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Amino acid sequence determination of Ancrod, the thrombin-like α-fibrinogenase from the venom of *Akistrodon rhodostoma*

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The thrombin-like serine protease and antithrombotic agent, Ancrod, was rapidly purified from the crude venom of *Akistrodon rhodostoma* by agmatine-Sepharose affinity chromatography followed by MonoQ anion exchange chromatography. N-Terminal sequencing and analysis of overlapping proteolytic fragments of purified Ancrod by automated Edman degradation in combination with tandem mass spectroscopy allowed the determination of the 234 amino acid sequence of the protease. Glycosylation sites at all five canonical *N*-linked glycosylation sites were inferred from the appearance of blank sequencer cycles in the amino acid sequence and were confirmed by mass spectroscopic analysis of the *N*-glycanase-treated peptides. Monoclonal antibodies raised against the denatured protein and HF-deglycosylated protein recognized Ancrod on Western blots. Sequence comparison to other thrombin-like serine proteases and reptilian fibrinogenases revealed a number of similarities, most notably the catalytic triad and many conserved cysteine positions.

Serine protease; Anticoagulant therapy; Affinity chromatography

1. INTRODUCTION

The venom of a number of snakes of the *Viperidae* family contain components with anticoagulant activity. These can act either through direct fibrinogenolysis or fibrinolysis or indirectly through activation of the host anticoagulant system by stimulating conversion of plasminogen to plasmin. An example of the former mode of activity is Ancrod, isolated from the venom of the Malayan pit viper *Akistrodon rhodostoma* [1]. This specific protease, instead of releasing fibrinopeptides A and B in thrombin-like fashion from the α-chain of fibrinogen, cleaves fibrinopeptides A, AP, and AY instead [2]. The aberrant fibrinogen is incapable of being cross-linked, forming easily dispersible clots. Host fibrinogenolytic and fibrinolytic mechanisms are also activated during this process resulting in a lowered circulating fibrinogen concentration. Ancrod thus acts as an apparent anticoagulant. Such a property has proven clinically useful over the past 20 years, and anticoagulant therapy with Ancrod has been shown to be at least as effective as conventional anticoagulants but may be safer in terms of reduced bleeding complications [3] appearing to induce the dissolution of preformed thrombi in a variety of clinical syndromes including hypercoagulable states [4], glomerular nephritis [5], and thrombotic stroke [6]. It has also shown efficacy in reducing re-occlusion in combination with classical thrombolytic treatment with streptokinase and urokinase [7].

The widespread clinical utility of Ancrod has been limited by (i) immunologic reaction in patients, and (ii) by availability and thus cost. While immunoreactivity could be due to trace contaminants in the commercial preparations, the heavy glycosylation (29% by weight; 96 moles of non-nitrogenous sugars, 60 moles of hexosamines, and 18 moles of sialic acid [8] could contribute to the reactivity. Although desialated (neuraminidase-treated) Ancrod is available (US Patent 4,585,653), recombinant production of Ancrod or a derivative could obviate production problems and control glycosylation. In this communication, the complete amino acid sequence of Ancrod rapidly purified from crude venom is elucidated. Preparation of monoclonal antibodies suitable for detecting unglycosylated Ancrod is also described. A preliminary account of this work has been presented (European Patent Application 90304432.9).

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2. MATERIALS AND METHODS

2.1. *Purification of Ancrod*

Ancrod was purified from lyophilized crude *Akistrodon rhodostoma* venom (Quality Venoms for Medical Research, Punta Gorda, FL) by a procedure similar to that described in [9], omitting the gel filtration step. Venom was fractionated by affinity chromatography on agmatine-Sepharose 4BCL prepared as described [9] in 50 mM Tris-HCl, 30 mM NaCl, pH 8.0, competitively eluting with 0.15 M guanidine-HCl in the same buffer. The eluate fractions containing the Ancrod activity were applied directly to a DEAE-Sephacel column equilibrated with 50 mM Tris-HCl, 30 mM NaCl, pH 8.0. The unabsorbed fraction containing the Ancrod was desalted into 10 mM Tris-HCl, pH

