foods, nutraceuticals, and even pharmaceutical uses. These peptides have displayed enormous potential for modulating numerous physiological functions, including reduced oxidative stress, increased immune responses, and control over blood pressure[5].Assessments of toxicity are crucial so that the bioactive peptides do not cause harmful effects once taken in adequate quantities.[6]Moreover, allergenicity tests should be performed to check whether any allergic reaction may be produced after ingesting these peptides. Given the increased awareness regarding food allergies, such knowledge will be crucial for their proper application in consumer products without compromising bioactivity [1].Response to these issues, this study adopts an in-silico method of examining the bioactivity of peptides produced from sheep milk proteins. Advanced computational tools have been used in this study to simulate the enzymatic hydrolysis of sheep milk proteins, targeting the major structures of caseins and whey proteins. In addition, through in-silico methods, the assessment of toxicity and allergenicity is conducted to screen peptides with risks on human health [3]. The main objective of this study is to examine the possibility of bioactivity in the peptides of sheep milk caseins and whey proteins while determining their safety. This work combines computational approaches to predict both bioactivity and toxicity, and thus, should contribute to the development of safe, innovative bioactive peptides as potential ingredients in functional foods, nutraceuticals, and pharmaceutical formulations [4].

2 Materials and methods

2.1 Materials used

- UniProtKB [7]
- ProtParam [8]
- BIOPEPdatabase [9]
- INNOVAGEN Peptide Property Calculator [10]
- APD3(Antimicrobial Peptide Database) [11]
- Allergen Online [12]
- SDAP (Structural Database of Allergenic Proteins) [13]

2.2 Methods

Some ovine milk proteins, representing a spectrum of caseins and whey proteins, to carry out in silico hydrolysis in this work. The caseins present here are αs1-casein, αs2-casein, β-casein, Serum Albumin, and κ-casein. β-lactoglobulin-1/B and α-lactalbumin are the selected whey proteins. These detailed primary sequences are found and published in the UniProt Knowledgebase, widely utilized as an all-in-one protein sequence resource. Further

characterization of the proteins was carried out by establishing their amino acid composition with the help of the ProtParam tool, which offers some vital statistics concerning the properties of the protein sequences. We hydrolyzed each of the selected ovine milk casein and whey proteins with two different proteolytic enzymes, namely pepsin and trypsin. Pepsin hydrolysis was carried out under certain conditions (EC 3.4.23.1; pH above 2 and at pH 1.3) to simulate the gastric environment, whereas trypsin hydrolysis (EC 3.4.21.4; pH conditions are not mentioned) was used as the secondary digestive enzyme. The possible biological activities of the resultant hydrolysates were verified from the BIOPEP database of cataloged peptides and their related bioactivities. Several important properties of the proteins and peptides were calculated using the INNOVAGEN Peptide Property Calculator, giving estimations of molecular weight, isoelectric point (pI), net charge at physiological pH 7, and peptide solubility. In parallel, we performed a detailed analysis of the Boman index of the generated peptides by using the Antimicrobial Peptide Database (APD3). This analysis was done on peptide sequences with a minimum length of five amino acids, which is crucial for the assessment of antimicrobial potential. To predict the toxicity of the peptides, we used the ToxinPred tool. Data are submitted via the batch submit option for peptides with less than 50 amino acids. For peptides of greater than 50 amino acids in size, we employed a protein scan approach. For the predictions, SVM algorithm was used and ensured that our threshold was set to 0.0, and the e-value cutoff was set to 10. Furthermore, for ascribing safety to the peptides for use as dietary ingredients, allergenicity assessments were performed using Allergen Online and the Structural Database of Allergenic Proteins (SDAP). Allergenicity was evaluated on stringent parameters:

1. Full FASTA sequence alignment with an E-value of 0.1 that targets sequences with more than 50% ID.

2. 80 amino acid sliding window alignment for finding the sequences with more than 35% identity (ID).

3. Obtain the short exact word matches from the sliding window alignments examining eight, seven, and six consecutive amino acids as proposed in the guidelines of the FAO/WHO(Food and Agriculture Organization/World health Organisation) on allergenicity. This broad assessment of hydrolysis as well as evaluation of potential biological activities, toxicity, and allergenicity ensures that the functional properties of the ovine milk proteins are examined exhaustively.

