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Purification of Regucalcin from the Seminal Vesicular Fluid: A Calcium Binding Multi-Functional Protein

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Abstract Regucalcin is a multi-functional protein having roles in calcium homeostasis as well as in anti-apoptotic, anti-prolific and anti-oxidative functions. Recently, it has been reported from the male reproductive tract, but its role in male reproduction needs further investigation; for which the native regucalcin of reproductive origin will be more appropriate. The gel exclusion chromatography followed by diethyl aminoethane cellulose chromatography and two-dimentional cellulose acetate membrane electrophoresis used for its purification are time consuming and less specific. Here, the regucalcin gene from buffalo testis has been cloned, expressed and purified in recombinant form, and subsequently used for raising hyper-immune serum. The Western blot of seminal vesicular fluid probed with anti-regucalcin polyclonal and monoclonal antibodies showed the presence of 28 and 34 kDa bands specific to regucalcin. Further, an affinity matrix has been prepared using anti-regucalcin polyclonal antibodies. An immuno-affinity chromatography method has been standardized to isolate regucalcin from seminal vesicular fluid. The initial complexity of the protein mixture in the seminal vesicular fluid has been reduced by a heat coagulation step. The purified protein on sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed a single band at 68 kDa that has been further confirmed as regucalcin by Liquid chromatography–mass spectrometry/mass spectrometry. The RGN purified from seminal vesicular fluid will be more appropriate for studying its possible role in male reproduction, especially sperm cell capacitation, hyperactivation, acrosome reaction and cryopreservation. The study can be applied in purifying regucalcin from different tissues or species with minor modifications in the methodology.

Keywords Buffalo · Regucalcin · Purification · Seminal vesicular fluid · Affinity chromatography

Abbreviations

His	Histidine
IAC	Immuno-affinity chromatography
PCR	Polymerase chain reaction
RGN	Regucalcin
rRGN	Recombinant regucalcin
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SUMO	Small ubiquitin-like modifier

1 Introduction

Regucalcin (RGN) is a calcium binding protein having multiple functions [1]. It maintains calcium homeostasis in the various cellular organelles through the regulation of Ca^{2+} transport activity [2] and functions as a multi-functional protein associated with many intracellular signaling pathways through the regulation of several Ca^{2+}-dependent enzymes [3]. Further, the RGN can mediate cell death and proliferation by modulating DNA synthesis and fragmentation [4, 5] and can influence the survival and apoptotic pathways [6–8]. Many studies demonstrated the antioxidant properties of RGN [7, 9, 10] by modulating the

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activity of enzymes required in the generation of oxidative stress and the antioxidant defense system [11–17]. In addition, RGN has been identified as the gluconolactonase enzyme in liver [18] which is involved in the synthesis of L-ascorbic acid that scavenges the free radicals [19].

A broad expression of RGN in all types of testicular cells, glandular epithelium of bulbouterine and prostate gland was confirmed by immunohistochemistry in rat, bovine and human [20, 21]. This common interspecies localization of RGN suggests its role in male reproductive physiology. In rat prostate, the overexpression of RGN resulted in inhibition of cell divsion and apoptotosis suggesting its role in maintaining prostate cancer [16]. It has also been detected in seminiferous tubular fluid [20] which is mainly secreted from Sertoli cells [22]. The semiferous tuibular fluid creates the ideal conditions for spermatogonial development [22, 23]. Thus, the broad expression of RGN along the male reproductive tract suggests that it has a significant role in male reproduction.

