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Purification and identification of transglutaminase from mouse coagulating gland and its cross-linking activity among seminal vesicle secretion proteins

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ABSTRACT

A 75-kDa protein secreted from mouse coagulating gland was purified to homogeneity by a series of isolation steps including ion exchange chromatography on a DEAE-Sephacel column and ion exchange high-performance liquid chromatography on a sulfopropyl column. It was identified to be Type IV transglutaminase (TG_4), based on the establishment of N-terminal sequences by automated Edman degradation together with partial sequences by MS analysis. Its cross-linking activity was tested on the reduced sample of mouse seminal secretion which contained seven major monomer proteins tentatively designated as SVS I–VII. The enzyme was able to cross-link any of SVS I–III but failed to cross-link the other SVS proteins with a M_r value less than 14 kDa. SVS I and SVS III showed comparable substrate activity, but were much weaker than SVS II during the TG_4 catalysis.

1. Introduction

Upon ejaculation, seminal vesicle secretion (SVS) constitutes the major portion of seminal plasma that is coagulated in a substantial number of mammalian species, including many myomorphic rodents, some moles, hedgehogs, marsupials, rabbits, stallions, boars and several primates [1]. The deposition of semen coagulum in animals such as rodents into the vagina at coitus results in the formation of a copulatory plug that occludes the vaginal barrel close to the uterine cervix. It should be noted that extirpation of seminal vesicle and coagulating gland from mice and rats prevents formation of the copulatory plug and this results in greatly reduced fertility [2,3], manifesting the indispensible roles of these two male accessory sexual glands in seminal coagulation.

Transglutaminase (TG; EC2.3.2.13) catalyzes protein cross-links via isopeptide formation [4]. This enzymatic reaction is generally believed to be essential for the formation of a semisolid gelatinous mass in human semen [5,6] or the seminal clotting in rodent semen [7,8]. Such enzyme activity has been illustrated in the human prostate [9] and in rat coagulating gland secretions (CGS) [10]. Although it has been shown that incubation of mouse SVS with TG of guinea pig liver (TG_2) result in protein cross-linking [8,11], TG_2 is not the actual enzyme involved in seminal coagulation during natural coitus. Rather, TG_4 from male sexual gland is responsible for this reproductive event. Therefore, it becomes a pre-requisite to purify TG_4 in order to study how it cross-links the SVS proteins. Mice are good experimental animals for this study from two view points. Firstly, purification of TG_4 from CGS in quantity is feasible. Secondly, some progress has been made on the analysis of the mouse SVS proteins that had been shown to consist of several minor proteins such as SVA [12], P12 [13], Ceacam 10 [14], and seven well-resolved monomer proteins designated SVS I–VII in the decreasing order of M_r values (95,000–8000) according to their mobility on reduced SDS–PAGE [15,16]. These results are an added advantage to identify the TG_4 substrates in the SVS. This work aims to purify and identify TG_4 from mouse CGS, and determine its protein substrates among mouse SVS proteins.

2. Materials and methods

The following materials were obtained from commercial sources: DEAE-Sephacel (Amersham Corp., Buckinghamshire, UK

Abbreviations: A25 peptide, biotin-TVQQEL; $BPNH_2$, 5-(biotinamido) pentylamine; CGS, coagulating gland secretion; DTT, dithiothreitol; GST, glutathione S-transferase; PBST, phosphate buffer saline containing 0.1% Tween 20; PMSF, phenylmethylsulphonyl fluoride; SVS, seminal vesicle secretion; TG_4, type IV transglutaminase.

