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ISOLATION, CHARACTERIZATION AND AMINO ACID SEQUENCE OF ECHICETIN ß SUBUNIT, A SPECIFIC INHIBITOR OF VON WILLEBRAND FACTOR AND THROMBIN INTERACTION WITH GLYCOPROTEIN Ib

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Summary. Echicetin is a dimeric protein isolated from the venom of *Echis carinatus* that is a potent inhibitor of von Willebrand Factor and thrombin binding to glycoprotein Ib. Here, we report isolation and amino acid sequence of the ß subunit of echicetin that contains 123 amino acids, including 7 cysteines, and shows similarity with amino acid sequences of botrocetin and Factor IXa/Xa binding protein. We provide evidence that biological activity of echicetin which resides in this ß subunit is relatively resistant to reduction of the molecule.

Echicetin, a protein isolated from *Echis carinatus* venom specifically inhibits agglutination of fixed platelets induced by several platelet glycoprotein Ib (GPIb) agonists such as human von Willebrand Factor (vWF), in the presence of botrocetin, bovine vWF and alboaggregins (1). Echicetin binds to GPIb, but unlike alboaggregins isolated from *Trimeresurus albolabris* (2,3) it does not induce platelet agglutination. Binding of ^{125}I-bovine vWF to platelets is strongly inhibited by echicetin. Echicetin significantly prolongs the bleeding time of mice, suggesting that it may inhibit vWF binding to GPIb in vivo as well as in vitro (1). Peng et al. (4) also demonstrated that echicetin is an inhibitor of α-thrombin-mediated platelet activation that results from thrombin binding to high affinity sites on GPIb. Echicetin

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inhibited platelet aggregation and release induced by low concentrations of thrombin (<0.2 units/ml) but it had no effect on platelet aggregation induced by high concentrations of thrombin (>0.2 units/ml) or by thrombin receptor peptide (4).

We reported previously that the ability of echicetin to inhibit platelet agglutination by bovine vWF was not altered following total reduction of the molecule (1). Here we report isolation of two subunits of echicetin by means of HPLC and demonstrate that inhibitory activity resides in its ß subunit.

MATERIALS AND METHODS

Echicetin. Echicetin was purified from *E.carinatus* venom (Sigma Chemical Co, St Louis, Mo) by combination of reverse phase and ion-exchange HPLC as described previously (1). About 70 µg highly purified echicetin was obtained from 1.0 mg venom.

Purification of α and ß chains of echicetin. Native echicetin (15.8 µM) was adjusted to pH 8.5 by adding 1 M Tris solution (pH 10.0). Reduction was performed at 37° C by adding DTT (6 mM) in 0.05 M Tris.HCl buffer, pH 8.6 for 30 min. An eight fold excess of iodoacetic acid in 0.05 M Tris.HCl buffer, pH 8.6, was then added and the sample mixture was allowed to incubate for 30 min at 22°C. The sample was applied on a C_{18} reverse - phase column and the fractions were eluted with 0 - 80% gradient of acetonitrile in 0.1% trifluoracetic acid (TFA) within 80 min. Homologous fractions were pooled from several preparations and the pools were re-dialyzed against 0.1% TFA in order to remove acetonitrile. Samples were concentrated by dialysis against polyethylene glycol 20,000 (PEG) to 1/4 of the original volume and then dialyzed against 0.1% TFA to remove PEG.

Protein concentration was measured by the BCA protein assay (Pierce Chemical Co) on microtiter plates with bovine serum albumin as a standard.

