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Purification and characterization of transglutaminases from the genital tract of the male rat

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ABSTRACT

In the genital tract of the male rat two different forms of the enzyme transglutaminase (TGase) could be identified and characterized. The coagulating gland and the dorsal prostate secrete a glycosylated and acylated TGase with a molecular weight of 65 000 dalton and p*I* value of 8.7.

This secretory form was purified to homogeneity using preparative isoelectric focusing and gel filtration on a Superdex 200 column.

Running fast protein liquid chromatographic gel filtration on a Superose 12 column in the presence of calcium ions, high-molecular-weight aggregates were physically formed which could only be eluted using drastic conditions (0.1 *M* sodium hydroxide). In the presence of 10 m*M* EDTA this tendency to aggregate was greatly diminished. Utilizing a Superdex 200 column for gel filtration, the secretory TGase was even eluted as a monomeric protein. Testicular TGase was isolated by ion-exchange fast protein liquid chromatography on a Mono Q and by gel filtration on a Superdex 200 column. This enzyme represents a tissue-type TGase with a molecular weight of 82 000 dalton and p*I* value of 5.25. Hydrophobic interaction chromatography on a phenyl-Superose column showed no further enrichment of the GTP-binding form of transglutaminase.

INTRODUCTION

Transglutaminases (TGase, EC 2.3.2.13, *R*-glutaminyl-peptide: amine-γ-glutamyl-transferase) are a class of enzymes widely distributed in animal tissues and body fluids. They catalyze an acyl transfer reaction between the γ-carboxamide group of peptide bound glutaminyl residue and a primary amino group of various acceptor substrates, with a concomitant release of ammonia [1,2]. Although different transglutaminases appear similar in their substrate specificity, several distinct forms of the enzyme have been identified [2]: two forms —transglutaminases such as the tissue-type form, purified from liver and erythrocytes [3,4] and epidermal TGase, which is also present in hair follicle [5,6], are found exclusively inside cells, whereas plasma factor XIII and the TGase of rodent seminal plasma are extracellular enzymes. Plasma factor XIII occurs as a zymogen in plasma, platelets and histiocytes [7] and is activated through a thrombin cleavage near the N-terminus. Coagulation of semen in rodents results from polymerization of seminal vesicle secretion proteins by transglutaminases of prostatic origin [8].

Studies in primary sequences as well as immunological comparisons revealed differences between the transglutaminases. All forms of the enzyme are calcium dependent, they need at least 0.1–1 m*M* Ca^{2+} for catalytic activity [9], and different concentrations of the cation are necessary for optimal activation. The active site of TGases contains an essential SH-group which is completely inactivated after alkylation [10].

Two forms of transglutaminases, which we identified in the genital tract of the male rat, were enriched utilizing different steps of purification procedures. Tissue-type TGase is expressed by stromal cells of the accessory sex glands as well as by myoid peritubular cells of the testis [11] and the secretory enzyme is present in epithelial cells of the coagulating gland and the dorsal prostate [12]. Because they tend to form physical aggregates and autocatalytic cross-linking even under physiological conditions, isolation of these TGases has been difficult. However, by using conventional and fast protein liquid

chromatography (FPLC) techniques we were able to purify the secretory TGase to homogeneity. The testicular enzyme, however, was still contaminated with lower-molecular-weight (32 000 and 24 000 dalton) proteins.

EXPERIMENTAL

Transglutaminase assay: estimation of purity

Enzymatic activity was measured by incorporation of [14C]putrescine (Amersham, Braunschweig, Germany) into N,N'dimethylated caseine (Sigma, Munich, Germany) [2,13]. Purity of the fractions was estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), analytical isoelectric focusing (IEF) and by immunoprint analysis, using either a specific antibody to prostatic secretory TGase [12] or a monoclonal antibody to guinea pig liver TGase (generous gift of Dr. P. J. Birckbichler, Ardmore, OK, USA).

