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Review

Transglutaminases: purification and activity assays

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Abstract

Transglutaminases (TGases) are a widely distributed family of proteins found in many tissues and body fluids of vertebrates. To date the following types have been distinguished: secretory, tissue, epidermal, keratinocyte, and hemocyte TGase as well as factor XIIIa and erythrocyte band 4.2. TGases are difficult to isolate, as they tend to form irreversible aggregates under native conditions. In this review, the isolation procedures for the different types of TGases are summarized. The most common chromatographic separation methods used for TGase purification are size-exclusion and ion-exchange chromatography. Additionally, other chromatographic methods (hydrophobic-interaction, affinity, adsorption chromatography) and electrophoretic techniques (preparative isoelectric focusing, sodium dodecyl sulphate polyacrylamide gel electrophoresis and zone electrophoresis) are described. Based on the enzymatic function of TGases (cross-linking of a primary amine and peptide-bound glutamine), several established activity assays are described.

Keywords: Reviews; Transglutaminase; Enzymes

Contents

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1. Introduction

Transglutaminases (TGases, EC 2.3.2.13, *R*-glutaminyl-peptide, amine-γ-glutamyl-transferase) belong to a class of enzymes which catalyze the acyl transfer reaction between the γ-carboxamine group of a peptide-bound glutaminyl residue and a primary amino group of various substrates, in a calcium-dependent reaction (Fig. 1). The result of this reaction is the formation of an irreversible cross-linked, insoluble supramolecular structure. TGases are widely distributed in tissues and body fluids. TGases were first discovered and characterized by Waelsch and co-workers [1,2]. To date, several distinct forms of enzymes have been described (Table 1).

The purification of TGases is known to be difficult, since they have a propensity to form irreversible aggregates under native conditions. TGase itself contains 38 glutaminyl and 42 lysyl residues which allow autocatalytical cross-linking (homoaggregates) during purification when calcium ions are present. In addition, the TGase can also catalyze the aggregation of various heteroaggregates in crude extracts. To avoid the purification of the above mentioned homo- or heteroaggregates, it is essential to use complexing reagents such as EDTA to prevent irreversible cross-linking during separation.

Although all types of TGases share functional and structural mutuality, they differ in their molecular and immunological characteristics, demanding different purification procedures. In most separation protocols, combinations of ion-exchange and size-exclusion chromatography have been described. Furthermore, other chromatographic methods such as hydrophobic-interaction, affinity, adsorption, and metal-chelating chromatography as well as electrophoretic separation methods like preparative isoelectric focusing, SDS-PAGE and zone electrophoresis have been applied (Table 2). The TGase enzyme activity assays are based on the function of the enzyme, i.e. to cross-link primary amines (e.g. lysine) and peptide-bound glutamine. This review represents a general view about the various isolation protocols for the different types of TGases and about the TGase enzyme activity assays (Table 3).

2. Isoforms of transglutaminases

Secretory TGase was originally identified in the secretion of the dorsal prostate and coagulating gland of rodents. The function of this TGase type is to form an intravaginal coagulation plug directly after copulation. The substrate used for this enzymatic reaction is SVS II, a monomer protein, originating from seminal vesicle secretion [3–8]. The secretory TGase is synthesized and processed in the cytoplasmic compartment and is secreted by an alternative export mechanism via apical blebs [9]. The secretory TGase is a glycoprotein, although it does not pass through the classical endoplasmic reticulum/Golgi route [8,10,11]. Furthermore, secret-

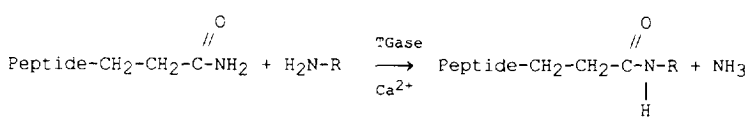

Fig. 1. Transglutaminase-catalysed reaction of γ-carboxamide group of the peptide-bound glutamine residue and a primary amine.

Table 1
Isoforms of transglutaminases

Enzyme	Synonyms	Source for enzyme purification
Secretory transglutaminase	Dorsal prostate protein-1(DP1)	Coagulating gland, dorsal prostate (guinea pig, rat)
Tissue transglutaminase	Liver-, cytosolic-, endothelial-, erythrocyte transglutaminase, transglutaminase type II	Liver (guinea pig, rat), testis (rat), erythrocytes (human), A431 tumor cells (human)
Factor XIII	Plasma transglutaminase, fibrin stabilizing factor, Laki-Lorand factor	Platelets, placenta, plasma (human)
Keratinocyte transglutaminase	Particulate transglutaminase, transglutaminase type I	Liver (rat), chondrosarcroma tumor (rat), squamous carcinoma cell line (human)
Epidermal transglutaminase	Bovine snout-, hair follicle transglutaminase, transglutaminase type III	Cow snout, skin (human, guinea pig)
Hemocyte transglutaminase	Limulus transglutaminase	Limulus hemocytes
Erythrocyte band 4.2	Erythrocyte membrane protein band 4.2	–

ory TGase was identified in human prostates. The function of human prostatic TGase is still unclear [12,13].