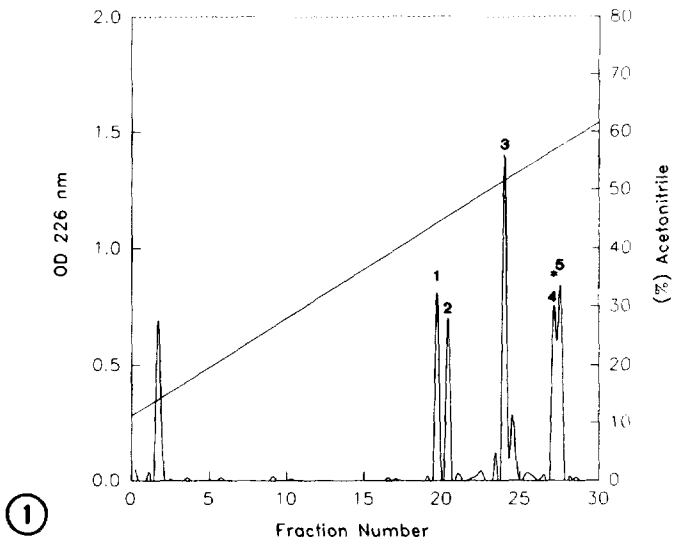
Amino acid sequence of ß chain of echicetin. The amino acid sequence of echicetin ß chain was established by automated N-terminal sequencing of the intact polypeptide chain and fragments produced by cleavage with endopeptidase Lys-C (Promega Biotech, 3% w/w in 10-20% acetonitrile, 0.2 M Tris-Cl, pH 8.6, 16 hrs, 37°C) or cyanogen bromide (final concentration 43 mg/ml in 6 M guanidine hydrochloride, 0.1 N HCl, 7% acetonitrile, 30 min, 37°C). Peptides were isolated from digests by reverse phase HPLC on 2.1 x 150 mm wide pore C-18 columns (Vydac, The Separations Group) equilibrated on 1% trifluoroacetic acid and developed with gradient of acetonitrile.

Preparation of platelet suspensions and measurement of thrombin - induced platelet aggregation and vWF - induced platelet agglutination. Human washed platelets were prepared according to Mustard et al (5) and suspended in Tyrode-albumin buffer containing calcium and magnesium. Aggregation was induced by α-thrombin (kindly donated by Dr John W Fenton II, New York State Dept of Health, Albany NY). Dose dependent inhibition of thrombin induced platelet aggregation by echicetin was measured in a Payton aggregometer. Formalin-fixed platelets were prepared according to Kirby (6). Agglutination of formalin - fixed platelets by bovine vWF and its inhibition by echicetin was measured as described by Peng et al (2).

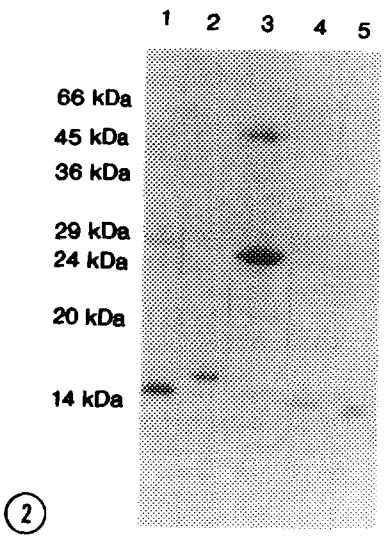
RESULTS AND DISCUSSION

After reduction and alkylation of echicetin, five fractions were eluted from the C_{18} column with a linear gradient of acetonitrile (Fig. 1). On a 12% SDS - polyacrylamide gel, fractions 1 and 2 revealed homogenous single chains with apparent molecular weight of 15 kDa and 16 kDa, respectively. Fraction 3 was unreduced as judged by an apparent molecular weight of 26 kDa. Fractions 4 and 5 showed apparent molecular weights of 13 kDa and 12 kDa, respectively. (Fig. 2).

NH_2 -terminal sequencing of the fractions revealed that fractions 1 and 2 had the same NH_2 terminus that corresponds to α echicetin. Fractions 4 and 5 also had the same NH_2 terminus corresponding to echicetin ß. Figure 3 shows the amino acid sequence of ß echicetin that contains 123 amino acids and 7 cysteines. We propose that one cysteine is involved in forming a bridge between two subunits of echicetin, whereas remaining cysteines may be involved in intramolecular S-S bridges. Amino acid sequence analysis also showed that three intramolecular bridges have been completely reduced and alkylated. The amino acid sequence of echicetin ß subunit shows 57% homology with the sequence of botrocetin, isolated from *Botrops jararaca* viper venom (7) and 61%



(1)



(2)

Figure 1. Fractionation of reduced and alkylated echicetin on reverse-phase HPLC.

Figure 2. SDS polyacrylamide gel electrophoresis of fractions eluted from HPLC. Fraction numbers are the same as in figure 1.

