THE PROTEIN COMPONENT OF HUMAN BRAIN THROMBOPLASTIN

Bjørklid, E., Storm, E. and Prydz, H.
Institute of Medical Biology, University of Tromsø, Tromsø, Norway

Received October 22,1973

SUMMARY: The protein component of human brain tissue thromboplastin (factor III) has been purified by deoxycholate (DOC) extraction, ultracentrifugation, gel filtration and finally repeated preparative polyacrylamide gel electrophoresis (PGE) in the presence of sodium dodecylsulphate (SDS). The final preparations gave one band in analytical PGE. Reduced and alkylated protein appeared as a band of molecular weight about 53 000 in SDS-PGE.

The protein had a low solubility in aqueous solutions in the absence of detergents. When recombined with an optimal amount of the phospholipid fraction of tissue thromboplastin (fraction B) the procoagulant thromboplastin activity was regained. Neither alone nor after recombination with phospholipid did the protein catalyze the hydrolysis of aminoacyl-β-naphthylamides or casein.

INTRODUCTION: Tissue thromboplastin (factor III) is a protein - phospholipid complex which initiates blood coagulation in the extrinsic system by activating factor VII. By extraction of crude thromboplastin preparations with pyridine (1) or sodium deoxycholate (2) and subsequent gel filtration (3) a protein fraction (factor III apoprotein) and a phospholipid fraction (fraction B) can be separated. Alone each fraction is inactive, when recombined they regain full procoagulant activity.

Nemerson and Pitlick (4) described the purification of a protein fraction from bovine lung which had high procoagulant activity when recombined with phospholipids and which also had peptidase activity (5), and they suggested that this activity was important for the activation of factor VII. In contrast, our earlier results (6) indicated that the activation of factor VII by tissue thromboplastin is reversible and therefore probably does not involve peptide bond cleavage. Further evidence in

Table 1

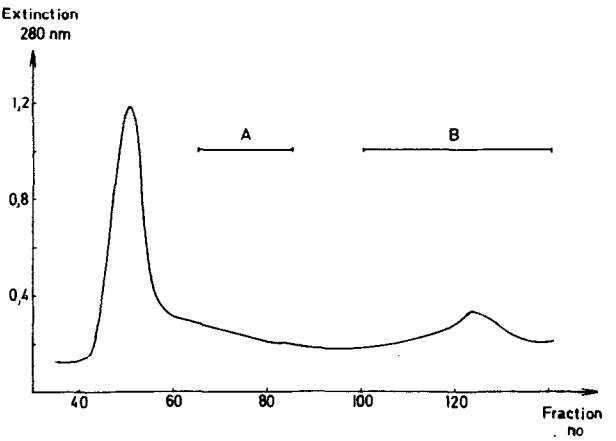
Peptidase activity of different thromboplastin preparations. Incubation was carried out at 37° C for 4 hr.

Preparations	Peptidase activity towards (nmoles/ml/hr)		
	Alanyl-β-naphthylamide	Leucyl-β-naphthylamide	Phenylalanyl-β-naphthylamide
Crude factor III	174	123	54
Factor III apoprotein	0	0	0
Factor III apoprotein + phospholipids	0	0	0
Factor III apoprotein + phospholipids + 1.0 mM or 0.05 mM Co^{++}	0	0	0

support of this view is given here. We have developed a procedure for the purification of factor III apoprotein from human brain. The preparations contained no detectable phospholipid and had no peptidase activity, but regained full procoagulant activity upon recombination with phospholipid. In addition, Nemerson and Esnouf (7) have also reported that the peptidase activity of their factor III preparations was not required for the procoagulant activity.

MATERIALS AND METHODS: Factor III apoprotein and phospholipid fractions were prepared from human brain (3) with the following modifications: Sodium deoxycholate extract (3) from half a brain was concentrated to 10 ml and submitted to gel filtration through a Sephadex G 100 column (100 x 5 cm) at a rate of 10 ml/hr. Fractions of 10 ml were collected.

Analytical and preparative polyacrylamide gel electrophoresis

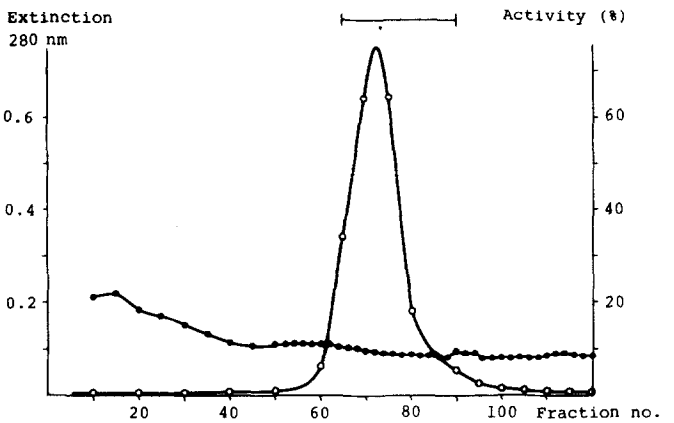


Legend to figure 1

Gel chromatography on a Sephadex G 100 column of a DOC extract from human brain microsomes. The bars indicate factor III apoprotein fraction (A) and phospholipid fraction (B).

in the presence of sodium dodecylsulphate (SDS-PGE). A discontinuous system with a running pH 9.5 (8) was used.

