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Sex-specific Epitopic Peptide Synthesis, Purification and Characterisation to Target Bovine Spermatozoa

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ABSTRACT

The present research was conducted during July, 2025 to September, 2025 at Division of Biochemistry, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India which mainly focused on the identification and synthesis of sex-specific peptide sequences associated with bovinespermatozoa. Initially, an extensive review was carried out using data obtained from various proteomic studies on bovine spermatozoa, where differentially expressed as well as specifically expressed proteins had been identified and systematically listed. Based on these earlier proteomic findings, SCAMP1 was selected as a potential candidate because it had been reported to be Y-chromosome-specific. To further investigate its immunogenic potential, epitope prediction and peptide design were performed using several bioinformatic tools, including DeepTMHMM and SEMA 2.0. These computational approaches helped in identifying suitable peptide regions with desirable antigenic and structural characteristics. Subsequently, the selected peptide sequence was synthesized through Fmoc chemistry-based solid-phase peptide synthesis employing the Multiple Antigen Peptide (MAP) format, which enhances peptide immunogenicity and stability. After synthesis, purification of the peptide was performed using High-performance Liquid Chromatography (HPLC) to obtain a highly purified product. Furthermore, structural characterization of the synthesized peptide was carried out using Circular Dichroism (CD) spectroscopy in order to evaluate its secondary structural conformation and confirm the integrity of the synthesized peptide. The present study aimed to investigate the potential of a sex-specific epitopic peptide and evaluate its possible application in the development of a novel sperm sexing technology. By identifying and characterizing sex-specific peptide epitopes, the study sought to provide a targeted and efficient approach for distinguishing X- and Y- spermatozoa.

KEYWORDS: MAP, proteomic, bovine, HPLC, spermatozoa, CD spectroscopy

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1. INTRODUCTION

Proteins on spermatozoa play a crucial role in fertilisation by facilitating sperm recognition and interaction with fertilisation-associated proteins on the oocyte surface. Spermatozoa originate from diploid spermatogonia and undergo successive meiotic divisions followed by spermiogenesis to develop into mature sperm cells. After becoming transcriptionally inactive, the spermatozoa are released into the lumen of the seminiferous tubules and subsequently transported through the epididymis, where they acquire progressive motility and functional maturation (Leahy et al., 2011). The differential expression of specific surface proteins between X- and Y-bearing spermatozoa has gained significant attention in reproductive biology. Identification of such sex-specific proteins may provide valuable molecular targets for selective sperm recognition and advanced sex-sorting strategies (Kinga et al., 2026). The selection of sperm types through sperm-sexing technology can accelerate livestock breeding programs and enhance the genetic potential of animals for improved lactation, reproduction, and overall productivity (Yodrug et al., 2025). Moreover, the significance of sexed semen extends beyond the farmers' perspective, as it also offers substantial benefits to both the dairy and beef industries (Rath et al., 2013). Basically, sex-specific sperm refers to X- or Y-chromosome-bearing spermatozoa present within a semen sample, which can be selectively utilised according to the need for offspring sex. Currently, fluorescence-activated cell sorting (FACS) is widely used for sperm sorting, with accuracy exceeding 90%, and has been extensively applied in commercial settings (Sharpe and Evans, 2009). This technique differentiates X- and Y-chromosome-bearing sperm based on differences in their DNA content. The DNA content between X and Y sperm varies by approximately 3.8% to 4.98%, depending on the cattle breed (Howes et al., 1997; Garner, 2006; Garner and Seidel, 2008). However, this technique is associated with several limitations, including dependence on fluorescent dyes, reduced fertility potential, sperm loss during processing, labour-intensive procedures, and the requirement for sophisticated machines (Carvalho et al., 2010; Garner et al., 2012). To address these limitations, there is a growing need for innovative, cost-effective, and accessible approaches for X-sperm enrichment. Proteomic technologies provide a promising platform for identifying differential protein expression between X- and Y-chromosome-bearing spermatozoa, thereby facilitating the development of alternative sperm separation strategies (Blecher et al., 1999). A deeper understanding of the plasma membrane proteins associated with X-and Y- chromosome bearing sperm may contribute to the advancement of non-invasive, efficient, and economically feasible sperm sexing technologies (Quelhas et al., 2021). There are a number of studies that have been done to identify a specific protein which can be identified as a sex-specific biomarker in case of bovine spermatozoa. Previous studies have suggested that distinct surface-associated antigens present on X- and Y-chromosome-bearing spermatozoa may serve as potential targets for their selective separation and targeting them by using antibodies can separate either sex spermatozoa (Sang et al., 2011; Chen et al., 2012; Yadav et al., 2017; Rahman and Pang, 2020). This concept is supported by the identification of the H-Y antigen, a male-specific antigen expressed in mammalian tissues, which has been explored as a potential immunological marker for differentiating sperm populations (Wachtel et al., 1988). Likewise, Shen and co-workers in 2021 identified differentially expressed proteins as well as specifically expressed proteins. Where he could identify SCAMP1 as Y-specific and CLRN3 as X-specific protein (Shen et al., 2021). Furthermore, studies conducted in India on bovine spermatozoa, particularly in Sahiwal cattle, identified several differentially expressed proteins within the sperm plasma membrane proteome using LC–MS/MS analysis, highlighting their potential importance in future reproductive and fertility-related investigations (Laxmivandana et al., 2021; Sharma et al., 2022). The present study aimed to identify sex-specific bovine sperm peptide sequences, synthesize them in multiple antigen peptide (MAP) format using solid-phase peptide synthesis, and characterize their structural properties through HPLC purification and circular dichroism (CD) spectroscopy.