3 Results and Discussions

3.1 Results:

3.1.1 UniProtKB Analysis on Ovein Proteins

Table 1. UniProtKB and INNOVAGEN Peptide Property Calculator data of ovein casein

Caseins	ID	Length	Molecular weight (Da)	Iso-electric point (Ip) (pH)	Net charge at pH 7	Peptide solubility
αs1	P67829	325	37,567	9.84	15.5	Good water solubility
αs2	P04654	223	26,332	7.80	1.3	Good water solubility
β	P11839	222	24,875	5.02	-6.6	Poor water solubility
κ	P02669	192	21,438	5.74	-2.8	Poor water solubility
Serum Albumin	P14639	607	69,188	5.72	-14.5	Good water solubility

Table 1 shows that α-caseins have higher solubility and charge, making them suitable for soluble applications, while β and κ-caseins are less soluble and less effective in such uses.

Table 2. UniProtKB and INNOVAGEN Peptide Property Calculator data of ovein whey

Whey	ID	Length	Molecular weight (Da)	Iso-electric point (Ip) (pH)	Net charge at pH 7	Peptide solubility
β-lactoglobulin -1/B	P67976	180	19,921	5.22	-4.2	Good water solubility
α-lactalbumin	A0A1V0CM03	44	4,901	5.41	-1	Poor water solubility
β-lactoglobulin	P67975	162	18,151	5.0	-5	Good water solubility

Table 2 shows that β-lactoglobulin-1 and β-lactoglobulin are highly soluble in water, while α-lactalbumin has lower solubility, making the former suitable for soluble applications and the latter for those requiring lower solubility.

3.1.2 Determination of Amino Acid Composition

The composition of amino acids was analyzed using the ProtParam tool, which provides detailed information about the amino acid sequence. This analysis helps in understanding the physical and chemical properties of the protein.

Table 3. Amino acid content of Ovine casein

Amino acid		Amino acid percentage in casein (%)				
		Serum Albumin	**αs1**	**αs2**	**κ**	**β**
Ala	A	8.4	7.0	5.4	10.9	3.6
Arg	R	4.1	2.8	3.1	3.1	1.4
Asn	N	2.3	4.7	5.4	4.7	1.8
Asp	D	7.2	3.3	2.7	3.6	1.8
Cys	C	5.8	0.5	1.3	1.6	0.5
Gln	Q	3.1	7.0	7.2	7.8	9.5
Glu	E	9.2	9.3	11.2	5.7	8.6
Gly	G	3.0	4.2	0.9	1.0	2.3
His	H	3.0	1.9	2.2	2.1	2.3
Ile	I	2.3	5.6	5.4	5.7	4.5
Leu	L	10.7	10.7	6.3	6.2	12.2
Lys	K	10.0	7.0	11.2	4.7	5.9
Met	M	0.8	2.8	2.2	2.1	3.2
Phe	F	5.1	3.3	4.0	3.6	4.1
Pro	P	4.6	7.9	5.4	10.4	15.3
Ser	S	4.8	8.9	6.7	6.8	6.3
Thr	T	5.3	1.9	6.7	7.3	5.0
Trp	W	0.5	0.9	1.3	0.5	0.5
Tyr	Y	3.5	5.1	5.4	4.7	1.4
Val	V	6.3	5.1	5.8	7.3	10.4

Notes: Values obtained from ProtParam.

Table 3 shows that sheep casein fractions like β-casein, αs1-casein, and αs2-casein are rich in hydrophobic and essential amino acids, enhancing bioactivity and stability, while serum albumin has a distinct amino acid distribution.

Table 4. Amino acid content of Ovine whey

Amino acid		Amino acid percentage in casein (%)		
		β-lactoglobulin-1/B	**α-lactalbumin**	**β-lactoglobulin**
Ala	A	11.1	6.8	9.9
Arg	R	1.7	0.0	1.9
Asn	N	3.9	0.0	4.3
Asp	D	4.4	4.5	4.9
Cys	C	3.9	2.3	3.1
Gln	Q	5.6	6.8	5.6
Glu	E	8.3	6.8	9.3
Gly	G	3.9	6.8	3.1
His	H	1.7	2.3	1.9
Ile	I	5.6	2.3	6.2
Leu	L	15.0	18.2	13.0
Lys	K	8.9	9.1	9.3
Met	M	2.8	4.5	2.5

Phe	F	2.2	6.8	2.5
Pro	P	4.4	2.3	4.9
Ser	S	3.3	6.8	3.7
Thr	T	4.4	4.5	4.9
Trp	W	1.1	0.0	1.2
Tyr	Y	1.7	2.3	1.9
Val	V	6.1	6.8	6.2

Notes: Values obtained from ProtParam.