Both analytical and preparative gels were composed of 10 % (w/v) acrylamide and 0.27 % (w/v) N,N'-methylenebisacrylamide. In the preparative gels the concentration of ammonium persulphate was reduced to 0.067 %. Analytical gels were stained in 0.05 % Coomassie Brilliant Blue R 250 (Schuchardt) in 20 % sulphosalicylic acid/ethanol (1 : 1, v/v) for 24 hr at 37°C, destained by diffusion for 6-8 hr in the solvent, restained for 1-2 hr at 37°C with 0.05 % Coomassie Brilliant Blue R 250 in 12.5 % trichloroacetic acid and finally transferred to 10 % trichloroacetic acid for storage (9). After reduction, alkylation (10) and concentration the purified factor III apoprotein was dialysed against 0.1 % SDS in 0.05 M Tris H_2SO_4 pH 6.1 (upper gel buffer). The standard proteins for the estimation of molecular weight, bovine serum albumin (Sigma), ovalbumin (Sigma), human γ-globulin (Nutritional Biochem.), and hemoglobin (Sigma) were prepared for electrophoresis by a procedure similar to that of Neville (8).



Legend to figure 2

Preparative SDS polyacrylamide gel electrophoresis of factor III apoprotein fraction from a Sephadex G 100 eluate.

The electrophoresis was carried out at 20°C to avoid precipitation of SDS. A constant current of 9 mA was applied, the elution rate was 1 ml/min, and 4 ml fractions were collected. Solutions of apoprotein III were concentrated by Aquacide II (Calbiochem) or by Diaflo PM-30 filtration.

Coagulation and peptidase activity. Tissue thromboplastin procoagulant activity was tested and standard curves established as described (11). Peptidase activity was measured by hydrolysis of aminoacyl-β-naphthylamides (Sigma and Koch-Light) (12), using the procedure of Pitlick et al. (5), and by hydrolysis of casein, using a modification of the procedure of Jacobsen (13).

Protein and phosphorous determination. Protein was measured by the fluorometric method of Böhlen et al. (14), using the reagent fluorescamine (Hoffman-LaRoche) with bovine serum albumin as standard.

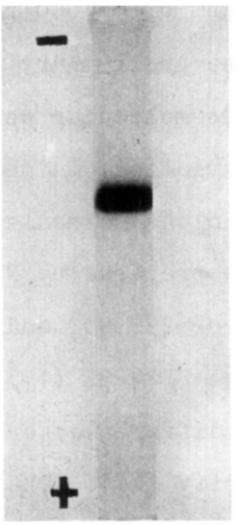
Lipid phosphorous was measured as described by Ames (15).

RESULTS: Due to its low solubility apoprotein III was purified in the presence of detergents. Partially purified apoprotein was isolated by gel chromatography in the presence of sodium deoxycholate (Fig. 1), and the protein fraction was submitted to preparative SDS-PGE. In preparative PGE the activity was eluted as a sharp peak, with no corresponding peak of OD_{280} (Fig. 2). The fractions constituting the first half of the peak (the bar in the figure) were pooled, concentrated to 30 ml, dialysed and submitted to a second preparative PGE under the same conditions. Now the fractions constituting the second half of the peak were pooled and used as the purified apoprotein. The yield from one brain was about 5 mg. Incompletely purified protein was recycled.

The purified apoprotein was submitted to analytical SDS-PGE. With unreduced protein only a single protein band was seen (Fig. 3). By slicing parallel gels, eluting, recombining and testing for coagulation activity it was confirmed that this band corresponded to the active protein. It had a molecular weight of 52 000 $\pm$ 6 000. In reduced and alkylated protein about 10 % impurities appeared behind the main band, which had a molecular weight of 53 000 $\pm$ 6 000. The similar molecular weights obtained in the two cases indicate that the protein is not composed of subunits interlinked by disulphide bridges.

The factor III activity of recombined apoprotein and phospholipid depends on the recombination ratio. The optimal ratio phospholipid (fraction B) : purified protein was 0.4 (w/w), i.e. a molar ratio of about 27.