Tissue TGase is distributed in numerous vertebrate tissues. The physiological function of this TGase type is still unknown. Some data indicate functions as a cross-linker for components of the extracellular space as well as in the cytosolic compartment [14]. In addition, there is some evidence that tissue type TGase plays a role in cell–matrix interactions [15] and modulates the regeneration of cells in tissue repair [16]. Furthermore, a role for tissue TGase in cell growth [17] and in the complex processes of the programmed cell death has been suggested [18,19].

Factor XIII consists of two a- and two b-subunits and is expressed as a zymogen in plasma, platelets and placenta [20]. The a-subunit (Factor XIIIa) contains the active site of the enzyme. Factor XIIIa is the last factor in the blood coagulation cascade. Synonyms are fibrin stabilizing factor and Laki–Lorand factor. It is activated by thrombin and calcium through limited proteolysis and catalyzes the polymerization of fibrin monomers into γ–γ-(fibrin) dimers and α-polymers during hemostasis [21]. In addition, factor XIIIa catalyzes the covalent linking of α-2-plasmin inhibitor to the α-chain of fibrin [22], as well as the cross-linking of factor V [23], fibronectin [24,25], lipoprotein-a [26], α-2-macroglobulin [27], plasmin-activator–inhibitor-2 [28], thrombospondin [29], vitronectin [30] and von Willebrand factor [31].

Hemocyte TGase is part of the coagulating system of the vertebrate hemolymph. The hemocytes circulating in the hemolymph of vertebrates contain an intrinsic coagulation system. This blood coagulating system is composed of several serine protease zymogens and a clottable protein, named coagulogen. The last serine protease zymogen to become activated is a clotting enzyme, termed hemocyte TGase, which converts the soluble coagulogen into an insoluble coagulin gel [32].

Keratinocyte TGase is a membrane-associated protein, which was first detected in keratinocytes [33]. Enzymes (particulate-associated TGase) identical to this described type were also identified in rat liver and chondrosarcoma tumors [34]. The epidermal TGase is a soluble proenzyme which is known as "hair follicle TGase" [35]. Both types of TGase are involved in the formation of the envelope of the stratum corneum during terminal differentiation. Further substrate proteins such as loricrin [36] are cross-linked to the cornified envelope by the epidermal isoform.

Erythrocyte protein band 4.2 is a membrane polypeptide sharing high sequence homologies to TGases, but with no TGase enzymatic activity. It is important for normal erythrocyte function, because in patients lacking band 4.2, erythrocyte disintegration is accelerated due to abnormally-shaped red blood cells, leading to anemia. More precise delineation of the function of erythrocyte membrane protein band 4.2. is yet to be determined [37,38].

Table 2
Purification of different transglutaminase types

Enzyme	Source	Species	Separation steps	Ref.
Secretory TGase	Coagulating gland	Guinea pig	AS-P IEC (DEAE-cellulose) SEC (Sepharose 6B)	[3,39]
		Rat	AS-P IEC (CM-cellulose)	5
	Dorsal prostate	Rat	IEC (DEAE-cellulose) SEC (Sephadex G-200) IEC (Phospho-cellulose)	[40]
	Coagulating gland/ dorsal prostate	Rat	AS-P Preparative IEF SEC (Superdex 200)	[7,8]
		Rat	AS-P SEC (Superdex 200) IEC (DEAE-EMD Fractogel 650 (s))	[41]
Factor XIII	Platelet	Human	IEC (DEAE-cellulose) SEC (Sephacryl S-300) HIC (phenyl-Sepharose CL-6B)	[52]
		Human	Immuno-AFC	[56]
	Placenta	Human	E-P IEC (DEAE-cellulose) AS-P SEC (Sephacryl S-300) HIC (phenyl-Sepharose CL-6B)	[57]
	Plasma	Human	IEC (DEAE-Sephacel/-Sepharose) SEC (Biogel A-5 m)	[58]
Hemocyte TGase	Hemocyte	Limulus	IEC (CM-Sepharose CL-6B) AS-P IEC (DEAE-cellulose) SEC (Sephacryl S-300) IEC (DEAE-Cosmogel) MCC (zinc-chelating-Sepharose 6B)	[32]
Keratinocyte TGase	Chondrosarcoma	Rat	IEC (DEAE-cellulose) AS-P SEC (Sephacryl S-300) ADC (Biogel HTP) IEC (DEAE-Sephacryl)	[34]
	Liver	Rat	Lubrol-WX IEC (DEAE-cellulose) SEC (Biogel A 0.5 m)	[34]
	Squamous carcinoma cell line	Human	Immuno-AFC	[59]
Epidermal TGase	Epidermis	Human	IEC (DEAE-cellulose) SEC (Sephadex G 75) IEC (CM-cellulose) SEC (Biogel A-0.5 m)	[60]
	Muzzle	Bovine	IEC (DEAE-Sephadex A 50) ZE SEC (Sephadex G 200)	[61]

Table 2 (Continued)