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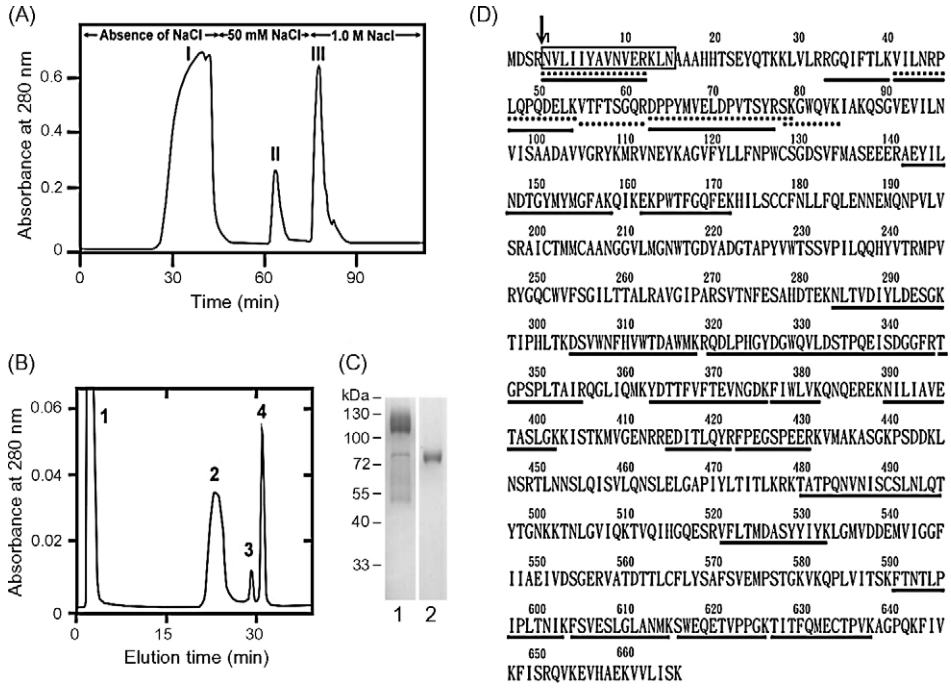

Fig. 1. Determination of partial sequences for a 75-kDa TG_4 purified from mouse CGS. (A) The soluble CGS was resolved into three fractions by ion exchange chromatography on a DEAE-Sephacel column. (B) Fraction II was further subjected to ion exchange high-performance liquid chromatography (HPLC) on a sulfopropyl (SP) column. (C) The purified protein was identified on an 8% reducing SDS–PAGE stained with Coomassie brilliant blue: lane 1, total CGS proteins and lane 2, peak 4 of (B). (D) The N-terminal sequences of peak 4 on (B) were determined by Edman degradation (box). Five peptide sequences were established from the MS-fit search for well-defined peaks on the MALDI-TOF spectrum of the trypsin-digested peak 4 (dotted lines), and 21 peptide sequences were obtained from the analysis by capillary column chromatography coupled with ESI-MS/MS for the trypsin digests on the MS spectral pattern (solid lines). All of these partial sequences matched perfectly to those found in the protein sequence deduced from the reading frame of the *TG_4* cDNA (GenBank accession number NP808579), which are specified by one-letter code numbered from the N-terminus. The cleavage point for the generation of the mature protein is indicated by arrow.

and Amersham Pharmacia Biotech, Uppsala, Sweden); Protein PAK SP 5PW column (Waters, Milford, MA); O-nitrophenyl-β-D-galactopyranoside and streptavidin-β-galactosidase (CALBIOCHEM, San Diego, CA); BCA protein assay kit and $BPNH_2$ (EZ-Link) (Pierce Chemical Co., Rockford, IL); PMSF (Sigma Chemical Co., St. Louis, MO); flat-bottom high binding 96-well enzyme immunoassay microtiter plates (catalog no. 442404) (NUNU, Roskilde, Denmark). The A25 peptide was synthesized according to the previous studies [17]. All other chemicals were reagent grade.

2.1. Preparation of SVS and CGS

Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA) and were maintained and bred in the animal center at the College of Medicine, National Taiwan University. The animals were treated according to the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14L:10D) at 21–22 °C and were provided with water and NIH-31 laboratory mouse chow *ad libitum*. Normal adult mice (8–12 wk old) were sacrificed by cervical dislocation. SVS and the CGS were squeezed individually into ice-cold 10 mM Tris–HCl, pH 8.0 in the presence of 1 mM PMSF and centrifuged at 8000 × *g* for 20 min at 4 °C to remove the precipitate.