2. MATERIALS AND METHODS

2.1. Selection of protein

The experiment was undertaken between July and September 2025 at the Division of Biochemistry, ICAR–Indian Veterinary Research Institute, Izatnagar. The selection of sex-specific proteins was carried out through an extensive review of previously published literature related to bovine sperm proteomics and sperm sexing technologies. Among the reported candidate proteins, SCAMP1 was identified as a Y-chromosome-specific protein by Shen and co-workers. Based on its reported specificity and potential relevance in distinguishing Y-bearing spermatozoa, SCAMP1 was selected as the target protein for the present investigation. The identification and characterization of such sex-specific proteins may contribute to the development of alternative molecular approaches for sperm sexing and reproductive biotechnology applications.

2.2. Epitope identification

The selected protein was further analysed to identify its potential epitope region for downstream applications. Initially, the FASTA sequence of SCAMP1 was retrieved from UniProt (https://www.uniprot.org/) for detailed

bioinformatic analysis. Subsequently, computational prediction tools, including DeepTMHMM and SEMA 2.0, were employed to characterise the structural features of the protein, such as transmembrane helices, extracellular (outside) regions, intracellular (inside) regions, and potential epitope-containing domains. Results of computational predictions are given in figure 1.

Based on these analyses, a suitable surface-exposed epitopic region with potential antigenic properties was identified and selected for further investigation. Following epitope prediction, the selected peptide sequence was subjected to physicochemical characterisation to evaluate important properties such as molecular stability, hydrophobicity, charge distribution, and antigenic potential. These analyses were performed to ensure the suitability of the selected epitope for peptide synthesis and subsequent experimental applications. The selected peptide details are given in Table 1.

Table 1: Selected sequence for peptide synthesis

Protein name	Peptide sequence	Localization
SCAMP1	(P1)- CVDPPRGVDFGLS (174-189)	Outside

2.3. Synthesis of MAP peptide

The selected peptide sequence was synthesized using Fmoc-based solid-phase peptide synthesis (SPPS) (Verma et al.,2020). Cysteine, identified as the core amino acid residue of SCAMP1, was initially coupled with di-Fmoc-L-lysine to generate a branched framework for the preparation of a Multiple Antigenic Peptide (MAP) (Figure 2). Before synthesis, Rink amide resin-AM (100–200 mesh; Novabiochem, Merck, Germany) was swollen overnight in N, N-dimethylformamide (DMF).

Initial deprotection of the resin-bound amino groups was carried out using 20% piperidine in DMF, followed by extensive washing with DMF and dichloromethane (DCM). Amino acid coupling was performed after pre-activation of Fmoc-protected amino acids in dry DMF using hydroxy benzotriazole (HOBt) and diisopropylcarbodiimide (DIPC) as coupling reagents. The activated amino acids were then allowed to react with the resin for 2 h at 25°C on a platform shaker to facilitate efficient coupling. Successive cycles of deprotection, activation, and coupling were repeated sequentially until completion of peptide synthesis. The efficiency of coupling reactions was periodically monitored using the Kaiser test.