Enzyme	Source	Species	Separation steps	Ref.
	Skin	Guinea pig	IEC (DEAE-cellulose) AS-P AFC (Heparin-Sepharose) GCP (Biogel A 0.5 m) IEC (S-Sepharose)	[62]
Tissue TGase	Liver	Guinea pig	IEC (DEAE cellulose) P-P IEC (DEAE-cellulose) P-P IEC (DEAE-cellulose)	[42]
		Guinea pig	IEC (DEAE-cellulose) P-P IEC (CM-cellulose) AS-P SEC (Agarose)	[43]
		Guinea pig	IEC (QAE-Sephadex) ADC (Biogel-HTP) AFC (phenylalanine-Sepharose)	[44]
		Guinea pig	Immuno-AFC	[45]
		Rat	IEC (DEAE-cellulose) SEC (Biogel A 0.5 m)	[34]
		Rat	AFC (casein-Sepharose)	[46]
		Rat	IEC (DEAE-Sepharose) ADC (Biogel-HTP) SEC (Superdex G 100)	[47]
		Rat	IEC (Mono-Q) Immuno-AFC	[48]
	Testis	Rat	AS-P IEC (Mono-Q) HIC (phenyl-Superose) SEC (Superdex 200)	[7]
		Bovine	IEC (DEAE-cellulose) IEC (Q-Sepharose) HIC (phenyl-Sepharose)	[49]
	Erythrocyte	Human	IEC (DEAE-cellulose) SEC (Biogel A 0.5 m Preparative SDS–PAGE	[50]
		Human	IEC (DEAE-cellulose) AS-P Immuno-AFC	[51]
		Human	IEC (DEAE-cellulose) SEC (Sephacryl S-300) AFC (Blue-Sepharose CL-6B) IEC (DEAE Biogel A)	[52]
		Human	IEC (DEAE-cellulose) SEC (AcA 44) AFC (Heparin-Sepharose) GP-HPLC (TSK 125)	[53]
		Human	IEC (DEAE-cellulose) IEC (Mono Q)	[54]
	A431 tumor cell	Human	IEC (DEAE-cellulose) AFC (Heparin-Agarose) AFC (casein-Agarose)	[55]

Table 3
Substrates for transglutaminase activity assays

Primary amine substrates	Glutamine substrates
5-(Biotinamido)pentylamine	Casein
Dansylcadaverine	β-Casein
Monodansylcadaverine	N,N-Dimethylcasein
Ethylamine	Biotinylcasein
Glycine-ethylester	Benzyloxycarbonyl-L-glutaminyl-glycine
Putrescine (^{14}C or ^{3}H)	

3. Purification of transglutaminases

3.1. Secretory transglutaminase

Secretory TGase was first purified from secretions of guinea pig coagulating glands. After centrifugation, the supernatant was precipitated with ammonium sulfate and subsequently the enriched TGase fraction was subjected to anion-exchange chromatography on DEAE-cellulose. After a repeated ammonium sulfate precipitation step, secretory TGase was finally purified to homogeneity by size-exclusion chromatography on Sepharose 6B. The coagulating glands of 40 guinea pigs yielded approximately 20 mg of TGase. The relative molecular mass of secretory TGase from guinea pig coagulating gland was 70 000 as determined by SDS-PAGE [3,39].

Secretory TGase originating from rat was enriched by Williams-Ashman et al. [5]. The extracts of coagulating gland homogenate or its secretion were precipitated by ammonium sulfate. TGase was further enriched using cation-exchange chromatography on CM-cellulose. Two distinct procedures to purify rat secretory TGase to homogeneity were described by Wilson and French [40] (a) and from Seitz et al. [8] (b):

(a) the supernatant of centrifuged homogenates of dorsal prostates was chromatographed on a DEAE-cellulose column. Fractions containing TGase were applied to size-exclusion chromatography on Sephadex G-200. TGase was finally isolated by phospho-cellulose chromatography. Approximately 5 mg of purified TGase was recovered from rat dorsal prostates obtained from 35 rats [40].

(b) the secretions of coagulating glands and dorsal prostates were precipitated by ammonium sulfate (25–50% saturation). TGase was isolated using preparative isoelectric focusing in a granulated Sephadex G-75 gel bed (Fig. 2a). Pharmalytes were removed subsequently by size exclusion on a Superdex 200 column ([7,8], Fig. 2b). Recently, we have modified this purification procedure. TGase enriched by ammonium sulfate precipitation was processed first by size-exclusion chromatography on Superdex 200 equilibrated with a calcium-free Tris buffer containing 10 m*M* EDTA (Fig. 3a). Contaminating secretory proteins were removed by subsequent chromatography of the TGase-containing fractions by ion-exchange chromatography using DEAE-EMD Fractogel 650 (s) (Piperazine buffer, pH 6.2, Fig. 3b, Fig. 4) [41]. The advantage of this procedure is a higher yield (5–10 mg TGase from the secretions of coagulating glands taken from 20 rats). Two isoforms of rat secretory TGase had been identified with a relative molecular mass of 65 000 [40,7,8] and isoelectric points of 8.7 and 7.8 [7,8].

3.2. Tissue transglutaminase

Several methods have been described for the purification of tissue TGase from guinea pig liver [42–45], and rat liver [34,46–48]. The starting point for each TGase purification was the supernatant of centrifuged liver homogenate, which was subjected first to anion-exchange chromatography with DEAE-cellulose [42,34,43], QAE-Sephadex [44], DEAE-Sepharose [47] or Mono Q [48]. Tissue TGase was eluted with 0.25 *M*–0.45 *M* NaCl using a Tris buffer containing EDTA and DTT at pH 7.5. For the subsequent purification steps several alternative methods are described:

(a) TGase was enriched by protamine precipitation and then rechromatographed on DEAE-cellulose. Both steps were repeated once [42].

