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

Huan-Chin Tseng[a,1], Han-Jia Lin[b,1], P.S. Sudhakar Gandhi[a], Chia-Yih Wang[a], Yee-Hsiung Chen[a,c,*]

[a] Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan
[b] Institute of Bioscience and Biotechnology, Center for Marine Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan
[c] Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

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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.

* Corresponding author at: Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei 106, Taiwan. Fax: +886 2 2363 5038.
E-mail address: bc304@gate.sinica.edu.tw (Y.-H. Chen).
[1] Equal contributions.

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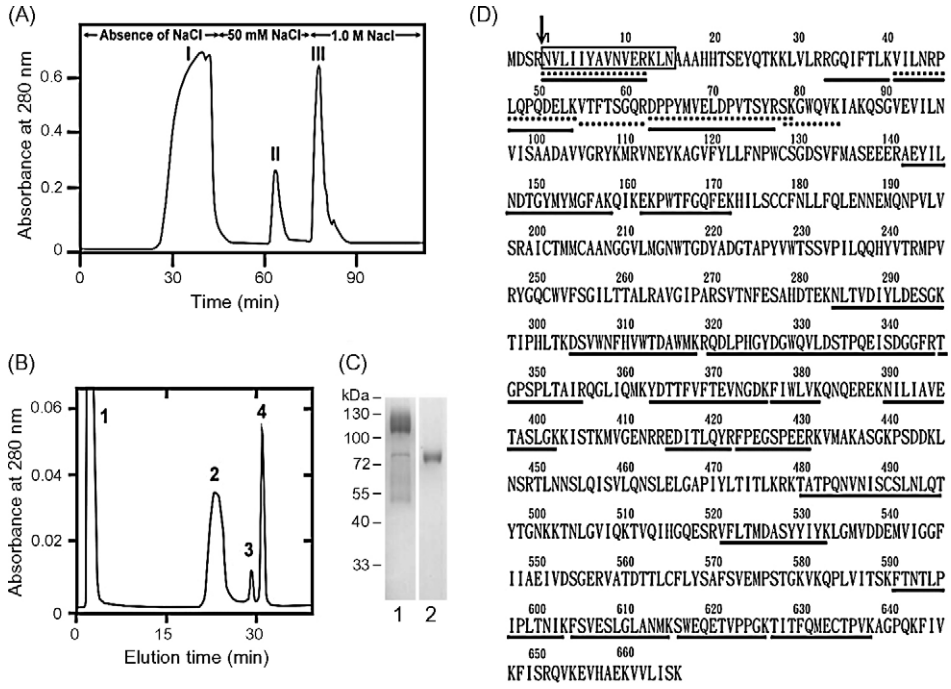

Fig. 1. Determination of partial sequences for a 75-kDa TG_4 purified from mouse CGS. (A) The soluble CGS was resolved into three fractions by ion exchange chromatography on a DEAE-Sephacel column. (B) Fraction II was further subjected to ion exchange high-performance liquid chromatography (HPLC) on 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