Purification of secretory TGase

Mature male Wistar rats (Ivanovas, Kisslegg, Germany) weighing 250–300 g were anesthetized and sacrificed by cervical dislocation. After dissection of the coagulating glands and the testes, the expressed residual secretions of the coagulating glands were extracted in physiological saline containing 5 m*M* Ca^{2+}. After centrifugation at 15 000 *g* for 20 min, TGase present in the supernatant was enriched by fractionated ammonium sulphate precipitation (25–50% saturation). Preparative IEF in a granulated Sephadex G-75 gel bed (Pharmacia/LKB, Freiburg, Germany) (pH gradient 3–8.5) eliminated contaminating secretory proteins. Pharmalytes (Pharmacia/LKB) were removed by gel filtration on a Superdex 200 column (Pharmacia/LKB). The apparent molecular weight of the native enzyme was estimated by FPLC gel filtration on a Superdex 200 or a Superose 12 (Pharmacia/LKB) column and the effect of 5 m*M* Ca^{2+} and 10 m*M* EDTA upon the elution profile of this TGase was tested.

Isolation of testicular tissue-type TGase

After removal of the testes the tunica albuginea and the testicular artery were discarded. The tissue was minced and subsequently homogenized in a pH 8.5, 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 0.33 *M* sucrose, 5 m*M* DTE, 50 m*M* KSCN, 1 m*M* EDTA (10% w/v) mixture. After centrifugation at 150 000 *g* for 45 min, the TGase present in the cytosol was pelleted by fractionated ammonium sulphate precipitation (50–60% saturation). The pellet was dissolved in distilled water, dialyzed overnight against the starting buffer (50 m*M* Tris–HCl, 1 m*M* EDTA pH 7.5, and subsequently applied to anion-exchange FPLC (Mono Q, Pharmacia/LKB). Fractions containing enzyme activity were applied to a Phenyl-Superose column (Pharmacia/LKB) which had been equilibrated with a pH 7.5, 50 m*M* Tris–HCl, 1 m*M* EDTA and 0.3 *M* NaCl mixture. Elution of the fractions was carried out in the presence of 1 m*M* GTP [14]. Samples containing enzyme activity were pooled, concentrated in a vacuum concentrator (Bachofer, Reutlingen, Germany) and separated by FPLC gel filtration on a Superdex 200 column.

RESULTS

Secretory transglutaminase

Secretory transglutaminase from the rat coagulating gland was identified by SDS-PAGE after preparative IEF for fractions 2–6 (Fig. 1a). Estimation of the enrichment of the enzyme and yield was affected by Pharmalyte contamination, which also impaired the determination of protein concentration. Therefore, Pharmalytes were removed by gel filtration on a Superdex 200 column. Compared with crude organ extracts, the enrichment factor was 4.5, although a yield of little more than 1% was obtained. SDS-PAGE revealed a single band of 65 000 dalton (Fig. 1b)) which was also seen after analytical IEF at p*I* 8.7 (not shown here). The influence of calcium ions upon gel filtration under native conditions was also investigated by applying crude TGase extract to a Superose 12 column. If the column was run in the presence of calcium ions (5 m*M*), only 8% of the TGase activity applied could be eluted in the void volume of the column (>300 000 dalton). Immunoprint analysis of this fraction revealed an autocatalytic cross-linking as well as a small degree of physical association of the enzyme. An additional 50% of the initial activity could be eluted if relatively strong conditions (0.1 *M* sodium hydroxide) were used. Activity appeared at an elution volume corresponding to 60 000–