Following synthesis, the resin beads were dried in a desiccator, and peptide cleavage was performed using a cleavage cocktail containing trifluoroacetic acid (TFE), thioanisole, ethanedithiol (EDT), triisopropylsilane (TIPS), phenol, and HPLC-grade water in an optimized ratio. The

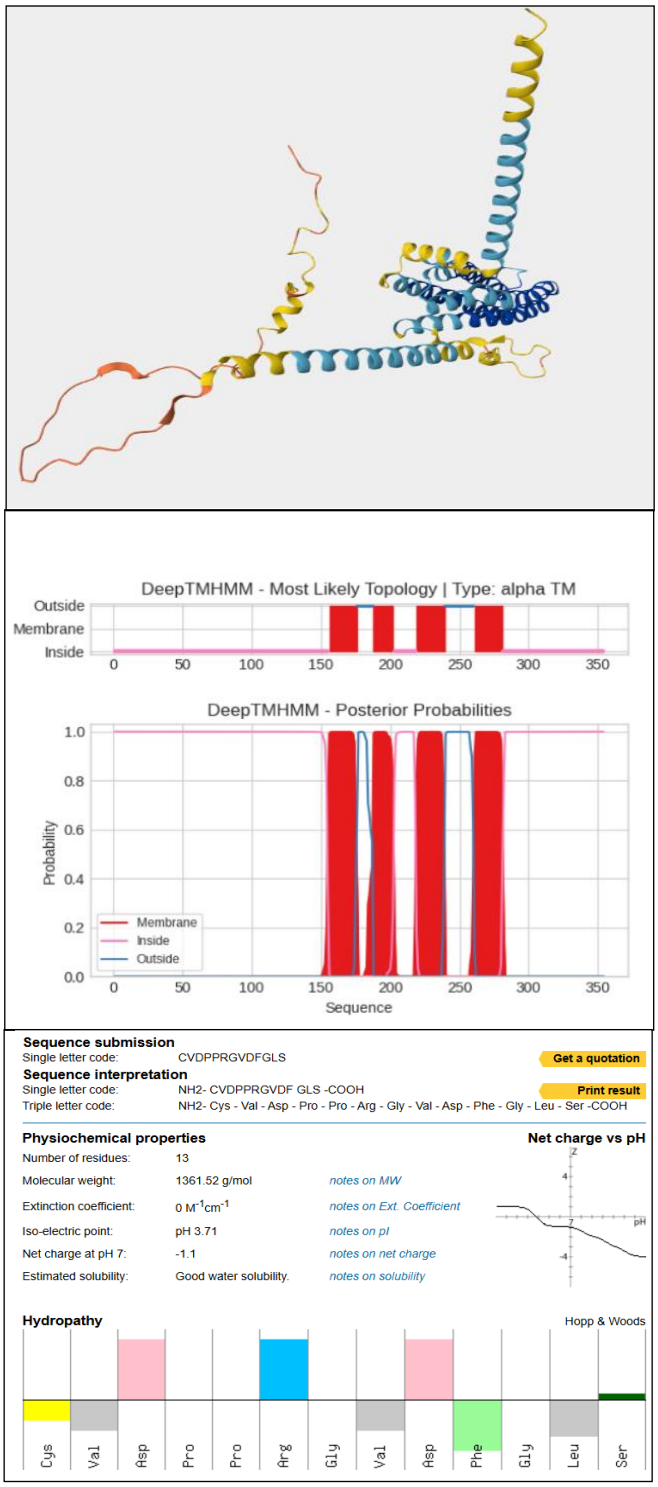

Figure 1: a) AlphaFold 3D structure of SCAMP1 taken from Uniprot(https://services.healthtech.dtu.dk/services/DeepTMHMM-1.0/), b) Transmembrane helix detection, c) Physiochemical property analysis of the selected peptide (PepCalc.com)

cleavage reaction was carried out for 6 hrs to release the peptide from the solid support. Subsequently, chilled diethyl

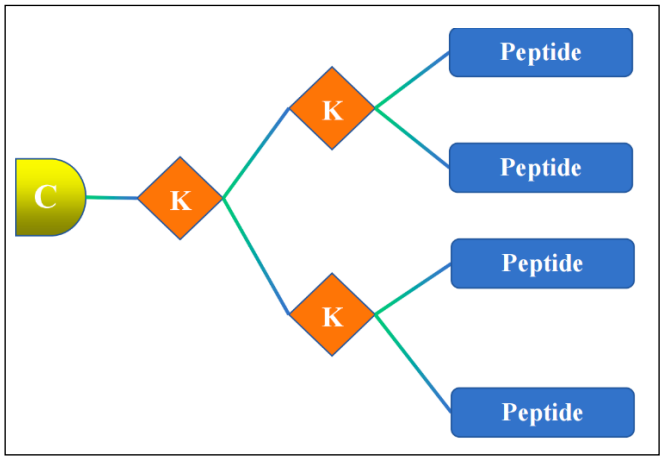

Figure 2: Structural representation of MAP

ether was added to precipitate the synthesized peptide, followed by overnight incubation at low temperature. The precipitated peptide was finally recovered by centrifugation at 6000 rpm for 10 min at 4°C.