2.2. Separation of CGS proteins

The soluble CGS collected from 50 mice was resolved by ion exchange chromatography on a DEAE-Sephacel column (12 cm × 2.6 cm) pre-equilibrated with 10 mM Tris–HCl, pH 8.0. After the non-retarded fractions were washed out, the column was sequentially eluted with 50 mM and 1.0 M NaCl in the same buffer at a flow rate of 1.0 mL/min. Fractions (2 mL) were collected and their absorbance at 280 nm was recorded (Fig. 1A). Fraction II of Fig. 1A was further subjected to ion exchange high-performance liquid chromatography (HPLC) on a sulfopropyl (SP) column (7.5 cm × 7.5 mm). The column was eluted with a linear gradient of 0–1.0 M NaCl in 25 mM sodium acetate, pH 6.0 at a flow rate of 1.0 mL/min for 40 min (Fig. 1B).

2.3. Assay for the substrate activity of SVS proteins during the TG_4 catalysis

Freshly prepared SVS was reduced in 50 mM Tris–HCl containing 10 mM DTT, pH 7.5 for 15 min at 37 °C. The reduced SVS was incubated with 1.6–100 μg/mL of TG_4 for 60 min at 37 °C. The reaction solution was then mixed with an equal volume of SDS–PAGE sample buffer containing 100 mM DTT and boiled before electrophoresis to detect the un-reacted protein components by resolving the proteins on a 12% reducing SDS–PAGE (6.5 cm × 10.5 cm × 0.075 cm). Based on the protein-staining pattern on the polyacrylamide gel, the amount of each un-reacted SVS protein component in one reaction was normalized to give a percentage (P) of its total amount present in the control. In terms of this approach, the value ($1 - P$) determined for each protein component reflected its percentage being cross-linked by the enzyme.

2.4. Solid-phase microtiter assay

We modified the solid-phase assay for the cross-linking activity of TG [18]. Microtiter plates were coated with 100 μL of a chimeric polypeptide of GST-fused SVS III residues 116–145 [11] (0–25 μg/mL) in 50 mM carbonate buffer, pH 9.6. After the unbound protein was discarded, the well was blocked with 0.3% GST in 50 mM Tris–HCl, pH 8.5 for 60 min, because we found that either

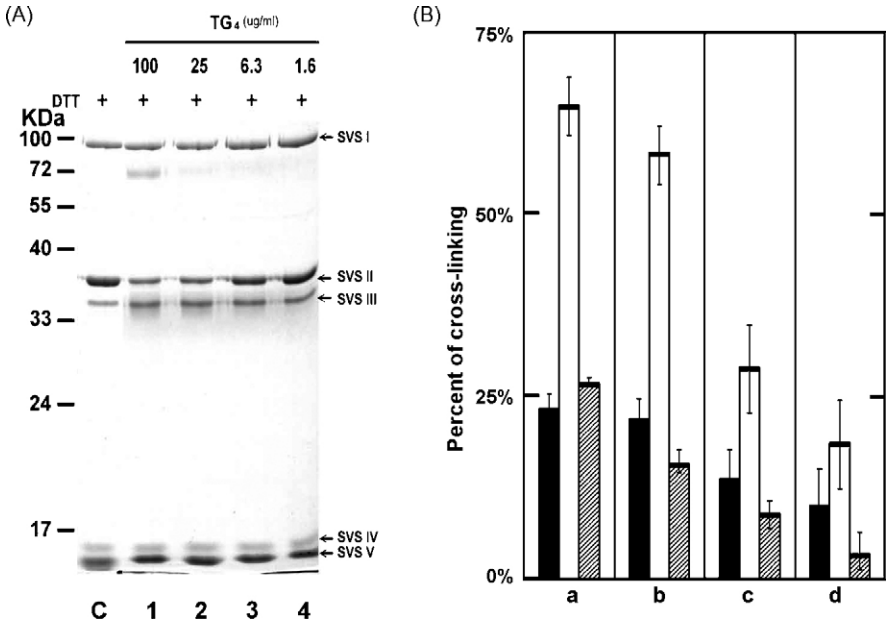

Fig. 2. The cross-links of SVS proteins by TG_4. (A) The reduced SVS was incubated with 1.6–100 μg/mL of TG_4 enzyme for 60 min at 37 °C. The un-reacted protein components were determined by a reduced SDS–PAGE. The gel was stained with Coomassie brilliant blue to reveal the protein bands; lane C was the control and (B) based on the staining pattern, the percentage of cross-linking was estimated from the catalysis at an enzyme dose of 100 μg/mL (panel a), 25 μg/mL (panel b), 6.3 μg/mL (panel c) and 1.6 μg/mL (panel d) for SVS I (solid columns), SVS II (open columns), and SVS III (hatch columns). The result represents the average of three determinations and error bars the S.D. (see Section 2).