(b) After enrichment by protamine precipitation, proteins were separated using cation-exchange chro-

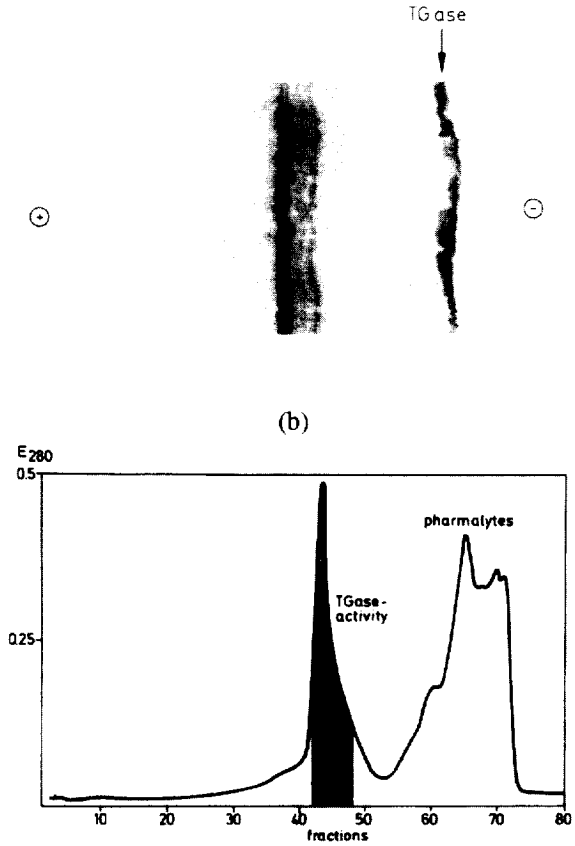

Fig. 2. Purification of rat secretory TGase using preparative IEF and SEC. (a) Filter paper print of rat coagulating gland secretion proteins, separated by preparative IEF on granulated Sepadex G-75 bed (pH 4.0 to 9.5). (b) SEC on Superdex 200 of fractions containing TGase.

matography with CM-cellulose. TGase was precipitated by ammonium sulfate and was finally isolated by size-exclusion chromatography using agarose [43].

(c) TGase-containing fractions were chromatographed using hydroxyapatite on Biogel-HTP [44,47] and finally purified by gel filtration chromatography using Superdex G 100 [47] or affinity chromatography with phenylalanine-Sepharose 4B [44].

(d) TGase-containing fractions were subjected to immunoaffinity chromatography using an antibody against cytosolic TGase [48].

(e) TGase was isolated using size-exclusion chromatography on Biogel A 0.5 m [34].

One-step purifications of guinea pig TGase [45]

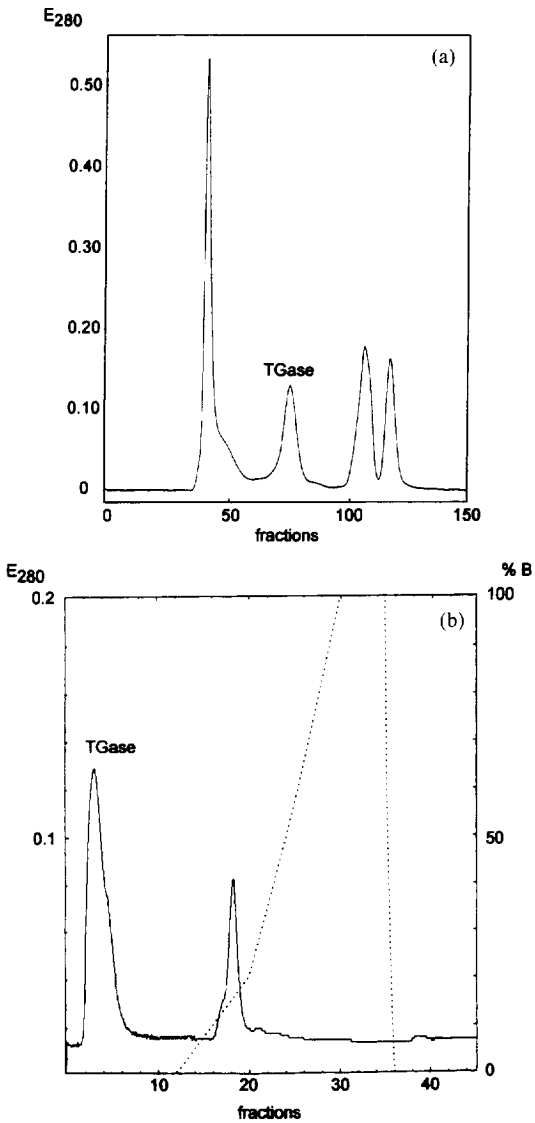

Fig. 3. Purification of rat secretory TGase using SEC and IEC. (a) SEC on Superdex 200 of ammonium-sulfate-precipitated secretion of rat coagulating gland. (b) IEC on DEAE-EMD-Fractogel 650 (s) of fractions containing TGase.

and rat TGase [46] have also been described. In these procedures, the supernatants of guinea pig liver homogenates were applied to an affinity column utilizing a monoclonal antibody against guinea pig liver TGase [45]. Extracts from rat liver were purified by calcium-dependent affinity chromatography using casein-Sepharose [46].