Table 4 shows that caseins have higher proline, glutamic acid, and leucine, contributing to their structure and bioactivity, while β-lactoglobulin-1/B and α-lactalbumin differ in amino acid composition, affecting their bioactive potential and functionalities. These variations in amino acid profiles highlight the role of protein source in influencing biological activity.

3.1.3 In-silico Hydrolysis of Ovein Proteins

Table 5. In silico proteolysis of ovein caseins in three different conditions and degree of hydrolysis

Casein	Hydrolysis condition	DH(%)
αs1	Pepsin pH 1.3	14.37
	Pepsin pH 2	70.36
	Trypsin	14.03
αs2	Pepsin pH 1.3	9.65
	Pepsin pH 2	69.3
	Trypsin	14.03
κ	Pepsin pH 1.3	9.64
	Pepsin pH 2	64.46
	Trypsin	7.61
β	Pepsin pH 1.3	15.85
	Pepsin pH 2	63.87
	Trypsin	7.04

Notes: DH: Degree of Hydrolysis

Table 5 shows that pepsin at pH 2 achieves the highest degree of hydrolysis in sheep casein, with αs1-casein having the highest DH, while lower DH is observed with pepsin and trypsin at pH 1.3, indicating the need for optimized enzymatic conditions for targeted peptide production.

Table 6. Quantity of peptides by size, released through in silico proteolysis of ovein whey in three different conditions and degree of hydrolysis

Casein	Hydrolysis condition	DH(%)
β-lactoglobulin-1/B	Pepsin pH 1.3	16.93
	Pepsin pH 2	74.86
	Trypsin	10.38
α-lactalbumin	Pepsin pH 1.3	25.58
	Pepsin pH 2	76.74
	Trypsin	9.30
β-lactoglobulin	Pepsin pH 1.3	15.15
	Pepsin pH 2	73.33
	Trypsin	10.90

Notes: DH: Degree of Hydrolysis

Table 6 shows that pepsin at pH 2 gives the highest DH for ovine whey proteins, while trypsin shows poor hydrolysis, highlighting the importance of optimizing enzymatic conditions for industrial use.

3.1.4 analysis of bioactivity and boman index of ovein peptides

- **For ovein casein:**

Beta Casein: Trypsin Digests: Boman Index Values ranging between -2.54 kcal/mol and 2.92 kcal/mol are a mix of low to moderate protein interaction peptides. One of the very low peptides is GPFPILV which indicates good bioavailability.
Pepsin Digests (pH 1.3): More variability was seen and values ranged from -1.13 to 4.93 kcal/mol. The peptides CSRRF (-5.79 kcal/mol) showed good interaction ability and therefore could be applied in therapeutic or inhibitory settings.
Alpha-S1 Casein: Most peptides had negative Boman Index values with LESQK at -3.27 kcal/mol and WYGQEK at -2.46 kcal/mol, indicating minimal interaction potential, making it ideal for bioactivity study.
Pepsin Digests (pH 1.3): Peptides like CSRRF (-5.79 kcal/mol) were again very different, having high interaction potential. The peptides with low Boman Index were generally spread in good order.

- **For whey protein**

Beta-lactoglobulin peptides lie within broader Boman Index values than that of the whey protein, thereby indicating diversified potential towards bioactivity.

DTDYKKYL peptides having Boman Index = 3.3 kcal/mol along with TPEVDNEALEK 3.01 kcal/ mol display high affinities toward possible roles as bioactive agents.
The Boman Index of Whey protein peptides shows only middle-range values but lacks in extremities of Boman index values. This was true for peptides, especially of HATQAEQL 2.07 kcal/mol and CEAFQK 1.97 kcal/mol which came with notable potential.

Enzymatic specificity affects peptide bioactivity potential:

•Pepsin digestion produces more high-affinity peptides in beta-lactoglobulin than trypsin.

•Trypsin digestion of whey protein produces lower-affinity peptides in general.

Practical Implication: The more the Boman Index value, the higher the potential of peptides DTDYKKYL, VRTPEVDNEAL, and TPEVDNEALEK to interact more effectively with biological proteins and might be targeted for further study in bioactivity or therapeutic application.