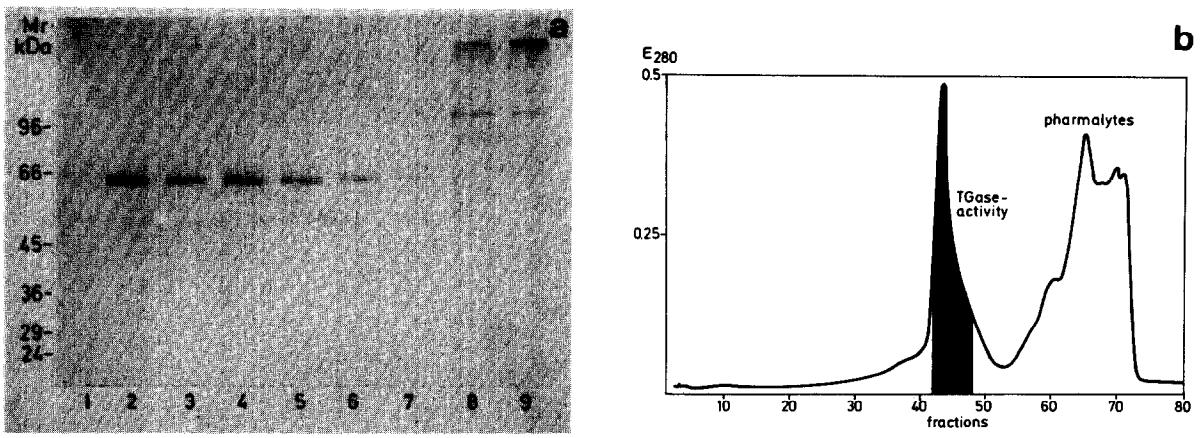

Fig. 1. (a) Analysis of fractions 1–9 from preparative IEF by SDS-PAGE (7.5–20% acrylamide) stained with Serva Blue G: identification of secretory TGase (65 000 dalton) in fractions 2–6. Molecular weight (M_r) indicated in kilodalton (kDa). (b) Gel filtration of pooled fractions 2–6 on Superdex 200 column and removal of Pharmalytes. Hatched area indicates fractions containing TGase activity (fractions 42–47).

70 000 dalton. If the column was run with calcium-free Tris buffer containing 10 m*M* EDTA, then about 38% of the applied TGase eluted at a volume corresponding to 200 000 dalton and an additional 20% eluted at a volume corresponding to a molecular weight of 60 000–70 000 dalton (Fig. 2a). A much different elution profile was obtained utilizing a column of Superdex 200 instead of Superose 12. Running the column with a buffer containing EDTA, TGase eluted in a separate peak (Fig. 2b) in a monomeric form, indicating the prevention of aggregation. Using FPLC gel filtration on a Superdex 200 column subsequent to ammonium sulfate precipitation, we obtained a highly purified enzyme with an enrichment factor of 5 and a yield of more than 30% (Fig. 3a). As a side effect, an additional

