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Purification of Human Brain Tissue Factor

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Key words

Tissue factor – Coagulation – Human brain

Summary

Tissue factor (thromboplastin or Factor III), a glycoprotein cofactor, is required for Factor VII to express its catalytic activity, thereby initiating the extrinsic as well as intrinsic pathway of blood coagulation. Human brain tissue factor was purified 2,500-fold to 98% homogeneity from 2% Triton X-100 extraction of acetone dried brain powder with an overall yield of 36%. The method was based upon affinity chromatography utilizing the high affinity binding of tissue factor to Factor VII noncovalently complexed to immobilized anti-Factor VII-agarose beads. The apparent molecular weight of the purified tissue factor is 45,000 as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its isoelectric point is 4.8–5.1 by column chromatofocussing and flat bed agarose isoelectric focussing.

Introduction

Tissue factor (TF)[1], an integral membrane glycoprotein, is a cofactor required for Factor VII to express its catalytic activity, thereby initiating both extrinsic (1) and intrinsic pathways (2) of blood coagulation. Radio-labeled Factor VII binds to endotoxin-stimulated leukocytes where TF is known to be generated, but not to normal leukocytes (3). Furthermore, both Factors VII and X bind to an immobilized TF only in the presence of calcium ions. Recently this high affinity binding of TF to Factor VII was exploited to purify TF by immobilizing Factor VII to agarose beads (4, 5). Covalent modification of Factor VII frequently results in drastic reduction or at worst complete abolition of activity. This is due to a lack of selectivity of covalent immobilization, which thereby alters binding affinity between TF and Factor VII. As an alternative to this disadvantage, we purified TF based on an affinity chromatography utilizing the high affinity binding of TF to Factor VII noncovalently complexed to immobilized anti-Factor VII agarose beads, and the method is described here.

1. The abbreviations used are: TF, tissue factor; TBS, tris buffer saline (50 mM tris, 0.1 M NaCl, pH = 7.4, 0.1% NaN_3, 20 mM benzamidine); TBS-Ca, TBS with 10 mM $CaCl_2$; EDTA, ethylene diamine tetraacetic acid; TBS-EDTA, TBS with 10 mM EDTA; MOPS, 2-(morpholino)-propane-sulfonic acid; CHAPS, 3-(cholamide-dipropyl-dimethylamino-1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. Unpublished results. 3. Unpublished results. 4. Unpublished results.

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Materials and Methods

Materials

Affi-Gel-10, Affi-Gel-15, acrylamide, bisacrylamide, and Triton X-100 were obtained from Bio-Rad. MOPS and SDS were from American Research Products, and cholate and deoxycholate from Calbiochem. Standard rabbit brain thromboplastin was from Difco, and CHAPS from Pierce Chemicals. Protein labeling reagents from Molecular Probes, Inc. and Tris, trypsin, soybean trypsin inhibitor, tweens, lubrols and all other chemicals were from Sigma. Fresh frozen human plasma was purchased from Central Jersey Blood Bank. Polybuffer, polybuffer exchanger, IEF agarose, and standard proteins for molecular weights and isoelectric points were from Pharmacia.

Methods

Clotting assay for tissue factor (TF) – All TF apoprotein was optimally relipidated with mixed brain lipids as described (6, 7). A two stage assay adopted in this laboratory (7, 8) is routinely used to determine the TF activity. 0.1 ml of TF containing sample is added to 0.2 ml of barium sulfate eluate (Factors VII and X) in ice bath and incubated at 37° C for 30 sec, and then 0.1 ml of 25 mM $CaCl_2$ is added to the above mixture. After 10 min 0.1 ml of this mixture is added to a second tube (2nd stage) containing 0.1 ml of 25 mM $CaCl_2$ and 0.1 ml of cephalin (1/150 dilution of 1 gram rabbit brain which was extracted by 40 ml chloroform and then dissolved in 33 ml of saline after complete evaporation of chloroform). Finally 0.1 ml of difluorophosphate (DFP) treated bovine plasma is added to the mixture, and clotting time is measured. One ml of full strength rabbit brain thromboplastin (1 gram/28 ml of saline with subsequent removal of debris by centrifugation) prepared by the manufacturer's instruction was arbitrarily defined as containing 1,000 units.