Furthermore, the *RGN* has been identified as androgen-target gene in rat testis [20] which might play a role in spermatogenesis directly or indirectly [24]. Recently, its expression has been used as a diagnostic tool for the illicit use of steroids in veal calves and beef cattle [25]. However, the scientific literature on effect of RGN in the male reproductive system of domestic animals, especially in semen and spermatozoa are very limited. The RGN has been shown to be present in multiple forms in rat liver and ejaculated buffalo spermatozoa [26, 27]. The protein can enter cells and modulate the activity of several enzymes, including protein kinases and phosphatases. Further, the role of RGN in the epidedimal maturation of spermatozoa has been established [28]. Now, it will be interesting to study its role in mature spermatozoa after ejaculation. Hence, the use of RGN purified from the seminal vesicular fluid (a major constituent of seminal plasma) would give more reliable data than RGN purified from other sources in studying its role in mature spermatozoa. Earlier, native RGN has been purified by gel exclusion chromatography followed by Diethylaminoethane cellulose chromatography [1] and by two-dimensional cellulose acetate membrane electrophoresis [29] from rat liver homogenate. Both of these processes are time consuming as well as less specific compared to immuno-affinity chromatography. As there are reports of tissue-specific isoforms of many genes, it is advisable to conduct fertility studies using protein of reproductive origin. In our study, we detected the secretion of RGN into the seminal vesicular fluid of buffalo in good concentration. Thus, the purification of this secreted protein will be more appropriate for fertility studies as the secretory forms are likely to have slight or major modifications compared to the cytosolic form. Further studies using the purified native RGN can be used to improve the fertility of buffalo where infertility is a major problem. The objective of the present study is to develop an immuno-affinity methodology to purify RGN from seminal vesicular fluid.

2 Materials and Methods

2.1 Collection of Seminal Vesicular Fluid

Vesicular fluid was collected from the seminal vesicles of buffaloes collected from slaughter house by cutting open the ducts of the gland and flushing with PBS. The fluid thus collected was centrifuged at 10,000×*g* for 20 min at 4 °C to remove all cell debri and stored at −20 °C.

2.2 Expression and Purification of Recombinant RGN

The total RNA was isolated from 100 mg of testis of an adult healthy buffalo from a slaughterhouse in Bareilly (Uttar Pradesh), India, using RNA isolation system (Promega, USA). Subsequently, cDNA was synthesized using Revert Aid^{TM} H-Minus First Strand cDNA Synthesis kit (Fermentas, USA). Gene specific primers were designed using Primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and were modified by the addition of 18 nucleotides at the 5′-ends of both the primers to ensure the cloning of the amplified gene into pRham^{TM} N-His SUMO Vector (Lucigen, USA) by homologous recombination (indicated in lower case). The forward primer 5′-cgcgaacagattggaggtGAGTGTGTTTTGCGGGAGAAC-3′ and reverse primer 5′-gtggcggccgctctattaTCCTGTGTAAGGATAGGGAGG-3′ were synthesized (Imperial Biomed, USA). The PCR (Polymerase chain reaction) mixture of 25 µL volumes, consisting of 1× *pfu* buffer with 20 mM $MgSO_4$, 0.2 mM of dNTP mixture, 50 pmol each of forward and reverse primers, 200 ng of buffalo testis cDNA as template and 2 U of *pfu* DNA polymerase enzyme. The reaction mix was then subjected to initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. After gel purification of the PCR amplicon, the transformation into *E. cloni* 10G competent cells was done using Expresso^{TM} Rhamnose Small ubiquitin-like modifier (SUMO) Cloning and Expression system (Lucigen, USA) as per the recommended protocol. The recombinant clones were selected on the basis of their kanamycin resistance indicated by their growth on Luria Bertanni-Kanamycin (30 mg/mL) plates and later the colonies were reconfirmed by colony PCR and sequencing using gene specific primers

following the above mentioned thermal cycling conditions. The sequence data are available in the NCBI GenBank database (KJ948109). Further, the sequence data was submitted to ExPaSy (http://web.expasy.org/cgi-bin/protparam/protparam) for protein parameter analysis.