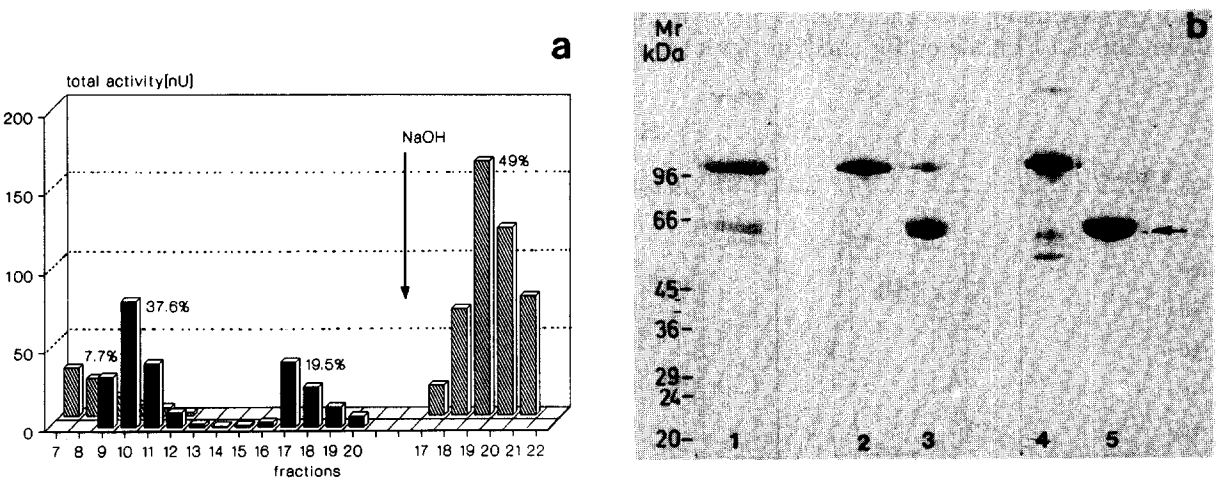

Fig. 2. (a) FPLC gel filtration of crude TGase extract on a Superose 12 column. Hatched areas represent elution with Tris buffer containing 5 m*M* Ca^{2+}. The major part of TGase is only eluted by 0.1 *M* sodium hydroxide (arrow). Black areas indicate elution of TGase as oligomers and monomers utilizing Tris buffer containing 10 m*M* EDTA. (b) Analysis of fractions in (a) containing high enzyme activity by SDS-PAGE. Lanes: 1 = crude coagulating gland extract; 2 = fraction 8 after elution with Tris/Ca^{2+}; 3 = fraction 19 after elution with 0.1 *M* sodium hydroxide; 4 and 5 = fractions 10 and 17 after gel filtration with Tris/EDTA.

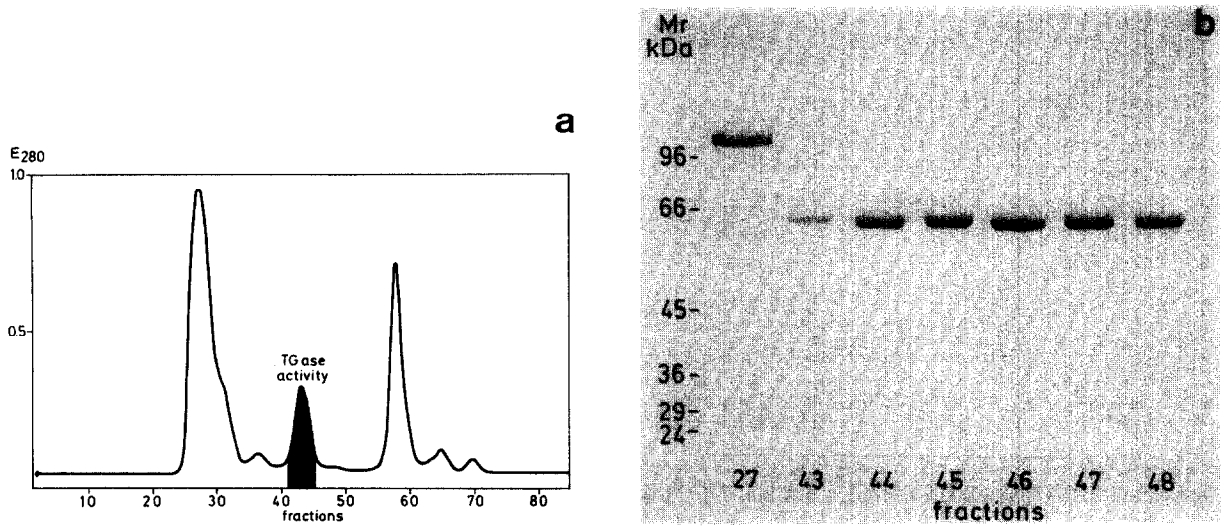

Fig. 3. (a) Gel filtration of secretory TGase on a Superdex 200 column after ammonium sulphate precipitation. Hatched fractions 43–48 contain enzyme activity. (b) SDS-PAGE of selected fractions in (a): in fraction 27, a secretroy protein of 100 000 dalton is highly enriched. Fractions 43–48 contain purified TGase.

secretory protein was isolated in a different fraction, corresponding to a molecular weight of more than 100 000 dalton (Fig. 3b, fraction 27).