Anti-Factor VII-Affi-Gel-10 – 5 ml of Factor VII immunized rabbit plasma were precipitated with ammonium sulfate to a 55% final saturation, and centrifuged at 10,000 g × 30 min. The pellet was resuspended in 5 ml of 25 mM phosphate buffer saline (PBS – 25 mM phosphate, 50 mM saline at pH = 7.4), and dialyzed overnight against the same buffer. The dialyzed solution was then applied to a 2 ml of preequilibrated protein A column and washed with PBS until optical density returned to baseline. The column was eluted with 50 mM acetic acid at pH = 3.0. The eluate was immediately neutralized with 2 M K_2HPO_4, and the active tubes were concentrated and dialyzed in 0.1 M MOPS buffer in an Amicon ultrafiltration system using PM-10 membrane. Five mg of purified anti-Factor VII in 10 ml of 0.1 M MOPS buffer were coupled to 2 ml of Affi-Gel-10 overnight at 4° C with gentle shaking according to the method described in the Bio-Rad Bulletin 1085, and unreacted sites were blocked with 0.1 M Tris buffer at pH = 7.5.

Crude barium sulfate eluate of fresh frozen human plasma was used for the source of Factor VII in complexing with anti-Factor VII Affi-Gel-10. Barium sulfate eluate was prepared according to Broze and Majerus (9) with a slight modification where elution of the vitamin K dependent factors with 0.15 M sodium citrate was replaced by 35% saturated ammonium sulfate solution. The eluate was concentrated and dialyzed in TBS-EDTA and reacted with anti-Factor VII-Affi-Gel-10 overnight with shaking at 4° C. The mixture was washed and equilibrated with TBS-Ca in 10 × 50 mm Bio-Rad econocolumn for further reaction.

Purification of TF apoprotein – 10 grams of acetone dried human brain powder were homogenized in 200 ml of TBS for 10 min in a Waring blender. The mixture was centrifuged at 12,000 g × 1 hr at 5° C, and the pellet was homogenized with TBS with 0.1% Triton X-100 for 10 min. and centrifuged at 12,000 g × 1 hr at 5° C. The pellet was resuspended in TBS

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with 2% Triton X-100. The mixture was blended for 10 min and centrifuged at 16,000 g × 1 hr. The supernatant, which is rich in TF activity, is stable at 4° C for several months.

Fifty ml of 2% Triton X-100 extracted TF, after adding $CaCl_2$ to 10 mM final concentration, were applied to a preequilibrated anti-Factor VII-Affi-Gel-10 column saturated with Factor VII. The column was washed thoroughly with TBS-Ca with 0.1% Triton X-100 and eluted with TBS-EDTA with 0.1% Triton X-100 in a fraction collector. The active tubes, which are not pure as judged by SDS-PAGE, were reapplied to a preequilibrated column without being further concentrated. After thorough washing with TBS-Ca with 0.1% Triton X-100, the column was eluted with TBS-EDTA with 0.1% Triton X-100. The active tubes were pooled and concentrated in an Amicon ultrafiltration system using PM-10 membrane.

Protein determination – A modified Lowry's method (10) was used in all experiments except for purified tissue factor. In order to determine purified tissue factor protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis was first carried out by the method of Laemmli (11). The Coomasie blue stained gel was read at 595 nm on a ACD-18 Densitometer of Gelman Sciences, Inc. using bovine serum albumin as a standard. Isoelectric points of TF were determined by chromatofocussing and flat bed isoelectrofocussing. In chromatofocussing, TF containing sample was applied to a 7 × 30 mm column preequilibrated with 50 mM imidazole buffer at pH = 7.0. After extensive washing with imidazole buffer the column was eluted with polybuffer 7/4 at pH = 4.0, and the activity was measured versus pH. Flat bed electrofocussing was performed on FBE-3000 of Pharmacia Fine Chemicals using isoelectric calibration kit and by a direct measurement of pH.