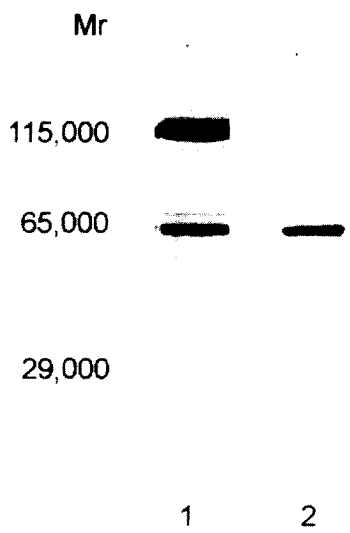

Fig. 4. SDS-PAGE of crude rat coagulating gland extract (lane 1) and secretory TGase purified by SEC and IEC (lane 2).

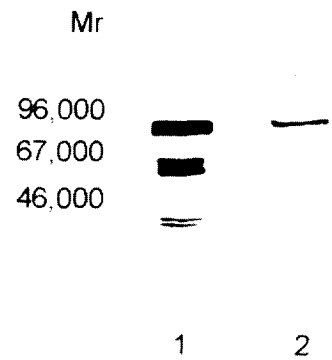

Fig. 5. Western blot analysis of fractions containing rat testicular tissue TGase (M_r 82 000) after IEC on Mono Q (lane 1) and SEC on Superdex 200 (lane 2).

Using the methods described above, 1–5 mg of pure TGase could be isolated from 100 g liver. Tissue TGase of liver origin has a relative molecular mass of approximately 85 000 as determined by SDS-PAGE.

Testicular tissue TGase has been purified from rat [7] and bovine [49] testes. TGase was obtained in an enriched form from rat testis homogenates by fractionated ammonium sulfate precipitation (50–60%) and subsequent anion-exchange chromatography on a Mono Q column. TGase was finally purified by hydrophobic-interaction chromatography using phenyl-Superose and size-exclusion chromatography using Superdex 200 (Fig. 5). The purified enzyme has a relative molecular mass of 82 000 using SDS-PAGE and has an isoelectric point of 5.25 [7]. TGase of bovine origin was isolated by anion-exchange chromatography using DEAE-cellulose and Q-Sepharose as well as hydrophobic-interaction chromatography with phenyl-Sepharose. Approximately 0.07 mg of protein was isolated from 50 g of bovine testis. The purified enzyme migrated as a single band on SDS-PAGE with a relative molecular mass of 80 000 [49].

Tissue-type TGase was also isolated from human erythrocytes [50–54] First, the supernatant of centrifuged human erythrocyte lysates was adsorbed in batches to an anion-exchange matrix of DEAE-cellulose. For the following purification of TGase different steps were used:

(a) After size-exclusion chromatography using Biogel A 0.5 m, TGase was finally purified by preparative SDS-PAGE with a total acrylamide concentration of 6 or 8.5% [50].

(b) The fraction containing TGase was applied to a size-exclusion AcA 44 column, followed by heparin-Sepharose chromatography. For final purification, TGase was chromatographed by size-exclusion HPLC using a TSK 125 column [53].

(c) TGase was isolated using size-exclusion chromatography with Sephacryl S-300, affinity chromatography on Blue-Sepharose CL-6B and finally anion-exchange chromatography using DEAE Biogel A [52].

(d) TGase was enriched using ammonium sulfate precipitation and finally purified by immunoaffinity chromatography [51].

(e) TGase was finally purified by anion-exchange chromatography on Mono-Q (Fig. 6a,b, [54]).

Approximately 1–2 mg of pure TGase could be isolated from hemolysate (ca. 20 000 mg protein) using the described methods. The pure enzyme has a relative molecular mass of 82 000, as established by SDS-PAGE.

In addition, a tissue type TGase was isolated from cultured human A431 tumor-cells. After cell homogenization, the next step used was anion-exchange chromatography on DEAE-cellulose. Subsequently, affinity chromatography on heparin-Agarose and on

casein-Agarose was performed. A 0.15-mg amount of pure TGase could be isolated from a cytosol fraction containing 650 mg total protein. The purified enzyme showed a single band on SDS-PAGE with a relative molecular mass of 83 000 [55].

3.3. Factor XIII

Factor XIIIa was obtained from human platelet lysates using anion-exchange chromatography with DEAE-cellulose, followed by size-exclusion chromatography on Sephacryl S-300 and hydrophobic interaction chromatography on phenyl-Sepharose CL-6B [52]. A single-step purification of platelet factor XIIIa was carried out by immunoaffinity chromatography using a monoclonal antibody against factor XIIIa [56]. It was possible to purify about 5 mg of factor XIIIa from crude platelets extracts (approximately 500 mg of total protein).