Testicular transglutaminase

Using anion-exchange FPLC on a Mono Q column, a highly enriched enzyme eluted at a single peak corresponding to a concentration of 0.3 *M* sodium chloride. Immunoprint analysis revealed a prominent band in the range of 82 000 dalton as well as smaller bands due to proteolysis of the enzyme (Fig. 4b). Nevertheless, an enrichment factor of 30 and a yield of more than 70% were obtained. All enzyme activity applied to the phenyl-Superose column appeared in the void volume and no activity was eluted by GTP. After application of concentrated fractions to a Superdex 200 column which was run in the presence of 1 m*M* EDTA, no enzyme

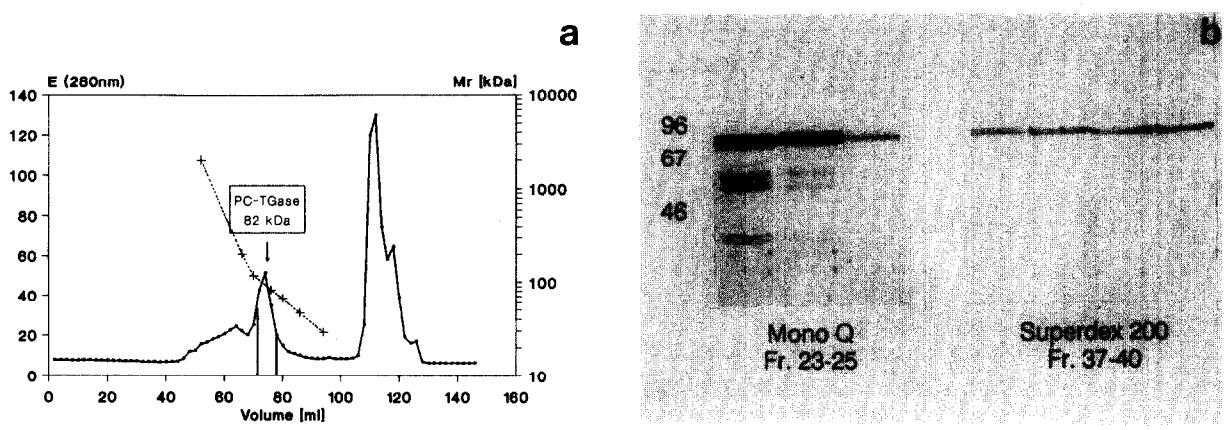

Fig. 4. (a) Gel filtration of testicular TGase on a Superdex 200 column after ion-exchange chromatography. Marked area represents enzyme activity and immunoreactivity. (b) Immunoprint analysis of fraction (Fr.) containing testicular TGase (82 000 dalton) activity after ion-exchange chromatography and gel filtration.

activity or immunoreactivity was detected in the void volume, indicating the absence of aggregates. Enzyme acitivity as well as immunoreactivity appeared as a single peak in a monomeric form corresponding to a molecular weight of 82 000 dalton as estimated by gel filtration (Fig. 4a) and immunoprint analysis (Fig. 4b). Although enzyme activity was low (1.5% yield), maybe due to the phenyl-Superose step, only two major contaminating protein bands with low molecular weights (about 32 000 and 24 000 dalton) were present as revealed by SDS-PAGE (not shown).