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1         10        20        30        40        50        60
.         .         .         .         .         .         .
NCLPDWSVYEGYCYKVFKERMNWADAEKFCMKQVKDGHLVSFRNSKEVDFMISLAFPMLK
61        70        80        90        100       110       120
.         .         .         .         .         .         .
MELVWIGLSDYWRDCYWEWSDGAQLDYKAWDNERHCFAAKTTDNQWMRRKCSGEFYFVCKCPA
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Figure 3. Amino acid sequence of ß echicetin.

homology with the sequence of Factor IXa and Xa binding protein isolated from *Trimeresurus flavoviridis* venom (8).

Table 1 compares an inhibitory effect of echicetin and its subunits on platelet aggregation induced by 0.07 units of thrombin and on agglutination of formalin-fixed platelets by 6.1 nM bovine vWF.

This experiment shows that biological activity of echicetin was present in the ß subunit of echicetin not in the α subunit. It is interesting that the purification of ß subunit resulted in a much more significant loss of inhibitory activity directed against thrombin-mediated platelet aggregation than in a loss of the inhibitory activity measured in platelet agglutination induced by bovine vWF. It is conceivable that echicetin's ability to block thrombin interaction with GPIb, that is only partially isolated with purified ß chain, may depend on the secondary structure of the whole molecule.

In conclusion, it is interesting that the ability of echicetin to inhibit vWF interaction with GPIb does not depend on

Table 1

Effect of echicetin subunits on thrombin - induced platelet aggregation and on platelet agglutination by bovine vWF

Antagonist	Platelet aggregation by thrombin*	Platelet agglutination by bovine vWF#
	IC_{50} μM	
Echicetin	0.042	0.015
α subunit	>1.40	>0.480
ß subunit	0.90	0.070

* Various concentrations of echicetin and its subunits were added to 500 μl platelet suspension (3 x 10^8 platelets per ml) one min prior to the addition of 0.07 u thrombin/ml.

\# Various concentrations of echicetin and its subunits were added to the suspension of formalin-fixed platelet one minute prior to the addition of bovine vWF (6.1 nM).

the conformation of the molecule maintained by S-S bridges. This is in contrast to disintegrin echistatin, another component of *E.carinatus* venom that blocks fibrinogen binding to glycoprotein IIb/IIIa receptor. Echistatin (9), like other disintegrins (10), lost almost completely their biological activity following reduction and alkylation of the molecule. Our observation, that the abilities of echicetin to block thrombin and bovine vWFis interaction with platelets do not decrease in parallel during purification of reduced ß echicetin, is consistent with the recent reports that the thrombin binding site and vWF binding site on the GPIb molecule are not identical (11,12).

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REFERENCES

1. Peng, M., Lu, W., Beviglia, L., Niewiarowski, S., and Kirby, E.P., (1993) Blood 81:2321-2328.
2. Peng, M., Lu, W., and Kirby, E.P.,(1991) Biochemistry 30, 11529-11536.
3. Peng, M., Lu, W., and Kirby, E.P.,(1992) Thrombosis and Hemostasis 67: 702- 707.
4. Peng, M., Mao, A., Lu, W., Kirby, E.P., (1993) Thrombosis and Hemostasis 69: 2332 (abstract).
5. Mustard, J.F., Perry, D.W., Ardlie, N.G., and Packham, M.A., (1972) Brit. J. Haematol. 22: 193-204.
6. Kirby, E.P., (1982) J. Lab. Clin. Med. 100: 963-976.
7. Fujimura, Y., Titani, K., Usami, Y., Suzuki, M., Oyama, T., Matsui, T., Fukui, H., Sugimoto, M., and Ruggeri, Z.M., (1991) Biochemistry 30, 1957- 1964.
8. Atoda, H., Hyuga, M., and Morita, T.J., (1993) J.Biol. Chem. 266, 14903 -14911.
9. Gan, Z.R., Gould, R.J., Jacobs, J.W., Friedman, P.A., and Polokoff, M.A., (1988) J. Biol. Chem. 263: 12397-12402.
10. Calvete, J.J., Schafer, W., Soszka, T., Lu, W., Cook J.J., Jameson, B.A., and Niewiarowski, S., (1991) Biochemistry 30: 5225-5229.
11. Cruz, M., Petersen, E., Turci, S.M., and Handin, R.I., (1992) J. Biol. Chem. 267: 1303-1309.
12. De Marco, L., Mazzucato, M., Massotti, A., and Ruggeri, Z.M., (1994) J. Biol. Chem. 269:6478-6484.