3% non-fat skim milk or 3% BSA in the same buffer as the blocking solution gave extremely high background. The plates were washed three times with 250 μL of Tris buffer containing 150 mM NaCl. The incorporation of $BPNH_2$ and A25 peptide into the recombinant polypeptide by TG_4 was continued in 50 mM Tris–HCl containing 10 mM DTT and 10 mM $CaCl_2$, pH 7.5 for 60 min. The reaction was stopped by 200 mM EDTA, pH 8.0 followed by three washes with PBS containing 0.1% Tween (PBST). Streptavidin-β-D-galactosidase (0.5 mg/mL) diluted 1:500 with PBST was added (100 μL/well) and incubated for 60 min at room temperature. The plate was washed four times with 250 μL of PBST prior to the addition of 150 μL of 0.1% O-nitrophenyl-β-D-galactopyranoside in 50 mM sodium phosphate containing 1.5 mM $MgCl_2$, pH 7.2. The enzyme hydrolysis was allowed to proceed for 15 min and then stopped by the addition of 150 μL of 1.0 M Na_2CO_3. The absorbance at 405 nm due to the formation of O-nitrophenol was determined. Reactions were performed in triplicate.

2.5. Analytical methods

Protein concentrations were determined using the BCA protein assay [19] according to the manufacturer's instruction. Proteins were resolved by reducing SDS–PAGE on a gel slab [20]. The protein in the gel slice was subjected to automated Edman degradation by means of a LC 492 Protein sequence System (Applied Biosystem) to determine the N-terminal sequences. Further, we determined the partial sequence of the protein by MS analysis. The protein sample was incubated with trypsin (0.1 mg/mL) in 25 mM NH_4HCO_3 at 37 °C overnight. The enzyme digests were dried. The resulting peptide samples were resuspended in 10% formic acid, desalted and concentrated using Zip-Tips (Millipore, Bedford, MA) for MS analysis.

A Q-TOF mass spectrometer was equipped with an electrospray probe (Micromass, Manchester, UK) and interfaced with a Series 1100 HPLC binary pump with an online degasser and an autosampler (Agilent Technologies, Wilmington, DE, USA). The system control, data acquisition and processing were carried out using Masslynx software (v 3.4) on a Microsoft Window NT system. Gradient chromatographic conditions were established using an Agilent Zorbax C8 (2.1 μm × 50 μm; 5 μm size) analytical column (Agilent Technologies). The trypsin digests were analyzed with ESI using MS/MS in the positive-ion acquisition mode. Nitrogen served as both the nebulizing and curtain gas. The ESI capillary (Micromass) was set at 3.5 kV while the MS analysis was conducted at a cone voltage of 52 V with a scan time of 1 s, inter-scan delay of 0.1 s and a scan range of 200–500 Da. The peptide sequences were established from the spectral peaks by a MS-fit search program and matched to the peptide sequences of mouse-specific proteins in a non-redundant protein database (NCBI) using the MASCOT on-line package.

3. Results

3.1. Purification and identification of TG_4 in mouse CGS

The soluble fraction of CGS was separated into fractions I–III by ion exchange chromatography on a DEAE-Sephacel column (Fig. 1A). Fraction II was further resolved into peaks 1–4 by ion exchange HPLC on a SP column (Fig. 1B). Peak 4 gave a single 75-kDa protein band on an 8% reducing SDS–PAGE, suggesting that it was purified to homogeneity (*cf.* lanes 1 and 2 of Fig. 1C). The reliable data from automated Edman degradation of this protein were assembled to a N-terminal sequence of ^{1}NVLIIYAVNVERKLN^{15}, which agrees totally with the mouse TG_4-deduced protein sequence at all positions (Fig. 1D, box). Further, we digested peak 4 with trypsin and determined the molecular mass of each fragment by MALDI-TOF. The spectral pattern revealed several well-defined peaks viz. peak a (*m/z* 832.3), peak b (*m/z* 895.4), peak c (*m/z* 1402.8), peak d (*m/z* 1662.9) and peak e (*m/z* 1997.0) with the ratio of molecular mass to charge cited within the parentheses. The MS-fit search result confirmed ^{78}SKGWQVK^{84} for peak a, ^{55}VTFTSGQR^{62} for peak b, ^{1}NVLIIYAVNVER^{12} for peak c, ^{41}VILNRPLQPQDELK^{54} for peak d and ^{63}DPPYMVELDPVTSYRSK^{79} for peak e based on their match