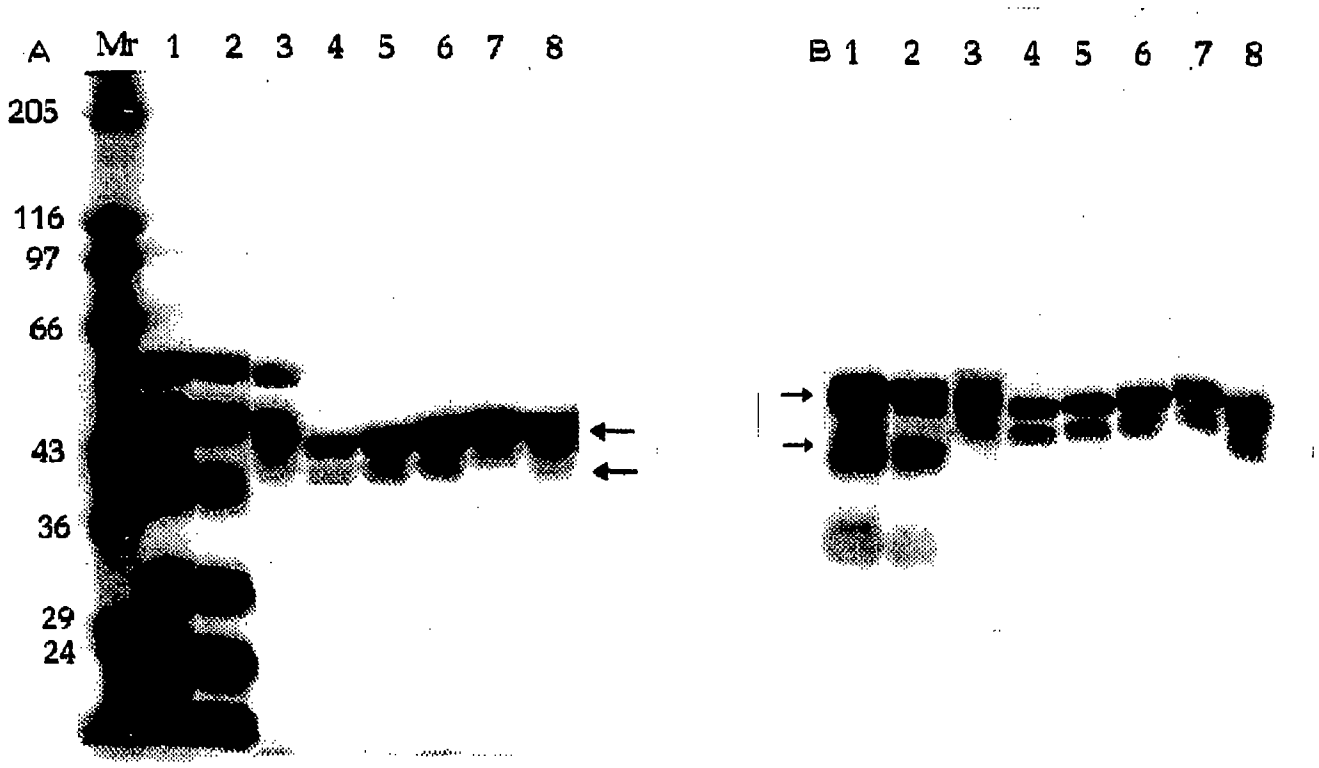

Fig. 1. SDS-PAGE analysis of the purification of Ancrod from *Akistrodon rhodostoma* venom. (A) Coomassie blue R-250-stained polypeptide profile. (Lane 1) Crude venom; (lane 2) flow-through from agmatine-Sepharose; (lane 3) flow-through from DEAE-Sephacel of agamatine-Sepharose eluate; (lanes 4–7) consecutive fractions from MonoQ column containing BAEE esterase activity; (lane 8) mixture of MonoQ fractions shown in previous four lanes. Arrows indicate the Ancrod polypeptides. (B) Western blot analysis of Ancrod through purification. Samples identical to those in (A) were transferred to 0.45 μm pore nitrocellulose (BioRad) and processed with a 1:200 dilution each of a mixture of monoclonal culture supernatants Anc1 and 3E1 which recognize whole protein and deglycosylated protein, respectively, as described in Methods. Arrows indicate the Ancrod polypeptides.

7.5, by ultrafiltration on an Amicon YM-10 membrane or by dialysis. Anion exchange chromatography was then performed at room temperature on a 1.5 ml TSK DEAE-5PW HPLC column (Rainen) or on a 1 ml MonoQ column (Pharmacia) eluting at 1 ml/min with a 0–1 M NaCl gradient over 20 min. Ancrod accounted for 1–2% of the venom protein.

Ancrod activity was routinely measured in partially purified preparations by following the increase in absorbance at 253 nm upon hydrolysis of benzoyl arginine ethyl ester (BAEE) [9]. Selective assays for Ancrod involved the clotting of fibrinogen solutions (soft clot), and the specific cleavage of the α-chain of fibrinogen monitored by SDS-PAGE [2].