One of the positive clones was cultured in Luria Bertanni-Kanamycin medium followed by induction with 0.2 % of Rhamnose after reaching the absorbance of the culture medium to 0.8 measured at 600 nm. In order to confirm the RGN expression, both induced and uninduced bacterial cultures were analyzed by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and followed by Western blotting using His Probe-HRPase conjugate (1:5000, Thermo Scientific, USA). The induced culture pellet was then resuspended in guanidine lysis buffer (6 M Guanidine HCl, 100 mM NaH_2PO_4, 10 mM Tris-Cl) and kept overnight on a dancing shaker at room temperature. Following lysis, the culture was centrifuged at 18,000×*g* for 15 min at 4 °C to remove the cell debris and the supernatant was filtered using a 0.44 µm disposable filter (Micro-POR, India). Subsequently, rRGN was purified using Nickel-nitrilotriacetic acid agarose column (Qiagen, USA) following the recommended protocol. A 10 mL final eluent was then concentrated to 2 mL using 10 kDa cutoff ultra-filter (Corning, USA). The SUMO-His tags were cleaved off using SUMO protease according to the manufacturer's instruction and tag free rRGN was purified and analyzed by SDS–PAGE. The quantification of purified rRGN was done using a Lowry's method [30].

2.3 Raising of Hyperimmune Sera against the Recombinant RGN

The polyclonal antibody against rRGN was produced by immunizing mice with an initial dose of 100 µg of rRGN in Freund's incomplete adjuvant followed by the booster doses of 50 µg of rRGN along with Freund's incomplete adjuvant on 14th, 21st and 28th day. Sera collected on the 7th day after the final booster was checked by Western blot at 1:100 dilution by loading both purified rRGN and seminal vesicular fluid. The specificity of the polyclonal sera was reconfirmed by comparing Western blot of seminal vesicular fluid with preimmune sera and commercially available monoclonal antibodies (ab67336, Abcam, UK) at 1: 1000 dilution.

2.4 Coupling of Anti-RGN Antibody to Cyanogen Bromide Activated Sepharose

The immunoglobulins were purified from the sera using an Immunoglobulin purification kit (GeNei, India) as per the manufacturer's instruction. The purified anti-RGN antibody was coupled to Cyanogen bromide activated Sepharose (Sigma, USA). The antibody purified was dialyzed against coupling buffer (0.1 M NaHCO3, 0.5 M NaCl, pH 8.3) at 4 °C. One g of Cyanogen bromide activated Sepharose 4B (Sigma, USA) was swollen in 100 mL of 1 mM HCl followed by washing with 250 mL of 1 mM HCl. The beads were then transferred to 15 mL centrifuge tube containing 10 mg dialyzed antibody. The mixture was allowed for overnight coupling at 4 °C with shaking. The supernatant after centrifugation at 2500×*g* for 15 min at 4 °C was taken out and its absorbance was measured at 280 nm to check the efficiency of coupling. The slurry was reconstituted with 0.2 M glycine in coupling buffer and kept at 4 °C for overnight in order to block the unbound sites. The antibody coupled matrix was then transferred to a 20 mL plastic column (BioRad, USA) and 0.2 M Glycine was allowed to pass through it followed by alternate washing of packed column with 30 mL of acetate buffer (0.1 M Sodium acetate and 0.5 M NaCl solution; pH 4.0) and borate buffer (0.1 M Sodium borate and 0.5 M NaCl solution, pH 8.0) and finally with 20 mL coupling buffer. The prepared column was stored at 4 °C in coupling buffer with 0.025 % Sodium azide.

2.5 Purification of RGN from the Seminal Vesicular Fluid by Immuno-affinity Chromatography (IAC)

The initial complexity of the protein mixture in the seminal vesicular fluid has been reduced by a heat coagulation step wherein a 1 mL of seminal vesicular fluid was heated at 60 °C for 10 min. The resulting pellet was discarded after centrifuging at 38,000×*g* for 20 min at 4 °C. Hereafter all the procedures for RGN isolation were carried out at 4 °C. The supernatant was diluted with 4 volumes of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and dialyzed against column equilibration buffer (50 mM Tris buffer, pH 7.4). The anti-RGN Sepharose column was activated by passing 35 mL of 2.0 M NaCl. The column equilibration was done by passing 30 mL of equilibration buffer and the optical density of dialyzed seminal vesicular fluid was taken at 280 nm to check the binding of RGN. The dialyzed seminal vesicular fluid was loaded on the top of the matrix and the protein was allowed to bind to the column by incubating overnight with end to end mixing. Washing of the column was done with 40 mL equilibration buffer. Isolation of RGN was done with elution buffer (0.2 M Glycine, pH 2.3–2.5) in 2 mL aliquots and immediately neutralized using 1 M Tris base. The optical density of elutes was taken at 280 nm and pooled fractions were concentrated by ultra-filtration column of 10 kDa cutoff

(Spin-X UF, Corning, England) to 1 mL volume. The protein concentration was estimated [30]. The protein, thus isolated was identified against protein molecular weight markers on SDS–PAGE [31] and confirmed on Western blot [32] probed with anti-RGN monoclonal antibody. The corresponding band from SDS–PAGE was cut and sent for Liquid Chromatography Mass Spectrometry/Mass Spectrometry analysis for protein identification (Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India).

