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STRUCTURAL AND FUNCTIONAL PROPERTIES OF A PROTHROMBIN ACTIVATOR FROM THE VENOM OF BOTHROPS NEUWIDI. J.W.P Govers-Riemslag, M.H.J. Knapen, G. Tans, R.F.A. Zwaal and J. Rosing. Department of Biochemistry, University of Limburg, Maastricht, The Netherlands

The prothrombin activator from the venom of Bothrops neuwidi has been purified to homogeniety by gelfiltration on Sephadex G-100, ion-exchange chromatography on DEAE-Sephacel and metal-chelate affinity chromatography on an Epoxy-activated Sepharose 6B column loaded with ZnCl . The overall purification was about 200-fold, which indicates that the crude venom contains about 0.5 weight % of the prothrombin activator. The venom activator is a single chain protein with an apparent molecular weight of 60,000 dalton. It readily activated bovine prothrombin with a Km of 37.7 uM and a Vmax of 120 umoles prothrombin activated per min/mg of purified venom activator. Venom-catalyzed prothrombin activation was not accelerated by the accessory components of the prothrombinase complex i.e. phospholipids plus calcium-ions and Factor Va. The venom activator does not require added calcium-ions for the expression of its prothrombin-converting activity. Calcium ions do, however, affect the catalytic activity of the venom activator. At 2 mM CaCl there is a 2-fold increase of the rate of venom-catalyzed prothrombin activation. However, at higher CaCl concentrations there is a gradual decrease of the activity of the venom activator. Gelelectrophoretic analysis of prothrombin activation indicated that the venom activator only cleaved the Arg 323-Ile 324 bond of bovine prothrombin since meizothrombin was the only product of prothrombin activation. The activator did not hydrolyze the chromogenic substrates S2222, S2337, S2238, S2366, S2302 or chromozym TH and its prothrombin converting activity was not inhibited by benzamidine, phenylmethylsulfonylfluoride, dansyl-glu-gly-arg-chloromethylketone and soybean trypsin inhibitor. However, chelating agents such as EDTA, EGTA and o-phenanthroline strongly inhibited the enzymatic activity of the venom activator. The activity of chelator-treated venom activator could, however, be restored by the addition of an excess CaCl . These results indicate that the enzyme from Bothrops neuwidi does not belong to the serine proteases but has the properties of a metal proteinase. Thus, the activator differs remarkably from Factor Xa, but strongly resembles the prothrombin activator from the venom of Echis carinatus, both structurally and functionally.

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PURIFICATION AND CHARACTERIZATION OF THE PROCOAGULANT OF THE VENOM OF TROPIDECHIS CARINATUS. P.P. Masci (1), A.N.Whitaker (1), J.J.Morrison (1), E.A.Bennett (2). Department of Medicine, University of Queensland, Princess Alexandra Hospital (1), and Department of Biochemistry, Queensland I stitute of Technology, Brisbane, Queensland, Australia.

Tropidechis carinatus is a venomous elapid snake distributed throughout Eastern Queensland. It has been considered as a tropical relative of Notechis scutatus and, similarly, the crude venom contains an indirect prothrombin activator, which will clot plasma provided that Factor V is present. Myotoxins and neurotoxins are also present. Envenomated patients regularly develop disseminated intravascular coagulation. The crude whole venom of T.carinatus was shown to have five major components by gel filtration, SDS PAGE and HPLC, and even more components by isoelectric focusing. The procoagulant eluted with a molecular weight of 55,000, being found in peak II on gel filtration on Sephadex-G150. The procoagulant was purified using a combination of Sephadex-G150 chromatography and ion-exchange on DEAE Sephadex-A50 and shown to migrate as a single band of molecular weight 55,000 by SDS PAGE. On reduction by β -mercaptoethanol this component was resolved into a heavy chain of molecular weight 30,000 and a light chain of 25,000. The procoagulant was shown to bind to con A-Sepharose 4B and Blue Sepharose 4B. Coagulation studies using this purified procoagulant confirmed a factor X_a-like activity activating prothrombin in the presence of factor V. The purified fraction is unstable in buffer solutions at 4°C, probably because of trypsin - like autodigestion. Ouchterlony studies of the procoagulants of T.carinatus and N.scutatus show both lines of homogeneity and spurring, indicating similarities but also significant differences between the two proteins. The purified procoagulant was lethal to adult rats, an IV injection of 10 µg killing in 1 - 2 minutes. Death was prevented by prior heparinization, suggesting that the procoagulant is toxic in the absence of neurotoxin and other components.