Factor XIII was also isolated from homogenates of human placentae. First, factor XIII was enriched by ethanol precipitation. The dissolved pellet was applied to a DEAE-cellulose anion-exchange column. After enrichment by ammonium sulfate precipitation (40%) factor XIII was purified using size-exclusion with Sephacryl S-300 and hydrophobic-interaction chromatography with phenyl-Sepharose CL-6B. Approximately 0.6 mg of enzyme could be isolated from four placentae [57].

Factor XIII from human plasma has been isolated using anion-exchange chromatography with DEAE-Sephacel or DEAE-Sepharose CL-6B. The purified factor XIII was activated by bovine thrombin and the activated a- and b-subunits were separated in the presence of calcium ions by size-exclusion chromatography using BioGel A-5 m [58].

Subunit a originating from platelets, placentae and plasma has a relative molecular mass of 80 000 as determined by SDS-PAGE.

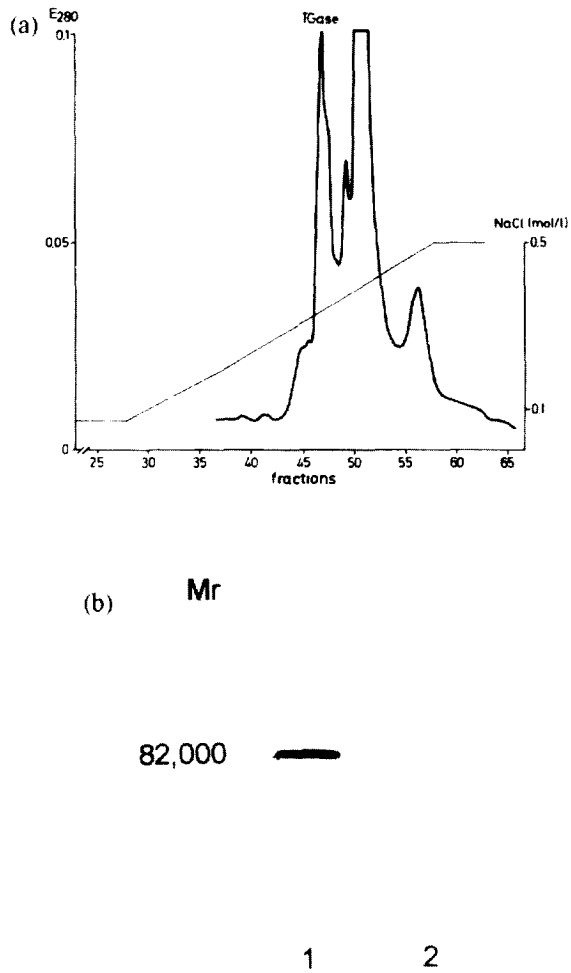

Fig. 6. (a) IEC on Mono Q of enriched tissue TGase from human erythrocytes after IEC on DEAE-cellulose. (b) SDS-PAGE (lane 1) and Western blot analysis (antibody against tissue TGase) (lane 2) of pooled fractions containing TGase.

3.4. Hemocyte transglutaminase

A further type of TGase has been isolated from limulus hemocyte lysate. The supernatant was applied to a cation-exchange chromatography column (CM-Sepharose CL 6B) using a Tris–acetate buffer, pH 7.5, containing EDTA. TGase was found in the unbound fraction. After ammonium sulfate precipitation (50%), the TGase-enriched pellet was bound to a DEAE-cellulose anion-exchange column followed by size-exclusion chromatography with Sephacryl S-300 and anion-exchange chromatography with DEAE-Cosmogel. Finally, fractions containing TGase protein were purified using a zinc-chelating Sepharose 6B column. A 1.6-mg amount of TGase was isolated from 32 g of hemocytes. The isolated TGase showed a single band on SDS-PAGE with a relative molecular mass of 86 000 [32].

3.5. Keratinocyte transglutaminase

TGase, identical to the keratinocyte type, was isolated from chondrosarcoma and rat liver (particulate-associated TGase [34]). Cells from a chondrosarcoma tumor were homogenized and subsequently centrifuged. The pellet containing the particulate TGase was homogenized a further three times in a sucrose buffer. The supernatants, containing the TGase, were chromatographed on an anion exchanger (DEAE-cellulose). Afterwards, TGase was enriched by ammonium sulfate precipitation (45%) and subjected to size-exclusion chromatography using Sephacryl S-300. The particulate TGase from chondrosarcoma cells was finally purified using hydroxyapatite chromatography with Biogel HTP and anion-exchange chromatography using DEAE-Sephacryl.

When rat liver was used as a source, the organs were homogenized and the pellets containing the particulate TGase rehomogenized as described above. The remaining particulate fraction from rat liver was homogenized three times with 1% Lubrol-WX in sucrose buffer. Supernatants were chromatographed on anion-exchange chromatography using DEAE-cellulose and size-exclusion chromatography with Biogel A-0.5 m. From the cytosolic fractions (the sucrose supernatants), most of the isolated TGase comprised the tissue type and to a lesser extent particulate-bound TGase was identified. From the detergent-extracted fractions, both TGase types (tissue and particulate-bound) were found in a ratio of 4 to 6.

Keratinocyte TGase had also been isolated from the non-ionic detergent extracts (0.3% NP-40) of the particulate fraction of a squamous carcinoma cell line, by means of a monoclonal antibody affinity chromatography [59].