3 Results

3.1 Cloning, Expression and Characterization of Recombinant RGN

The 917 bp sized PCR product of *RGN* was successfully amplified (Fig. 1a) that includes the primer flanking sequence for cloning of the amplified gene into pRham^{TM} N-His SUMO Vector (Lucigen, USA). The recombinant clone upon sequencing showed 881 bp of *RGN* sequence (GenBank Accession KJ948109). In protein expression studies, one of the selected clones showed optimum expression of 51 kDa fusion protein after 10 h of post-induction on SDS–PAGE (Fig. 1b) and Western blot (Fig. 1c). After purification, the elutes of the expressed recombinant fusion protein showed the specific protein band on SDS–PAGE (Fig. 1d). The SUMO-His tags of the fusion rRGN (Recombinant regucalcin) were cleaved off using SUMO protease and the cleaved protein showed a molecular weight of 33 kDa on SDS–PAGE (Fig. 2a) and Western blotting (Fig. 2b).

3.2 Characterization of RGN Hyperimmune Sera

The hyperimmune sera raised against the rRGN was characterized by Western blotting wherein it showed a specific immunoreactive band of expected 33 kDa size with rRGN protein. The Western blot of seminal vesicular fluid probed with polyclonal and monoclonal antibodies showed the presence of 28 and 34 kDa immunoreacting bands specific to RGN as shown in Fig. 3a, b. The Western blot using preimmune sera with the same dilution failed to give any immunoreactive band, confirms the specificity of the polyclonal sera.