2.4. Purification of peptide by using reverse phase HPLC

The dried peptide was collected and dissolved in HPLC water with 0.01% TFE before purification. The dissolved peptide was injected into a loop of a C18 semi-analytical column for purification in an HPLC binary system, Shimadzu make. The peptides were purified with different gradients of acetonitrile. The chromatograms at 220 nm and 280 nm showed a single major, sharp peak along with some minor peaks for the synthesised peptide, a characteristic feature indicating purity. Afterward, the peak was collected and dried using a vacuum concentrator (Eppendorf).

2.5. Characterization of peptide by using CD spectroscopy

To investigate the secondary structural configuration of the synthesized peptide, Circular Dichroism (CD) spectroscopy analysis was performed under different solvent environments. The peptide was dissolved in varying ratios of water and trifluoroethanol (TFE) to evaluate its conformational behaviour in both polar and non-polar conditions. The peptide solutions were prepared in 100% water, 75% water: TFE (v/v), 50% water: TFE (v/v), and 25% water: TFE (v/v). These solvent systems were selected to assess the influence of solvent polarity on peptide folding and structural stability.

CD spectra were recorded over the far-UV region, typically between 190 and 260 nm, using a quartz cuvette with an appropriate path length under controlled temperature conditions. The obtained spectra were analysed to determine the presence of characteristic secondary structural elements such as α-helices, β-sheets, β-turns, or random coil conformations. Changes in ellipticity values and spectral minima in different solvent compositions provided insight into solvent-induced conformational transitions of the peptide. The gradual increase in TFE concentration allowed the evaluation of peptide structural adaptation in increasingly hydrophobic environments, thereby helping to understand the stability and folding propensity of the synthesized peptide under different physicochemical conditions.

3. RESULTS AND DISCUSSION

Sex-specific proteins have emerged as promising targets in the field of immunological sperm sexing, which is increasingly being explored as an alternative to conventional flow cytometry–based sperm sorting techniques. Unlike flow cytometry, immune-based approaches have the potential to provide a less invasive, cost-effective, and more fertile method for sperm separation. In the present preliminary study, the identification and synthesis of a sex-specific peptide sequence represent an important step toward the development of targeted immunological tools for bovine sperm sexing. The synthesized peptide may serve as a potential antigen for the generation of highly specific antibodies against sex-associated sperm surface proteins. The selected protein SCAMP1 was claimed as a Y-specific protein by researchers (Shen et al., 2021). To identify a potential sex-specific epitopic region within the SCAMP1 protein, several bioinformatic prediction tools were employed for detailed structural and antigenic analysis. Computational analyses enabled the identification of extracellular and intracellular domains, along with the prediction of transmembrane helices within the protein sequence. Such analyses are essential for selecting surface-exposed regions that may serve as effective antigenic targets for antibody generation. Similar computational approaches have previously been utilized for epitope prediction and antigen design in reproductive and immunological studies (Saha and Raghava, 2006; Jespersen et al., 2017).

The selection of the peptide sequence for synthesis was based on multiple physicochemical and antigenic parameters, including molecular weight, amino acid composition, water solubility, and pH stability. The finally selected peptide sequence fulfilled these essential criteria, exhibiting favourable solubility and an appropriate molecular weight, thereby indicating its suitability for downstream immunological applications, particularly antibody production. Surface-accessible and hydrophilic peptide regions are generally considered more immunogenic and effective for raising specific antibodies (Hopp and Woods, 1981).

The selected peptide was synthesized using Fmoc-based Solid Phase Peptide Synthesis (SPPS) in the form of a Multiple Antigenic Peptide (MAP). The MAP strategy significantly enhances peptide immunogenicity by increasing molecular size and epitope density without requiring carrier

proteins. This multimeric arrangement improves antigen presentation and promotes stronger immune responses, making it highly suitable for antibody generation and immunological studies (Tam, 1988).

Following synthesis, purification of the peptide was performed using High-Performance Liquid Chromatography in reverse phase (HPLC). The chromatographic profile (Figure 3) exhibited a distinct and sharp peak, indicating successful synthesis and high purity of the peptide preparation. The purified peptide fraction corresponding to the major peak was subsequently collected for future applications, including antibody production and the development of immunological sperm sexing approaches. The generation of antibodies against sex-specific sperm surface peptides may provide a promising platform for developing alternative immune-based sperm sorting technologies with improved efficiency and reduced cellular damage compared to conventional flow cytometry-based methods.