The apoprotein had low solubility in aqueous solutions. This was probably not due to tightly bound phospholipids, since no phosphate was detected (i.e. $<$ 3 % phospholipid). The protein itself may be highly hydrophobic.



Legend to figure 3

Analytical SDS polyacrylamide gel electrophoresis of purified factor III apoprotein.

Under conditions where crude tissue thromboplastin hydrolyzed alanyl-β-naphthylamide, phenylalanyl-β-naphthylamide or leucyl-β-naphthylamide, purified factor III apoprotein alone or recombined with fraction B to give a thromboplastin of similar high procoagulant activity had no detectable peptidase activity (Table 1). Small amounts of cobalt ions have been reported to stimulate membrane bound aminopeptidases (16) and particularly the tissue factor characterized by Pitlick et al. (5). With the purified apoprotein, however, the presence of cobalt in the incubation mixture did not provoke any peptidase activity (Table 1). No proteolysis was observed by incubation of casein with apoprotein III, alone or recombined with phospholipids, at 37°C for up to 10 hr. Crude factor III lost its peptidase activity after exposure to 0.1 % SDS. The activity was not restored by addition of cobalt.

DISCUSSION: The stability of factor III apoprotein in SDS made the use of preparative SDS-PGE convenient for purification.

The estimated molecular weight of 52 000 for the unreduced protein is in good agreement with the preliminary results of Hvatum and Prydz (3). The fact that the molecular weight was not significantly changed by reduction and alkylation, indicates the presence of only one polypeptide chain in the molecule. This is in contrast to the bovine tissue factors prepared by Nemerson and Pitlick (4), which had molecular weights of 220 000 and 330 000 and appeared as 2 or 3 bands in analytical SDS-PGE (17). The difference might be due to tissue or species difference, or to differences in preparation procedure or purity (18). The optimal recombination ratio (w/w) for phospholipid : protein reported here (0.4) is much lower than the ratio (7.5) obtained by Nemerson and Pitlick (4). This may be due to different composition of the phospholipid mixtures used. The phospholipids used by us (fraction B) were a partially purified fraction.

Crude tissue thromboplastin hydrolyzed alanyl-β-naphthylamide, whereas purified apoprotein or thromboplastin prepared from it with high procoagulant activity had no detectable peptidase activity. These results imply that peptidase activity is not necessary for the extrinsic activation of factor VII. This is in accordance with our previous results (6), which showed that factor VII activated by tissue thromboplastin was inactivated by purified phospholipase C and reactivated by renewed exposure to tissue thromboplastin. It appears unlikely that an activation process involving hydrolysis of peptide bonds would be reversible in this way.

REFERENCES

1. Deutsch, E., Irsigler, K. and Lomoschitz, H., Thromb.Diath. Haemorrhag. (Stuttg.) 12, 12 (1965)
2. Chargaff, E., Moore, D.H. and Bendich, A., J.Biol.Chem. 145, 593 (1942)

3. Hvatum, M. and Prydz, H., Thromb.Diath.Haemorrhag. (Stuttg.) 21, 217 (1969)
4. Nemerson, Y. and Pitlick, F.A., Biochemistry 9, 5100 (1970)
5. Pitlick, F.A., Nemerson, Y., Gottlieb, A.J., Gordon, R.G. and Williams, W.J., Biochemistry 10, 2650 (1971)
6. Østerud, B., Berre, Å., Otnæss, A.-B., Bjørklid, E. and Prydz, H., Biochemistry 11, 2853 (1972)
7. Nemerson, Y. and Esnouf, M.P., Proc.Nat.Acad.Sci. USA 70, 310 (1973)
8. Neville, D.M. Jr., J.Biol.Chem. 246, 6328 (1971)
9. Chrambach, A., Reisefeld, R.A., Wyckoff, M. and Zaccari, J., Anal.Biochem. 20, 150 (1967)
10. Griffith, I.P., Biochem. J. 126, 553 (1972)
11. Hvatum, M. and Prydz, H., Biochim.Biophys.Acta 130, 92 (1966)
12. Goldbarg, J.A. and Rutenberg, A.M., Cancer 11, 283 (1958)
13. Jacobsen, C.D., Scand.J.Clin.Lab.Invest. 16, 372 (1964)
14. Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S., Arch. Biochem.Biophys. 155, 213 (1973)
15. Ames, B.N., Meth.Enzymol. Vol. VIII, 115 (1966)
16. Emmelot, P. and Visser, A., Biochim.Biophys.Acta 241, 273 (1971)
17. Nemerson, Y. and Pitlick, F.A. in Progress in Hemostasis and Thrombosis 1, ed. Spaet, T.H., Grune & Stratton, New York 1972
18. Maddy, A.H., Dunn, M.J. and Kelly, P.G., Biochim.Biophys. Acta 288, 263 (1972)