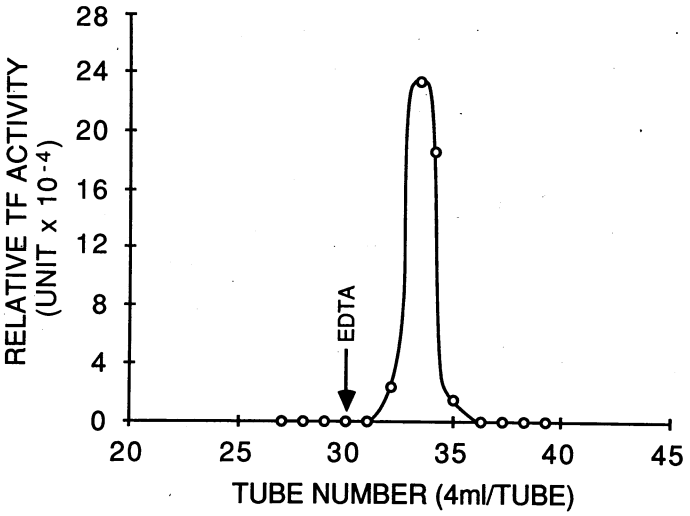

Fig. 1 2% Triton X-100 extract was applied to a 10 × 40 mm column of Factor VII-anti-Factor VII-Affi-Gel-10 in TBS-Ca (50 mM tris, 0.1 M NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, 0.1% NaN_3 at pH = 7.4) at a flow rate of 8 ml/h. After extensive washing with 100 ml of the same buffer at a flow rate of 16 ml/h, the bound tissue factor was eluted with TBS-EDTA (50 mM tris, 0.1% NaCl, 10 mM EDTA, 0.1% Triton X-100, 0.1% NaN_3 at pH = 7.4) at a flow rate of 8 ml/h

Results

All the extraction buffers contained 0.1% NaN_3 and 5 mM EDTA and the buffers for affinity chromatography contained 0.1% NaN_3 and 20 mM benzamidine. The whole human brain was frozen at −70° C within 24 h post mortem, and 50 grams of the frozen brain was homogenized with 200 ml of cold acetone in a Waring blender and filtered in a Whatman No. 2 filter paper in a glass funnel. The pellet was again homogenized with 200 ml of cold acetone, and the procedure was repeated until no acetone soluble materials were detected. The pellet was dried at room temperature overnight and stored at −70° C. The acetone dried human brain powder at −70° C is stable for years. Before extracting with 2% Triton X-100 the acetone dried brain powder was washed first with TBS, and then with TBS with 0.1% Triton X-100. With 0.1% Triton X-100 detergent, about 10% of total tissue factor was extracted. The remaining tissue factor was extracted with 2% Triton X-100. 2% Triton X-100 solubilized tissue factor is stable at 4° C for several months.

The average tissue factor activity of 200 ml of 2% Triton X-100 extraction of 10 gr of acetone dried human brain was 10 million units. Neither prolonged homogenization time nor higher concentration of detergent improved extraction. $CaCl_2$ powder was then added to a 2% Triton X-100 solubilized tissue factor to a final 10 mM concentration, and the mixture became turbid. Fifty ml of the turbid solution without centrifugation were applied to a 10 × 40 mm column of Factor VII-anti Factor VII-Affi-Gel-10 at a flow rate of 8 ml/h. The column was then washed with 50 ml of TBS-Ca with 0.1% Triton X-100 at a flow rate of 16 ml/h. Extensive washing with 200 ml of TBS-Ca with 2% Triton X-100 showed similar results. Tissue factor protein was eluted with TBS-EDTA with 0.1% Triton X-100 at a flow rate of 8 ml/h (Fig. 1). The active tubes were pooled and concentrated in an Amicon ultrafiltration apparatus using PM-10 membrane, and further concentrated by Amicon Centricone (MW cut-off = 30,000). SDS-polyacrylamide gel electrophoresis showed purified tissue factor to be about 5% of total protein (Fig. 2B). This partially purified tissue factor of the first run was reconstituted with $CaCl_2$ without being concentrated and reapplied to a reequilibrated column with TBS-Ca. No tissue factor activity was detected in the breakthrough and wash-out, and elution with TBS-EDTA with 0.1% Triton X-100 produced the same elution chromatogram as the first one with more than 90% recovery of the activity. The SDS-gel electrophoresis shows a single band at a position corresponding to the molecular weight of 45,000 with three faint minor bands at molecular weights 88,000, 78,000 and 30,000 respectively (Fig. 2 E). Repeated purification by the same method eliminated the proteins of molecular weight 78,000 and 30,000, but not the one of 88,000 which might be a dimeric form of tissue factor protein. The densitometer scanning shows the tissue factor protein to be 98% homogeneous (Fig. 3).