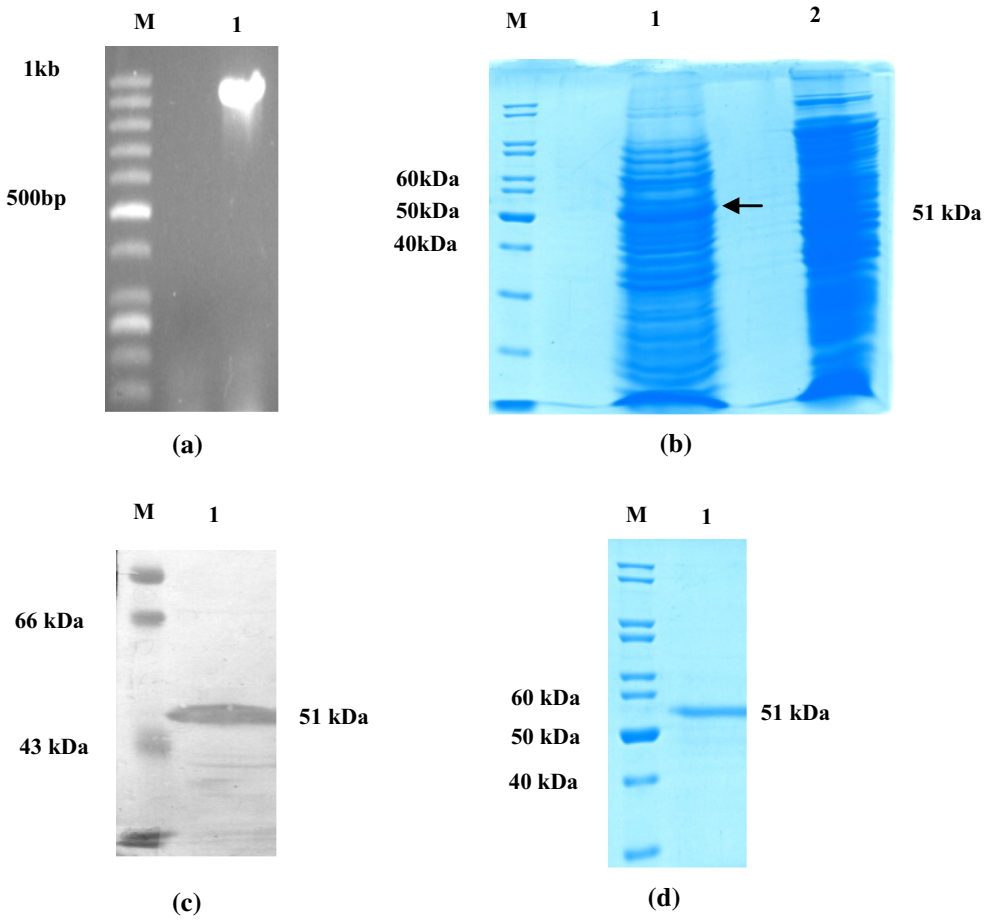

Fig. 1 **a** 1 % agarose gel showing PCR amplicon of buffalo *RGN* gene amplified from cDNA of buffalo testis. Lane M: 50 bp DNA ladder, Lane 1: *RGN* amplicon of 917 bp; **b** 12 % SDS–PAGE showing expression of *rRgn* in *E. cloni* 10G cells. Lane M: Unstained marker, Lane 1: Culture pellet after 10 h post-induction using rhamnose, Lane 2: Uninduced culture pellet; **c** Western blot showing the immune reactive band of recombinant RGN probed with Ni-activated Horse Reddish Preroxidase conjugate. Lane M: Pre-stained protein marker, Lane 1: ~51 kDa rRGN; **d** 12 % SDS–PAGE showing purified rRGN fusion protein using Nickel-nitrilotriacetic acid column. Lane M: Unstained marker, Lane 1: Purified rRGN fusion protein

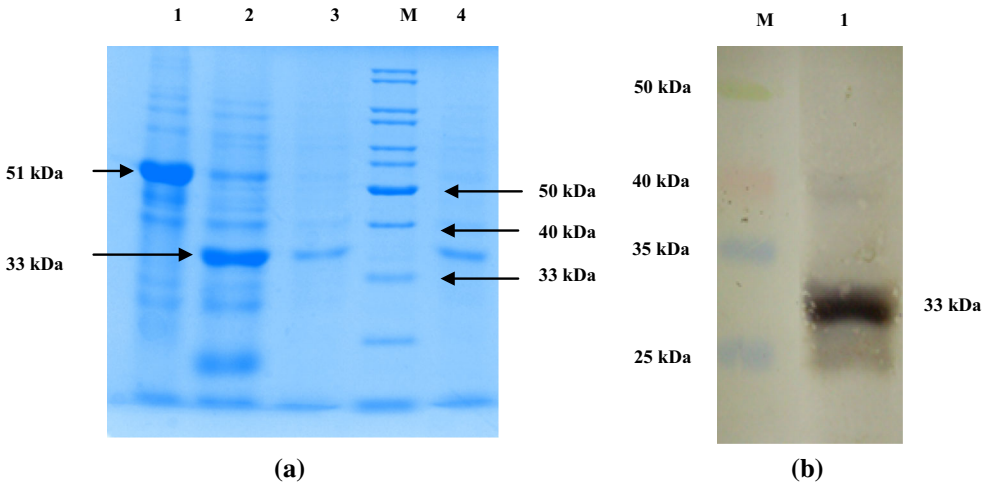

Fig. 2 **a** SDS–PAGE showing cleavage and purification of *rRgn*. Lane M: Unstained protein marker. Lane 1: Uncleaved rRGN fusion protein, Lane 2: rRGN of ~33 kDa after cleavage, Lane 3: Flow through containing purified rRGN of ~33 kDa, Lane 4: Wash containing purified rRgn of ~33 kDa; **b** Western blot showing an immune reactive band of rRGN probed with anti-RGN polyclonal Ab. Lane M: Pre-stained protein marker, Lane 1: Cleaved rRGN