DISCUSSION

Purification of transglutaminase is known to be difficult because of its tendency to form aggregates even under native conditions, and especially in the presence of calcium ions. The enzymatic function of TGases consists of covalent cross-linking of glutaminyl and lysyl residues [2]. Since transglutaminases themselves contain a sufficient number of these amino acids, an autocatalytic cross-linking instead of a physical aggregation is conceivable. For further elucidation of this problem, two different TGases were isolated from the genital tract of the male rat: (i) an extracellular protein secreted by the coagulating gland and (ii) a cytosolic enzyme expressed by myoid peritubular cells of the seminiferous epithelium [15]. Although both forms share structural and functional properties, such as calcium dependence in the millimolar range and presence of an essential SH-group at the active site, significant differences are observed at the molecular and immunological level. The prostatic enzyme which is exported by the unusual pathway of apocrine secretion [12] has a molecular weight of 65 000 dalton and a p*I* value of 8.7. Furthermore it is membrane-bound by a phosphoinositol anchor which prevents autocatalytic cross-linking and obstruction of the excretory ducts [16]. The testicular enzyme, in comparison has a higher molecular weight (82 000 dalton) and is anionic (p*I* 5.25). Moreover, it is closely related to the tissue-type form expressed by rat and guinea pig livers as revealed by immunohistochemistry and immunoprint analysis. Like other tissue-type TGases, its activity is inhibited by micromolar concentrations of GTP [17] and it binds to GTP–agarose columns [18]. As described above the secretory enzyme was isolated to homogeneity. Gel filtration in presence of calcium ions resulted in a great loss of enzyme and therefore the yield of purification was low. When using FPLC (Superose 12 column, buffer containing calcium ions) more than 40% of applied enzyme were cross-linked autocatalytically. Approximately 49% aggregated physically and could only be eluted using drastic conditions (0.1 *M* sodium hydoxide), whereas 8% of enzyme activity appeared in the void volume. Using a Tris buffer containing 10 m*M* EDTA to bind endogenous calcium, 38% of aggregated prostatic TGase eluted at a volume corresponding to 200 000 dalton and 20% at a volume corresponding to 60 000–70 000 dalton, the latter indicating the presence of monomers. A significant improvement concerning the elution profile of the enzyme was obtained by changing the matrix for gel filtration. Using FPLC on a Superdex 200 column in the presence of EDTA, aggregation of the protein was significantly dimished and it eluted as a monomeric pure enzyme with a high specific activity. We therefore presume that, in contrast to Superose 12, Superdex 200 gel matrix does not induce physical aggregation of the secretory TGase.

TGase activity present in testicular homogenates and Sertoli cell cultures has been reported previously [19] and an effect of transglutaminase substrates on the cellular sequestration and processing of follicle-stimulating hormone (FSH) has been proposed [20]. Furthermore, TGase activity in bovine calf testicular membranes is thought to participate in the interaction of FSH with its receptor [21]. Utilizing a monoclonal antibody to tissue-type TGase we have demonstrated that in the testis only interstitial cells and myoid peritubular cells express the tissue-type form [15]. We therefore conclude that TGase activity present in Sertoli cell cultures is due to contamination by peritubular or interstitial cells. For further characterization of the enzyme, tissue-type TGase was isolated from testicular homogenates using ion-exchange FPLC on a Mono Q column, and gel filtration on a Superdex 200 column in the presence of 1 m*M* EDTA. Neither on Mono Q nor on Superdex 200 column did enzyme activity or immunoreactivity appear in the void volume —indicating the absence of covalently linked or physically aggregated TGase. Using ion-exchange FPLC subsequent to ammonium sulphate precipitation, the enzyme eluted as a single peak corresponding to 0.3

M sodium chloride. An enrichment factor of 30 and a yield of more than 70% were achieved. Running reversed-phase FPLC on a phenyl-Superose column showed no further purification of the GTP-binding enzyme. After gel filtration on a Superdex 200 column, testicular TGase eluted as a single peak with low specific activity corresponding to a native molecular weight of about 80 000 dalton. Using immunoprint analysis, the enzyme was shown to consist of a monomeric form of 82 000 dalton, which is antigenically unrelated to the prostatic secretory TGase.

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