In order to determine the role of lipids in the tissue factor binding to Factor VII-anti-Factor VII-Affi-Gel as well as to Factor VII, whole brain lipids (7) were added to 2% Triton extract as well as all the buffers used in the affinity chromatography at a concentration of 100 μg/ml. Since the result in the presence of lipids was identical to the one without lipids even after excessive washing with 2% Triton X-100 TBS-Ca, where unbound or loosely bound lipids seemed completely washed out, lipids appear unimportant for tissue factor-Factor VII binding.

When 6 million units of tissue factor in 2% Triton X-100 solution were applied to the antigen-antibody-Affi-Gel column in order to fully saturate the column, which maximizes the binding, excessive amounts of tissue factor were found in the breakthrough and wash-out totaling approximately 5–5.2 million units. Elution with TBS-EDTA results in a partially purified tissue factor activity of 400,000 units. However, the second run over the same column results in a 90% recovery of activity. Based on this observation overall yield of the tissue factor activity is 36–45% from the starting material of 2% detergent extract. Protein determination of purified tissue factor in a high concentration of detergent is a difficult task by a modified Lowry's method (9). Because Triton X-100 does not pass an Amicon PM-10 or PM-30 membrane, protein measurement of concentrated tissue factor is prohibitively inaccurate. To circumvent this problem, purified tissue factor protein was determined by a densitometer of a Coomasie blue stained gel using bovine serum albumin as a

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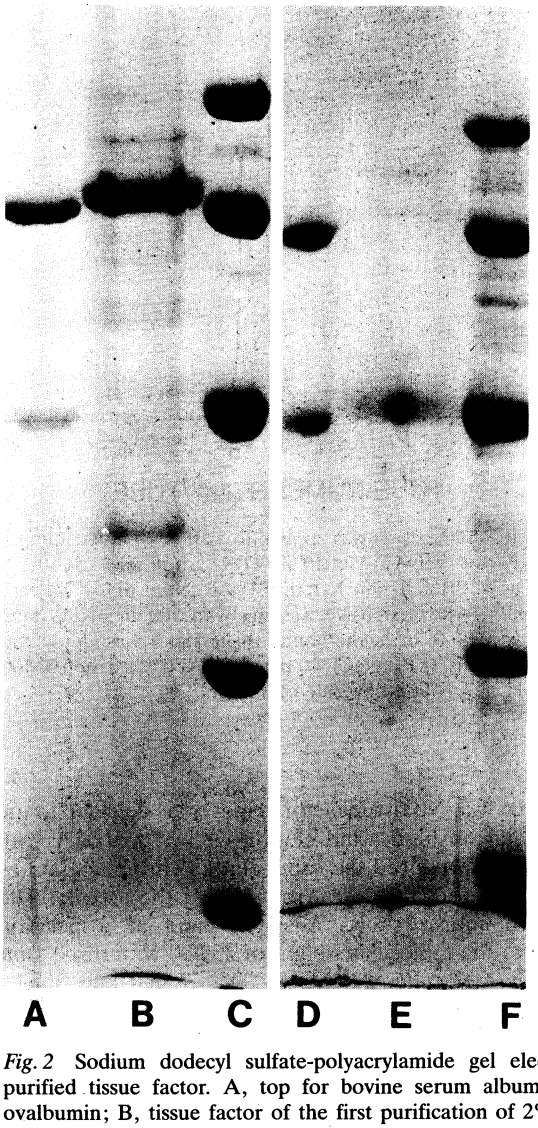