with the theoretical mass established for each peptide, as predicted from the trypsin digestion of *TG_4*-deduced protein sequence (Fig. 1D, dotted lines). In addition, several trypsin digests from the mass spectrum were analyzed using capillary column LC ESI-MS/MS to establish 21 peptide sequences that are in complete alignment with the deduced protein sequence (Fig. 1D, solid lines). Overall, peak 4 consisting of 666 amino acid residues is derived from the *TG_4* gene with post-translational cleavage at the peptide bond between R and N in the signal peptide.

3.2. The TG_4 protein substrates in SVS

The cross-linking activity of TG_4 was tested on the SVS proteins. SVS was reduced with DTT before incubation with the enzyme. Following the enzyme reaction, further cleavage of protein disulfide bonds by DTT was tracked on a reducing SDS–PAGE in order to detect the residual proteins that were not cross-linked by the enzyme. In the absence of enzyme, the staining intensity of each protein component on the polyacrylamide gel (lane C in Fig. 2A) represented its total amount available for the enzyme catalysis. Since an isopeptide-linked complex could be distinguished from the un-reacted proteins based on their molecular size, a high level of enzyme-catalyzed cross-links with increased enzyme dose lead to the presence of a lowered amount of un-reacted proteins when detected by SDS–PAGE. Precisely, the above pattern was observed with each of the SVS I–III proteins after incubation with the enzyme (*cf.* lanes 1–4 of Fig. 2A). Although SVS IV could be cross-linked by tissue type transglutaminase (TG_2) according to the previous study [21], it was not cross-linked even at a high dose of TG_4 (100 μg/mL). Obviously, SVS IV was not a good TG_4 substrate. TG_4 failed to cross-link any of the other SVS proteins with a M_r value less than 14 kDa. Based on the protein-staining pattern on the polyacrylamide gel, the amount of each un-reacted SVS I–III in one reaction was normalized to give a percentage (P) of the total amount present in the control. In terms of this approach, the value $(1 - P)$ determined for each protein component reflected its percentage being cross-linked by the enzyme. As shown in Fig. 2B, TG_4 showed unequal cross-linking activity for SVS I–III. Their catalytic behavior manifested that SVS I and SVS III showed comparable substrate activity but they were much weaker than SVS II during the TG_4 catalysis.

Transformation of glutamine residue by deamination to an activated acyl group, that facilitates the nucleophilic attack by a ε-amino group of a lysine residue, is the key step to initiate the TG-catalyzed protein cross-link. SVS III consists of 252 amino acid residues. Lin et al. have demonstrated the five tandem repeats QXK(S/T) in residues 116–145 of SVS III as the TG cross-linking site, where X represents an aliphatic residue [11]. The peptide sequence QXK(S/T) is characteristic of a TG substrate both as an acyl donor and acyl acceptor according to their study. We applied a method of solid-phase assay to measure the TG_4 cross-linking activity for incorporation of two biotinylated substrates, $BPNH_2$ as an acyl acceptor and A25 peptide as an acyl donor, into a GST-fused polypeptide for the residues 116–145 of SVS III coated on microtiter plates. We detected a very low level of incorporating either $BPNH_2$ or A25 peptide to GST alone, but a certain level of their incorporation into the chimeric polypeptide, indicative of the substrate activity arising from the part of QXK(S/T) sequences in which the cross-linking extent of $BPNH_2$ to the glutamine residues was much higher than that of A25 peptide to the lysine residues (Fig. 3).