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NEW CHROMOGENIC SUBSTRATES FOR THE DETERMINATION OF COAGULATION AND FIBRINOLYSIS ENZYMES. W. Stüber, D. Schiwek, U. Becker, N. Heimburger. From the research laboratories of Behringwerke, Marburg, F.R.G.

A new type of chromogenic substrates based on derivatives of phenoxazine is presented. Particularly interesting is the blue dye 7-amino-3-diethylamino-8-methylphenoxazine (ADMP) with a molar extinktion coefficient of about 80,000 at 624 nm. Peptides were linked to the aminogroup of the dye and red coloured substrates were obtained with a λ_{max} value of about 540 nm. On account of the distinct difference of the λ_{max} values and the negligable influence of the absorption peaks of the acylated and the free dye this chromophore is suitable for the synthesis of substrates. Besides the spectral properties of these new chromogenic peptides we determined their characteristics using serine proteases involved in the process of coagulation and fibrinolysis. In comparison to para-nitroaniline substrates the introduction of the relatively bulky ADMP into the peptide sequence led to products with superior properties in respect of sensitivity and specificity.

It was found that the ADMP substrates are particularly favourable for the determination of thrombin, urokinase and activated protein C in the presence of other proteases.

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HUMAN HEPATOCYTES CONTAIN HIGH MOLECULAR WEIGHT POLYPEPTIDES OF FACTOR VIII. J. Ingerslev (1), B. Sloth Christiansen (1), A. Bukh (3), S. Stenbjerg (1), T. Munck Jørgensen (2) and C. Munck Petersen (1). Departments of Clinical Immunology and Clinical Chemistry (1), and Urology (2), University Hospital Aarhus, and Institute of Medical Microbiology (3), University of Aarhus, Denmark.

Human hepatocytes were isolated by the two-step collagenase technique applied on distal left liver lobe. Homogenous and large cells were isolated revealing hepatocyte characteristics by light-microscopy. Hepatocytes were washed repeatedly in albumine buffer (5%), resuspended in the same buffer and sonicated using a cell density of 0.75×10^6 cells/ml. In some cases cells were separated from non-viable cells by flotation on a linear Percoll gradient. Supernate material after sonication was subjected to ELISA for VIII:Ag using human antibodies and vWf:Ag by polyclonal antibodies. Freshly isolated cells contained at least 0.25 IU/ 0.75×10^6 hepatocytes, whereas the vWf:Ag was below 0.01 IU/ 0.75×10^6 cells. The material obtained from sonication was further studied using fast protein liquid chromatography by Mono-Q HR 5/5 revealing a single peak of VIII:Ag eluting in the same position as the high molecular weight polypeptides of VIII:Ag of high purity FVIII derived from the plasma source. Isolated hepatocytes also were cultivated at 37°C in medium RPMI 1640 supplemented with Ultroser G (4%), glutamine and antibiotics. Cells secreted increasing quantities of albumin, fibrinogen and protease-inhibitors. The supernatants also contained VIII:Ag in quantities ranging from 0.04 - 0.17 IU/ml after 24 hours, but no further secretion was observed. No vWf:Ag could be detected. Cells harvested and sonicated after 30 hours of culture only contained 0.04 IU/ 0.75×10^6 cells. Our results shows, that VIII:Ag is present in freshly isolated human hepatocytes and that only traces of vWf:Ag is found. A hepatocyte site of production of VIII is speculated. These very preliminary findings do not permit conclusions concerning active synthesis of VIII in hepatocytes. Further studies are underway.