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified tissue factor. A, top for bovine serum albumin, bottom for ovalbumin; B, tissue factor of the first purification of 2% Triton X-100 extract of human brain by a Factor VII-anti-Factor VII-Affi-Gel-10; C, molecular weight standards from top to bottom phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000); D, same as A; E, tissue factor of second purification by the affinity chromatography; F, same as C

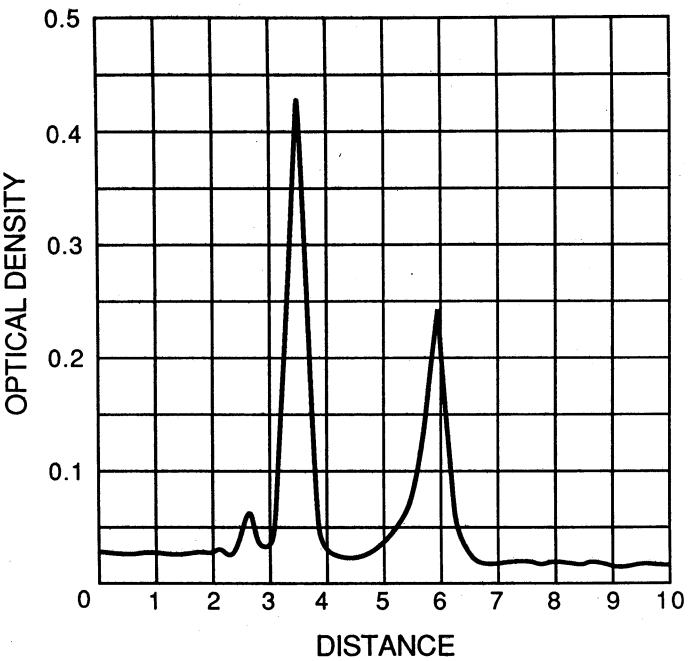

Fig. 3 Densitogram of Coomasie blue stained electrophoresis gel by ACD-18 Densitometer of Gelman Sciences, Inc. The first major peak at distance 3.5 is for 4 μg of bovine serum albumin, and the second major peak at distance 5.90 is for 480,000 units of purified tissue factor from acetone dried human brain. A small peak at 2.6 may be a dimeric form of tissue factor protein as judged by molecular weight

Table 1 Purification of human brain tissue factor

	TF activity ($\times 10^3$)	Protein	Specific activity unit/μg	Purification -fold
2% Triton X-100 extraction	60/ml	840 μg/ml	71	1
Affinity column (2nd)	480	2.7 μg	177,800	2,500

standard (Fig. 3). The result shows a 2,500-fold purification of tissue factor from 2% Triton X-100 extract of human brain acetone dried powder (Table 1).

Discussion

Purification of tissue factor has been a difficult task for a long period of time. Solubilization with 2% deoxycholate with subsequent removal of lipids by gel filtration and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis result in a relatively good purification of tissue factor apoprotein (12, 13). The activity of SDS denatured tissue factor after electrophoresis is fully recovered if the gel is eluted in a cholate-deoxycholate buffer (1.5% cholate, 0.5% deoxycholate, 50 mM Tris, pH = 7.4). However, it has been observed that during or after concentration, lyophilization or storage the tissue factor activity is gradually reduced[2]. It is possible that if the lipids directly supporting the tertial structure of the active form of tissue factor are dissociated from tissue factor-lipids complexation, the active form of tissue factor undergoes a slow conformational change leading to a permanent loss of activity. The nonionic detergent on the contrary does not readily dissociate the structural lipids from tissue factor apoprotein, thereby preserving the integral structure of tissue factor. Two percent Triton X-100 appears to be better than other nonionic detergent such as lubrols and tweens.