3.1.5 Analysis of Toxicity of Peptides Released through In-silico Hydrolysis

- **For ovein casein**

In silico hydrolysis of ovein caseins under three different conditions (pepsin at pH 1.3 and trypsin digestion) resulted in the generation of fourteen peptides identified as toxins. These toxic peptides originated primarily from alpha-S1 casein, serum albumin, and kappa casein sequences. The presence of these peptides highlights the

potential for hydrolysis processes to produce bioactive compounds, including those with toxic effects.

1. Alpha-S1 Casein (Pepsin, pH 1.3):
MGIGRHCNKL

2. Serum Albumin (Pepsin, pH 1.3):
AKTCVADESHAGCDKSL,RETYGDMADCCEKQEPERNECF,QECCQAEDKGACL
,EDCCAKEDPHACYATVF,VDKCCAADDKEGCF

3. Serum Albumin (Trypsin):
ETYGDMADCCEK, YNGVFQECCQAEDK,ECCHGDLLECADDR, EYEATLEDCCAK,
CCTESLVNR, CCAADDK

4. Kappa Casein (Trypsin):
ICCEK

The above mentioned peptide sequences show toxicity in ovein casein.

- **For Ovein Whey**

In-silico hydrolysis of ovine whey with pepsin at pH 1.3 and trypsin showed no toxic peptides, suggesting that hydrolysis is safe for potential food or nutritional applications.

3.1.6 Analysis of Allergenicity of Peptides Released through In-silico Hydrolysis

- **For casein**

- **Analysis of allergenicity of αs1 ovein casein:**

In-silico hydrolysis of αs1 casein identified a peptide (AAN05083, Sal k 2.0101) with allergenic potential (alignment score: 128, E-value: 0.0031), requiring further validation for safe food and therapeutic use.

- **Analysis of Allergenicity of αs2 ovein casein:**

In-silico hydrolysis of αs2 casein identified allergenic peptides such as P02663 and AAA30479 (Bos d 10.0101, Bos d 8), which further requires experimental validation to be safe for food and medicinal use.

- **Analysis of Allergenicity of β ovein casein**

In-silico hydrolysis of β-casein identified peptides with significant allergenic potential, such as AAA30480 and P02666 (Bos d 8, Bos d 11.0101), showing high alignment and low E-values. Other sequences, like BAA11251 (Tri a gliadin), pose moderate allergenic risks, requiring further validation for food and therapeutic safety.

- **Analysis of Allergenicity of κ ovein casein**

The peptides produced from in-silico hydrolysis of κ casein indicate a great allergenic potential, as sequences P02663 and AAA30479 are linked to Bos d 10.0101 and Bos d 8, meaning that there is a significant risk of allergy. AAA30429 scored lower, but it remains an

allergen. Therefore, there is a need for more experimental testing on their safety for consumption and therapeutic purposes.

- **Analysis of Allergenicity of Serum Albumin of ovein casein:**

The peptides from ovine casein, including those linked to Bos d 6, show strong allergenic potential and require further testing for safety. The study highlights the need to focus on conserved allergens for universal treatments and species-specific ones for tailored therapies.

  - **For Whey**

- **Analysis of Allergenicity of β-lactoglobulin-1/B of ovein whey:**

In-silico hydrolysis of β-lactoglobulin-1/B revealed peptides with high allergenic potential, particularly sequences like P02754 and AAA30411, associated with Bos d 5 allergen, showing extremely low E-values (7.2e-71) and high scores. Other allergens, including Can f 2 (O18874) and Fel d 4 (AAS77253), also demonstrated significant allergenicity with E-values ranging from 2.6e-12 to 2.4e-10. Moderate allergenic potential was observed in sequences like P02761 (Rat n 1) and AAA39768 (Mus m 1.0102). These findings underscore the need for experimental validation of β-lactoglobulin peptides for safe use.

- **Analysis of Allergenicity of α-lactalbumin of ovein whey:**

The in-silico analysis of α-lactalbumin has detected several potentially allergenic peptides. Their sequences related to Bos d 4 indicated high alignment scores and low E-values, pointing toward strong allergenic potential. The second sequence for Bos d 4 revealed a moderate risk. However, the Equ a 6.01 had scores less than these two and indicates reduced allergenic risk.

- **Analysis of Allergenicity of β-lactoglobulin of ovein whey:**

In-silico hydrolysis of β-lactoglobulin in ovine whey identified allergenic peptides like P02754 and AAA30411 (Bos d 5) with high scores (alignment: 947–1024, E-value: 1.9e-65) and moderate potential in others like O18874 (Can f 2). These findings necessitate further validation to ensure safety for consumption and therapeutic applications.Overall, the analysis underscores Bos d 5 (domestic cattle) as a dominant allergen characterized by high alignment quality, Bits Scores, and favorable E-values which collectively suggest substantial allergenic potential and relevance in allergic.