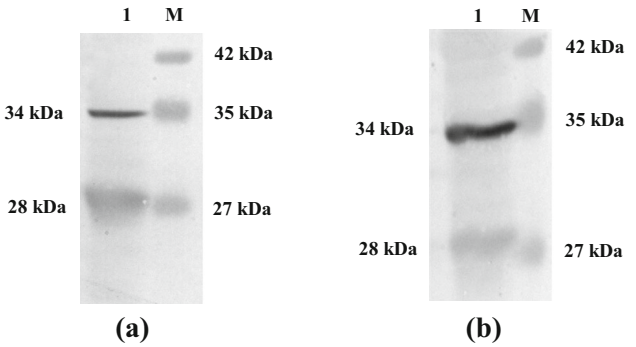

Fig. 3 Western blot of seminal vesicular fluid showing specific immune reactive bands at 34 and 28 kDa of native RGN. **a** Probed with anti-RGN polyclonal Ab. Lane M: Pre-stained marker, Lane 1: Seminal vesicular fluid; **b** Probed with anti-RGN monoclonal Ab. Lane M: Pre-stained marker, Lane 1: Seminal vesicular fluid

3.3 Purification and Characterization of RGN from the Seminal Vesicular Fluid Using Immuno-Affinity Chromatography

The concentration of purified anti-RGN antibodies was estimated to be 5.4 mg/mL. The coupling efficiency of anti-RGN antibodies to the Cyanogen bromide activated Sepharose was found to be 97 %. The presence of RGN in the supernatant of seminal vesicular fluid after heat treatment was confirmed by Western blot, wherein the RGN specific bands viz., 28, 34 and >51 kDa bands were observed only with supernatant (Fig. 4a). The supernatant thus obtained was passed through the anti-RGN IAC column and elution was carried out using 0.2 M glycine buffer in 2 mL aliquots. Subsequently, the elutes of anti-RGN IAC after loading on SDS–PAGE showed a single band at ~68 kDa (Fig. 4b). The protein was further confirmed by Liquid Chromatography Mass Spectrometry/Mass Spectrometry (Table 1).

4 Discussion

There are previous reports of expression of rRGN of rat [33], buffalo (27) and human [34]. Here, we had expressed buffalo RGN in a prokaryotic system to raise the hyper-immune serum which was subsequently used for generating IAC matrix for purifying the native RGN. A 917 bp PCR product had been amplified, including primer flanking sequence of 36 bp and actual coding 881 bp DNA sequence of buffalo *RGN*. The amplified buffalo *RGN* gene contained an open reading frame of 294 amino acids, which is 5 amino acids truncated from N-terminal-portion of the native RGN. The formation of low molecular weight isoforms of RGN having 28 and 24 kDa by proteolytic cleavage at the N-terminal end is reported [26], which oversees the possibility of retaining functionality even after the processing of the N-terminal region. The expected molecular weight of the actual rRGN protein sequence devoid of tag sequence is ~32.7 kDa as predicted by ExPASy (http://web.expasy.org/cgi-bin/protparam/protparam). The free 6xHis-SUMO tag at the N-terminus of the expressed protein with an engineered cleavage site; has an expected molecular weight of 12 kDa, but move as ~15–18 kDa. Thus, the total molecular weight of expressed rRGN is ~50.7 kDa.

Commercially available polyclonal and monoclonal anti-RGN antibodies are against recombinant human RGN.