Recently tissue factor apoprotein has been purified by use of affinity chromatography based upon immobilization of Factor VII to agarose beads by way of N-hydroxysuccinimide esters (4, 5).

However, we have observed that the binding capacity of tissue factor to a Factor VII-Affi-Gels critically depends on the nature and degree of coupling of Factor VII to the beads, and that a workable experimental condition is difficult to establish. Although N-hydroxysuccinimide ester group of Affi-Gels primarily couples with the primary amino group of protein, it is known to compete with other functional groups such as sulfhydryls. It was found in our laboratory that covalent modification of Factor VII with fluorescein isothiocyanate, a selective coupling reagent

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to the primary amino groups, and that the activity of the labeled Factor VII is reduced by 50–90% depending on the degree of coupling. Other nonselective labeling reagents drastically reduced the activity[3]. On the other hand, immobilization of Factor VII by way of antibody-coupled agarose beads gave a consistently reproducible purification of tissue factor. The advantage of this affinity chromatography is that (1) it is not necessary to use the purified Factor VII. A crude barium citrate eluate of human plasma is sufficient as a source of Factor VII. The other contaminants do not bind to the anti-Factor VII-Affi-Gel. Purification of Factor VII is a difficult undertaking because of the trace amount of Factor VII in plasma, (2) this antigen-antibody solid phase column is extremely stable. More than 30 consecutive experiments over 3 months showed no sign of deterioration of the binding capacity of tissue factor. When the column begins to deteriorate, it is easily regenerated by incubating with fresh barium citrate eluate of vitamin K dependent factors after stripping the column with 3M potassium thiocyanate (3). It is unnecessary to pretreat 2% Triton X-100 extraction with glycine ethyl ester Affi-Gel as employed by Broze et al., or to posttreat tissue factor by tryptic digestion as well as by gel filtration chromatography as done by Guha et al. It is worthy of note that elution of tissue factor with 0.1%. Triton X-100 gave the same result as that with 2% Triton X-100. Since Triton detergent does not readily pass the Amicon ultrafiltration membrane PM-10 or PM-30, concentration of tissue factor eluent on a membrane filtration in a high concentration detergent buffer poses a problem unless the detergent is removed prior to concentration. Neither washing nor elution buffer contained lipids in our experiments, and it is expected that only tightly bound structural lipids are retained with tissue factor in the affinity column. Additional lipids in the 2% extraction and washing buffer did not enhance binding capacity of tissue factor. Perhaps the amount of lipids is not critical in tissue factor-Factor VII binding contrary to the important role of lipids in clotting activity (6). Lipids may not be required in tissue factor-Factor VII binding. Fig. 2 shows that besides the major tissue factor band there are two faint but distinct bands in the SDS-PAGE corresponding to molecular weights of 88,000 and 78,000 (Fig. 2 E). One broad but faint band at 30,000 was observed (Fig. 2 E) but fell in the noise level in the scanning densitometer (Fig. 3). Further purification by repeated application of tissue factor to the affinity column eliminated the bands at 78,000 and 30,000, but not the one at 88,000, which may be a dimer as judged by a molecular weight criterion (Fig. 2, D and F). It is interesting to note that a substantial amount of a dimeric form has been observed in purified tissue factor (4). It is also important to note that both human brain and human leukocyte tissue factors partially purified by gel electrophoresis have been associated with multiple molecular weight forms (14)[4]. Guhar et al. also noted that tryptic digestion of tissue factor lowers the molecular weight without loss of tissue factor activity. Purification by hydroxyapatite shows that elution with phosphate buffer distinguishes the two forms of tissue factor activities. Based on the molecular weight determination by the gel densitometer our purification yields 2,500-fold from a starting material of 2% Triton X-100 extraction, which roughly corresponds to the results previously reported (1,950-fold by Guhar et al. and 2,000-fold by Broze et al.). This affinity chromatography by a Factor VII-anti-Factor VII-Affi-Gel-10 is simple and stable, and warrants a large scale purification.

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