All described keratinocyte TGases have a relative molecular mass of 92 000 as determined by SDS-PAGE.

3.6. Epidermal transglutaminase

Epidermal TGase has been isolated from human hair follicle-free epidermis [60], cow muzzle [61] and guinea pig skin (proenzyme, [62]). The supernatants of homogenized epidermis of human, bovine or guinea pig origin were subjected to anion-exchange chromatography with DEAE-cellulose [60,62] or DEAE-Sephadex A 50 [61].

Human epidermal TGase was then purified using size-exclusion chromatography with Sephadex G 75, followed by cation-exchange chromatography with CM-cellulose. For the final purification, fractions containing human epidermal TGase underwent size-exclusion chromatography using Biogel A-0.5 m. From 50 g of callus, 2 mg of pure TGase were isolated [60].

Guinea pig epidermal TGase was enriched by precipitation with ammonium sulfate (75%). The enriched TGase was applied to a heparin-Sepharose column. The following final purification steps were performed using size-exclusion chromatography with Biogel A and cation-exchange chromatography using S-Sepharose. Approximately 14 mg of TGase was purified from 200 g of skin powder [62].

Epidermal TGase from cow muzzle was finally purified using zone electrophoresis and size-exclusion chromatography with Sephadex G 200. [61]. The proenzyme of epidermal TGase has a relative molecular mass of 78 000, while the active enzyme of 50 000 as determined by SDS-PAGE [62,60].

4. Chromatographic and electrophoretic behavior of transglutaminases

Secretory TGase in rat coagulating gland has been separated into two forms which migrate to the cathode using electrophoresis on agarose gels at pH 7.4. Rat tissue TGase and human factor XIII orientate towards the anode under the same conditions [4]. These electrophoretic behaviors are in agreement with the above described chromatographic behavior using anion-exchange chromatography. Rat secretory TGase elutes in the unbound fraction using a buffer with pH 7.5 [40], whereas the other TGase isoforms remain bound at this pH. Using isoelectric focusing, secretory TGase focused in crude coagulating gland extracts at pH 7.8, whereas the pure enzyme revealed an additional isoelectric point of pH 8.7. As both isoforms migrate identically on SDS-PAGE, modification through proteolytic cleavage is unlikely. However, the observed shift to the basic net charge could be due to the loss of some substituted groups.

In addition, after FPLC chromatofocusing of enriched fractions containing rat secretory TGase, both isoforms were separated and identified. Rat testicular tissue TGase focused at a pH of 5.25 [8].

As TGase has a propensity for autocatalytical cross-linking during purification under native conditions, the influence of calcium ions upon size-exclusion chromatography was investigated. Therefore, crude TGase extracts of rat origin were processed by size-exclusion chromatography using a Superose 12 column. More than 40% of the applied enzyme was cross-linked autocatalytically if the column was run with 5 m*M* calcium. Additionally, 49% of the enzyme aggregated physically and could only be eluted using comparably strong conditions (0.1 *M* NaOH). Only 8% of the enzyme activity was detectable in the void volume. Using a calcium-free Tris buffer containing 10 m*M* EDTA, 38% of aggregated TGase eluted at a volume corresponding to M_r 200 000 and 20% at a volume corresponding to M_r 60 000–70 000. Using FPLC with Superdex 200 instead of Superose 12 the yield was significantly improved, and the aggregation of the protein markedly diminished [7].

In addition, the posttranslational modification of secretory TGase has an influence upon autoaggregation. After treatment with phospholipase C, deacylated secretory TGase aggregated autocatalytically into oligomers greater than M_r 200 000. The oligomers are unable to enter the stacking gel (Fig. 7).

The relative molecular masses of the different isoforms determined by SDS-PAGE are summarized in Table 4.

Fig. 7. Western blot analysis of secretory TGase of rat coagulating gland. Monomer protein with M_r 65 000 (lane 1). Autoaggreagation af TGase to oligomers>M_r 200 000 (lane 2) after deacylation using phospholipase C.

5. Transglutaminase assays

5.1. Fluorometric and radioactive assays

TGase activity assays are based on the enzymatic function of the enzyme, i.e. the covalent binding of a primary amine to a peptide-bound glutamine in a calcium-dependent reaction. In most assays used, a lysine analogue (radiolabeled (putrescine ^{14}C or ^{3}H, [63,64]) or fluorescent-labeled primary amine derivatives) is incorporated into a protein acceptor [65,66].

5.1.1. Fluorometric chromatographic and electrophoretic assays

A fluorescent amine such as monodansylcadaverine is incorporated into casein or a synthetic peptide-like benzyloxycarbonyl-L-glutaminylglycine [65,66]. The fluorescent cross-linked product is separated from the initial substrate by electrophoresis, ion-exchange chromatography, thin-layer chromatography, size-exclusion chromatography [65] or reversed-phase HPLC [66].

In addition, activity staining on agarose gels has been described. After electrophoresis filter papers soaked with calcium, monodansylcadaverine and N,N-dimethylcasein were applied on the gel. After fixation of the proteins with ethanol–acetic acid and subsequent gel drying, the incorporation of the dansyl group was visualized with an UV lamp [67].