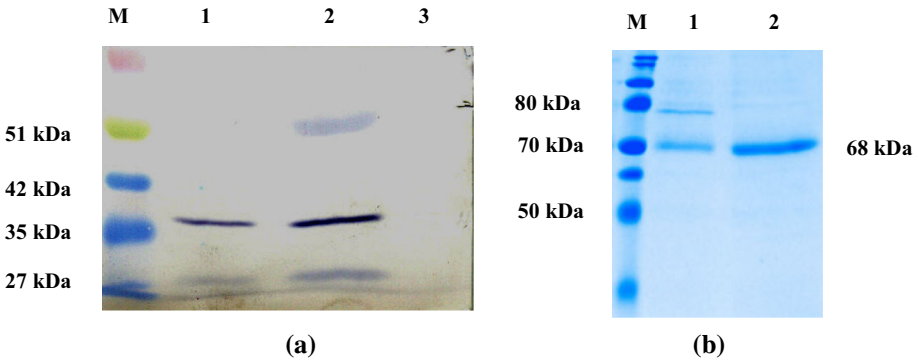

Fig. 4 **a** Western blot analysis of RGN after heat treatment of seminal vesicular fluid. Lane M: Pre-stained protein marker, Lane 1: Seminal vesicular fluid before heat treatment, Lane 2: Supernatant of heat treated seminal vesicular fluid, Lane 3: Pellet of seminal vesicular fluid after heat treatment. **b** 12 % SDS–PAGE of elutes of anti-RGN immune-affinity chromatography. Lane M: Protein marker, Lane 1–2: Elutes of anti-RGN affinity column

Table 1 Data of LC MS/MS of elute of anti-RGN immune affinity chromatography

Peptide sequence	% Coverage	Theoretical molecular mass (Da)	Theoretical pI	Protein score	Protein identified
PVSSVALR VTVDAPVSSVALR PEAGGIFK GLLQQPEAGGIFK	8.7 %	33,878.07	7	110.048	Regucalcin

It has generally been advised to develop homologous antibodies for each individual species under investigation in order to obtain more reliable quantitative data. The hyperimmune sera raised against the rRGN showed specific immunoreactivity with the recombinant protein as well as with the native RGN in the seminal vesicular fluid upon Western blotting. However, the preimmune sera did not show specific reactivity. The appearance of similar specific bands of 34 and 28 kDa of native RGN in seminal vesicular fluid on Western blot probed with both commercially available monoclonal and raised polyclonal antibodies further confirms the specificity of the raised antibody. This is the first report of the presence of RGN in the seminal vesicular fluid. Similar isoforms of RGN are reported in rat liver tissue [35].

The heat purification step which was used in purifying RGN from rat liver [1] also worked well for seminal vesicular fluid. Both 34 and 28 kDa specific RGN isoforms appeared in the supernatant fraction, while both were not detected in the pellet. These results are in line with what have been reported in rat liver and cell lines [26]. Thus, the complexity of the protein mixture in the seminal vesicular fluid was reduced by the heating step which was subsequently used for IAC. The elutes in SDS–PAGE showed a single band at 68 kDa in Coomassie R-250 staining. The acidic pH, used for elution of the protein from the IAC has been speculated to facilitate a conformational change in the protein favoring the dimerisation [36, 37]. Further, this protein had been confirmed as RGN by Liquid Chromatography Mass Spectrometry/Mass Spectrometry and has an average molecular weight of 33,878.07 Da suggesting that the 68 kDa protein is a dimeric form of RGN.

The spermatozoa needs to undergo many physiological (Capacitation, hyperactivation and acrosome reaction) and non-physiological (Freezing and thawing) adaptations before fertilization wherein the calcium levels play an important role. Intracellular calcium levels increase in sperm during capacitation [38], hyperactivation [39] and the zona pellucida—induced acrosome reaction [40, 41]. Recent research suggests that the calcium regulatory mechanisms during capacitation [38] and the acrosome reaction [41] are complex, involving intracellular stores and voltage-dependent calcium channels. Disruption of capacitation and/or the acrosome reaction would severely compromise the fertilizing potential of spermatozoa [40]. The semen is commonly subjected to cryopreservation (a non-physiological process) for future breeding purposes. The cryopreserved sperm show elevated intracellular calcium levels that may be due to the reduced capacity to maintain normal concentrations of this cation [42–44] resulting in the poorer fertility of post-thaw sperm. The RGN being a calcium regulating protein, needs to be studied for its role in spermatozoa after ejaculation and during fertilization. The RGN purified from the seminal vesicular fluid will be more appropriate for studying its possible role in male reproduction, especially sperm cell

capacitation, hyperactivation, acrosome reaction and cryopreservation. This study can be beneficial for researchers in purifying RGN from different tissues with minor modifications in the methodology, which in turn help in further understanding the role of RGN in male reproduction.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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