3.2 Discussions

The properties and profiles of hydrolysis by ovine casein, whey proteins have put an emphasis on the former in food and pharmaceutical use. Caseins, notably αs1-casein, have a high rate of solubility at acidic pH, thus favorable for dairy-based foods or production of bioactive peptides. Whey proteins-β-lactoglobulin-are soluble with important biological activity and useful dietary supplements and functional foods. The enzyme capable of hydrolyzing in the most effective way proved to be pepsin. Acidic pH ensures that the yield of bioactivity peptides is maximal, with β-casein possessing exceptional versatility. The critical points of toxicity and allergenicity, raised concerning some peptides derived from αs1-casein and serum albumin, indicate the need for strong evaluation of safety.

4 Conclusion

This study is of immense value in describing the functional properties, bioactivity, and safety profiles of ovine casein and whey proteins. Such extensive analysis of protein characteristics, hydrolysis patterns, and peptide bioactivity indicates that these proteins may have multiple applications in food and pharmaceutical industries. Both caseins and whey proteins present unique solubility, charge stability, and amino acid profiles, which can be employed to create functional foods, bioactive peptides, and nutritional supplements. Results also highlight the need for optimization of the hydrolysis conditions toward the generation of bioactive peptides with therapeutic potential. However, the results do point out important considerations in the aspect of toxicity and allergenicity of some peptides, especially those from casein proteins. This calls for additional studies on safety, toxicological evaluation, and testing for allergenicity of these peptides, especially in terms of human consumption and use in therapy. It thus implies that ovine caseins and whey proteins have great potential as bioactive ingredients with a range of industrial applications. As long as the safety issues are taken into consideration and resolved, it will facilitate further development and deployment of these proteins for the creation of new food products and health supplements.

Acknowledgment

This study does not involve human participants, animals, or sensitive data requiring ethical approval or consent to participate. The data and materials used in this study were obtained from freely available online tools and resources and can be accessed through the respective web platforms. The authors declare that they have no competing interests. No specific funding was received for this research work.

References

1. Hervé Bernard Stéphane Hazebrouck, Nicolas Gaiani and Karine Adel-Patient Allergen Risk Assessment for Specific Allergy to Small Ruminant's Milk: Development of Sensitive Immunoassays to Detect Goat's and Sheep's Milk Contaminations in Dairy Food Matrices [Journal]. - [s.l.] : Frontires, 2021. - **Vol. 2.**
2. Molik Zuzanna Flis and Edyta Importance of Bioactive Substances in Sheep’s Milk in Human Health [Journal]. - [s.l.] : MDPI, 2021.
3. Muhammad Najmi Mohammad Fauzi Aisyah Aqilah Abu Bakar, Liyana Amalina Adnan, Tg Ainul Farha Sequencing and Annotated of Goat's Milk using [Journal]. - 2021. - **Vol. 7.**
4. Richard J. FitzGerald Maria Cermeño, Mohammadreza Khalesi, Thanyaporn Kleekayai Application of in silico approaches for the generation of milk protein-derived [Journal]. - [s.l.] : ELSEVIER, 2020. - **Vol. 64**.
5. Sapna Rani Km Pooja, Gaurav Kumar Pal Exploration of potential angiotensin converting enzyme inhibitory peptides generated from enzymatic hydrolysis of goat milk proteins [Journal]. - [s.l.] : ELSEVIER, 2017.
6. Simona Almášiová Róbert Toman, Martina Pšenková Toxic Elements in Sheep Milk, Whey, and Cheese from the Environmentally Burdened Area in Eastern Slovakia and Health Risk Assessment with Different Scenarios of Their Consumption [Journal]. - [s.l.] : MDPI, 2024.
7. UniProtKB -URL: https://www.uniprot.org
8. ProtParam- URL: https://web.expasy.org/protparam/
9. BIOPEPdatabase-URL: https://biochemia.uwm.edu.pl/biopep/start_biopep.php

10. INNOVAGEN Peptide Property Calculator-URL: https://pepcalc.com/
11. APD3(Antimicrobial Peptide Database) -URL: https://aps.unmc.edu/
12. Allergen Online-URL: http://www.allergenonline.org/
13. SDAP (Structural Database of Allergenic Proteins) -URL: https://fermi.utmb.edu/