Table 4
Relative molecular masses of the different transglutaminase types determined by SDS–PAGE

Enzyme	M_r
Secretory TGase	65 000–70 000
Tissue TGase	80 000–85 000
Factor XIIIa	80 000
Hemocyte TGase	86 000
Keratinocyte TGase	92 000
Epidermal TGase	
Proenzyme	78 000
Active enzyme	50 000

5.1.2. Radioactive assays

Radiolabeled putrescine (^{14}C or ^{3}H) is incorporated into a protein substrate, e.g. casein. The separation of unreacted amine and cross-linked protein is conveniently done by precipitation. The increase of radioactivity is measured as TGase activity [63,64].

5.2. Enzyme-linked colorimetric and immunochemical assays on microtiter plates

More recently, colorimetric assays are described which use biotinylated amine substrates such as 5-(biotinamido)pentylamine [68–70] or dansylcadaverine derivatives [71,72] for incorporation into casein. Biotinylated products are visualized by streptavidin-β-galactosidase [68,69] or streptavidin-alkaline phosphatase [70]. Bound dansylcadaverine is detected using a monoclonal antibody against the dansyl moiety [71,72]. The enzymatic TGase reaction can either be carried out in tubes [71,68,69] or directly in microtiter plates coated with casein [70,72]. In a sandwich system biotin-labeled casein can also be used as a substrate for the TGase cross-linking reaction with casein bound to microtiter plates. The biotinylated cross-linked product is visualized using avidin with alkaline phosphatase conjugated [73].

Immunochemical tests are an alternative way of quantifying plasma factor XIII and tissue TGase. Factor XIII binds to monoclonal antibodies attached to a microtiter plate against the factor XIII subunit a or b. The bound factor XIII is detected using biotinylated antibodies [74]. Tissue TGase binds to a polyclonal IgG against human TGase immobilized to microtiter plates. The bound TGase is visualized using a monoclonal antibody [75].

5.3. Photometric assays

Photometric assays have been described using ethylamine [76] or glycine-ethylester [77–79] as substrates for the incorporation into modified β-casein [76] or a specific synthetic peptide [77–79]. The released ammonia is detected in a combined reaction: catalyzed by glutamate dehydrogenase, ammonia is incorporated into α-ketoglutarate under NADH consumption. The decreasing NADH concentration is monitored at 340 nm. These assays are predominately used to determine plasma factor XIII to diagnose factor XIII deficiency.

5.4. Comparison of the different methods

The most sensitive methods for accurate quantification of TGase activity are radioactive and fluorometric assays. However, to date many laboratories try to avoid working with radioisotopes. Furthermore, fluorometric assays require special instruments for the detection of the fluorescent products. Colorimetric and photometric assays are an alternative to radioactive or fluorometric assays. These assays are fast, reproducible and sensitive. All substrates are commercially available and special equipment is not necessary. Up to 95 samples in parallel can be handled using a colorimetric assay.

6. Perspectives

To date all current popular chromatographic purification methods (ion-exchange, size-exclusion, hydrophobic-interaction, affinity, and adsorption chromatography) were described for TGase isolation. Nevertheless, during recent years protein purification procedures have improved remarkably. One or two decades ago it took a long time and a lot of effort to purify proteins, because conventional chromatography columns usually ran for several hours or days. Using recent methods for protein purification, such as FPLC or Smart systems and modern chromatography media, it is possible to run columns in 1 or 2 h with a high reproducibility. Since column size, solvent consumption, as well as the void volume of the instruments is small, it is possible to separate proteins even in the micromolar range. Short purification times and improved chromatography matrices with low self absorption capacities have meant better yields for TGase, since self-aggregating and cross-linking of contaminating proteins is diminished. At present, new electrophoretic methods are being developed, such as automated capillary electrophoresis. However, at the moment this method is only usable for analytical and not for preparative applications. At present, manufacturers are trying to improve this method for preparative applications also.

Purified TGase can be applied to the following three areas:

1. Clinical aspects: factor XIIIa is an important protein for wound healing. Patients with factor XIIIa deficiency have a pathological hemostasis and insufficient wound healing. Substitution with factor XIIIa isolated from placentae guarantees normal blood coagulation, wound healing and placenta retention.
2. Tissue TGase can be used as a cross-linker to bind glutamine-containing peptides or polypeptides to NH_2-columns, in order to get new affinity matrices, or for the covalent attachment of antigen and antibody after blotting procedures. In addition, it can be utilized as an inductor of cell adhesion in cells cultured on substrates (e.g. fibronectin or collagen). Furthermore, proteins can be labeled with dansyl- or radioactive-residues for direct analytical demonstration.
3. TGases have been also used in the food industry to cross-link proteins (e.g. casein and soybean globulins) or to supplement food proteins with the amino acid lysine. Since protein solutions can be gelatinized by TGase, the enzyme can be used as a stabilization factor of emulsions in many processed foods.

7. List of abbreviations

AFC	Affinity chromatography
ADC	Adsorption chromatography
AS-P	Ammonium sulfate precipitation
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E-P	Ethanol precipitation
FPLC	Fast protein liquid chromatography
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
MCC	Metal-chelating chromatography
P-P	Protamine precipitation
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
TGase	Transglutaminase
Tris	Tris(hydroxymethyl)-aminomethane
ZE	Zone electrophoresis

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