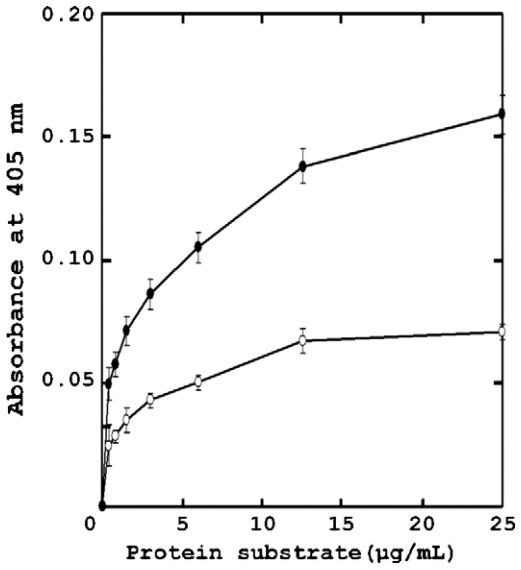

Fig. 3. Incorporation of $BPNH_2$ and A25 peptide into an immobile protein by TG_4. A recombinant GST-fused polypeptide comprising residues 116–145 of SVS III was coated on microtiter plates. To each well, 100 μL of a reaction buffer containing 0.5 mM $BPNH_2$ or A25 peptide and 2.5 μg of TG_4 was added and incubated at 37 °C for 60 min. Incorporation of $BPNH_2$ (closed circle) and A25 peptide (open circle) into the protein substrate was measured by ELISA using streptavidin-β-galactosidase. The enzyme activity in the hydrolysis of O-nitrophenyl-β-D-galactopyranoside was determined by measuring the absorbance at 405 nm (see Section 2). The data represents the average of three determinations and error bars the S.D.

4. Discussion

Since TG_4 is the actual enzyme involved in the seminal coagulation during the natural coitus, our study about its purification from CGS and the characterization of its cross-linking activity among SVS proteins are essential for further study of the protein cross-links in the formation of semen coagulum. Esposito et al. suggested TG_4 from rat coagulating gland secretion as a NH_2-terminally blocked protein [10]. This is in contrast to mouse TG_4, which has an asparagine as its NH_2-terminal residue. Nonetheless, the protein sequences of these two rodent enzymes share more than 86% identity. Based on the three-dimensional crystal structure of human TG factor XIII established by Pedersen et al. [22], the active site C^{314} of this protein molecule is situated in a catalytic triad arrangement along with H^{373} and D^{296}. They proposed a role of thiolate–imidazolium ion pair formed by C^{314} and H^{373} as a part of the catalytic mechanism. The stabilization of this ion pair is achieved by the hydrogen bonds positioned between H^{373} and one O_δ atom of D^{296} and between C^{314} and the O_η of Y^{560}. Murthy et al. [23] proposed the indole group of a conserved tryptophan residue in the TG family, namely W^{241} of TG factor XIII, as an essential role in the stabilization of the oxyanion intermediates in the enzyme-catalyzed transamidation. These five active residues of factor XIII are conserved in the TG_4 molecule in which W^{220}, C^{255}, H^{314}, D^{337} and Y^{501} are matched by protein sequence alignment in terms of catalytic activity and active site.

SVS II and *SVS III* in $2H_3$ locus of mouse chromosome arise from a common evolutionary origin [24]. Like SVS III, the peptide QXK(S/T) is tandem-repeated 20 times within residues 82–250 of SVS II containing 375 residues. It is possible that the tandem repeated QXK(S/T) is exposed on the carboxyl side of GST. The higher reactivity of glutamine residues as compared with the lysine residues in the TG_4 catalysis (Fig. 3) manifests that the peptide QXK(S/T) in SVS II and SVS III was more active as an acyl donor rather than an acyl acceptor for the TG_4 cross-linking once the two protein molecules were unfolded. The fact that TG_4 substrate activity of SVS II was much higher than that of SVS III may be attributed to the high content of QXK(S/T) in SVS II. SVS I–III are monomer proteins that had been tentatively assigned on reducing SDS–PAGE

during the analysis of mouse SVS proteins [15,16]. However, a previous study suggested that they may not be present in mouse SVS as monomers but restricted into high molecular weight complexes (HMWCs) formed by inter-polypeptide disulfide bonds [25]. Dispersion of the protein components from the complexes by reduction with DTT may result in their unfolding to expose the TG_4 cross-linking sites. It awaits future study to assess how the disulfide cross-links among SVS I–III in HMWCs affect their TG_4 substrate activity.

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