The secondary structural behaviour of the synthesized peptide was analysed using Circular Dichroism (CD)

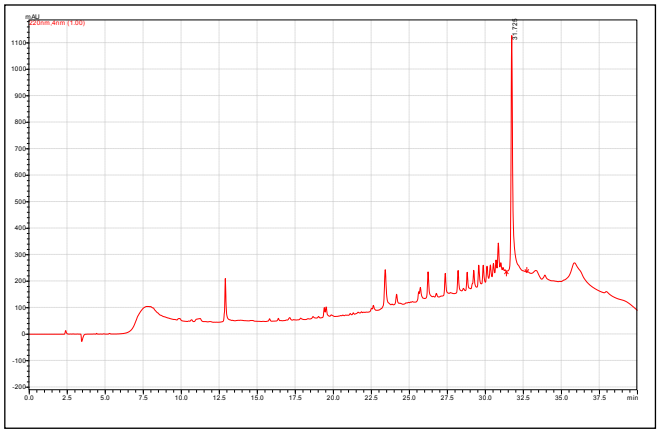

Figure 3: HPLC chromatogram showing a distinct sharp peak @ 31.7 min corresponding to the purified synthesized MAP (10 mg ml^{-1})

spectroscopy in different solvent systems comprising varying ratios of water and trifluoroacetic acid (TFE). The CD spectra were recorded in 100% water, 75% water: TFE (v/v), 50% water: TFE (v/v), and 25% water: TFE (v/v) to evaluate the influence of solvent polarity on peptide conformation (Figure 4).

The obtained spectra demonstrated noticeable variations in molar ellipticity with changes in solvent composition, indicating solvent-dependent conformational alterations of the peptide. In 100% water, the peptide exhibited comparatively lower negative ellipticity values with a broad minimum around 198–205 nm, suggesting the predominance of a random coil or unordered conformation in highly polar conditions. Similar spectral characteristics

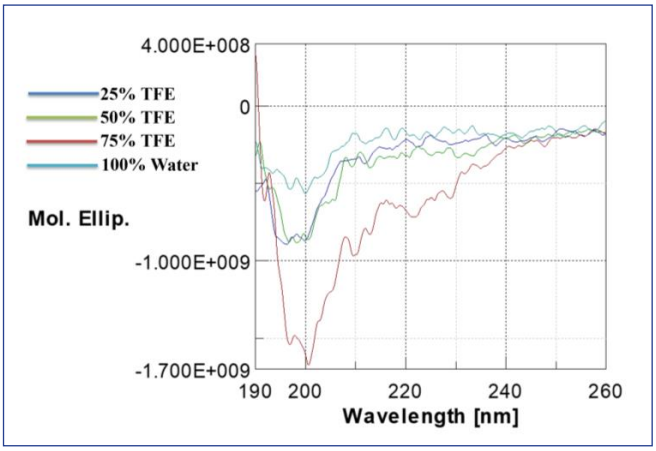

Figure 4: Circular dichroism (CD) spectra of the synthesized MAP under different solvent environments containing varying concentrations of trifluoroethanol (TFE)

were observed in 50% TFE and 25% TFE conditions, although slight changes in ellipticity indicated partial structural rearrangement of the peptide.

Among all solvent conditions, the peptide dissolved in 75% TFE displayed the highest negative molar ellipticity, particularly in the region between 195–205 nm. The pronounced negative band observed in this region suggests enhanced stabilization of ordered secondary structural elements under comparatively hydrophobic solvent conditions. The increased intensity of the negative ellipticity indicates that higher TFE concentration promoted conformational folding and structural organization of the peptide.

Furthermore, gradual spectral shifts observed with increasing TFE concentration demonstrated the sensitivity of the peptide structure to solvent polarity. The transition from aqueous to TFE-rich environments likely reduced peptide-solvent hydrogen bonding interactions and facilitated intramolecular interactions responsible for structural stabilization. Overall, the CD spectral analysis confirmed that the synthesized peptide undergoes solvent-dependent conformational modulation, with higher TFE concentrations favouring a more ordered secondary structural arrangement compared to purely aqueous conditions. Figure 4 shows the spectra result of synthesised MAP in different dilutions.

4. CONCLUSION

The present study represents an initial step toward the development of a novel immune-based sperm sexing approach through the identification and synthesis of a sex-specific peptide using solid-phase peptide synthesis (SPPS). Identification of extracellular epitopic regions is crucial, as these sequences can serve as effective targets for

antibody generation against specific sperm populations. The synthesized peptide may therefore act as a potential antigen for the production of sex-specific antibodies, which could further contribute to the development of alternative immunological sperm sorting techniques beyond conventional flow cytometry-based methods.

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