2.2. *Preparation of Ancrod for sequencing*

The Ancrod was prepared for reduction and alkylation by desalting on a reverse-phase Brownlee Phenyl column (2.1 × 100 mm) using a linear 0–100% acetonitrile gradient in 0.1% trifluoroacetic acid over 30 min. Reduction and alkylation were performed for 1 h under N_2 at 37°C in 6 M guanidine-HCl, 1 M Tris-HCl, pH 8.6, containing 10 mM EDTA and 20 mM dithiothreitol. 4-Vinylpyridine was added to 50 mM and the incubation continued at room temperature for 30 min. The reduced and alkylated protein was re-isolated by HPLC. Amino acid analyses of Ancrod were performed on an ABI 420A Amino Acid Analyzer system by Nora Geddy. Hydrolyses at 110°C and 150°C gave similar results in four separate analyses.

2.3. *Generation of peptides for sequencing*

Endoprotease Lys-C peptides were generated by incubation with sequence grade Lys-C (1:100) (Boehringer Mannheim) in 0.1 M Tris-HCl, pH 8.5, for 40 h at room temperature. Endoproteinase Asp-N (Boehringer Mannheim) digestions (1:100) were performed similarly for 15 h at room temperature. CNBr cleavage was performed on 12 μg of reduced, alkylated Ancrod in 10% trifluoroacetic acid containing 25 μg of CNBr at room temperature in the dark under N_2 for 23 h. After lyophilization the CNBr peptides were redissolved in 6 M guanidine-HCl for HPLC separation.

Peptides were isolated by HPLC on a Hypersil ODS column (2.1 × 150 mm; The Separations Group) eluting with a 90 min linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (Lys-C peptides), or a 60 min 0–80% linear gradient (CNBr peptides). Asp-N peptides were separated on an Aquapore Phenyl column using a 0–40% linear acetonitrile gradient in 0.1% trifluoroacetic acid over 60 min. Peptides including the C-terminal peptide were confirmed using tandem mass spectrometry and Fourier transform mass spectroscopy by Janice Alexander in the laboratory of Dr. Don Hunt at the University of Virginia, Charlottesville. Automated Edman degradations were performed using the Applied Biosystems 477A liquid-pulse sequencer. Phenylthiohydantoin-amino acids were identified on an Applied Biosystems 120A PTH analyzer.

2.4. *Monoclonal antibody production*

Monoclonal antibodies were raised by standard methods [10,11] against repurified commercially available Ancrod (Knoll and Sigma). To reduce potential toxicity, 50 μg/ml phenylmethylsulfonyl fluoride was incubated with the Ancrod for 30 min at 37°C followed by dialysis. No BAEE esterase activity was observed after this treatment.

Hybridoma supernatants were screened initially against Ancrod coated on Immunolon 2 plates (Dynatech) and positive clones against preparative SDS-PAGE-separated Ancrod polypeptides transferred to nitrocellulose (Western blots) with a Miniblotter 45 slot blot apparatus (Immunetics, Cambridge, MA). All antibodies obtained recognized both proteins (M_r 48,000 and 44,000) of SDS-PAGE-separated Ancrod. Chemical deglycosylation of Ancrod with HF [12] collapsed the two bands to a single narrow band of M_r ≈29,000. The venom protein was resistant to enzymatic deglycosylation even after removal of sialic acid with neuraminidase, necessitating the use of anhydrous HF. Antibodies specific for the polypeptide backbone were selected by screening against the chemically deglycosylated protein and verified by their reactivity on a Western blot. Immunoblots were processed and detected with an alkaline phosphatase-conjugated second antibody as described previously [13]. Hybridomas were cloned by limiting dilution.

3. RESULTS AND DISCUSSION

Fig. 1 shows the purification of Ancrod from crude *A. rhodostoma* venom monitored by SDS-PAGE. Most venom proteins are removed by the agmatine-Sepharose affinity chromatography (lane 2). Residual contaminants are subsequently removed by ion exchange FPLC. Ancrod separates into two broad microheterogeneous zones of protein (Fig. 1A; arrows) coincident with immunoreactivity (Fig. 1B) at M_r around 48,000 and 44,000. Several monoclonal antibodies recognizing different epitopes failed to distinguish between the forms. Such a pattern is not unexpected for a heavily glycosylated protein such as Ancrod (≈30% carbohydrate [8]. Elution from FPLC anion exchange media partially separates sub-bands of material suggestive of different charge states, possibly due to differential sialic acid content (lanes 4–7). Lane 8 shows a pool of the FPLC-separated fractions with the resultant apparent 'smearing' of the bands. Catalytic activity was observed for all species. These two bands co-eluted on reverse-phase chromatography and were indistinguishable in amino acid sequence. Removal of carbohydrate by HF treatment collapses protein and immunoreactivity into a single band of M_r 29,000 (data not shown), which is consistent with the size predicted from the amino acid sequence.

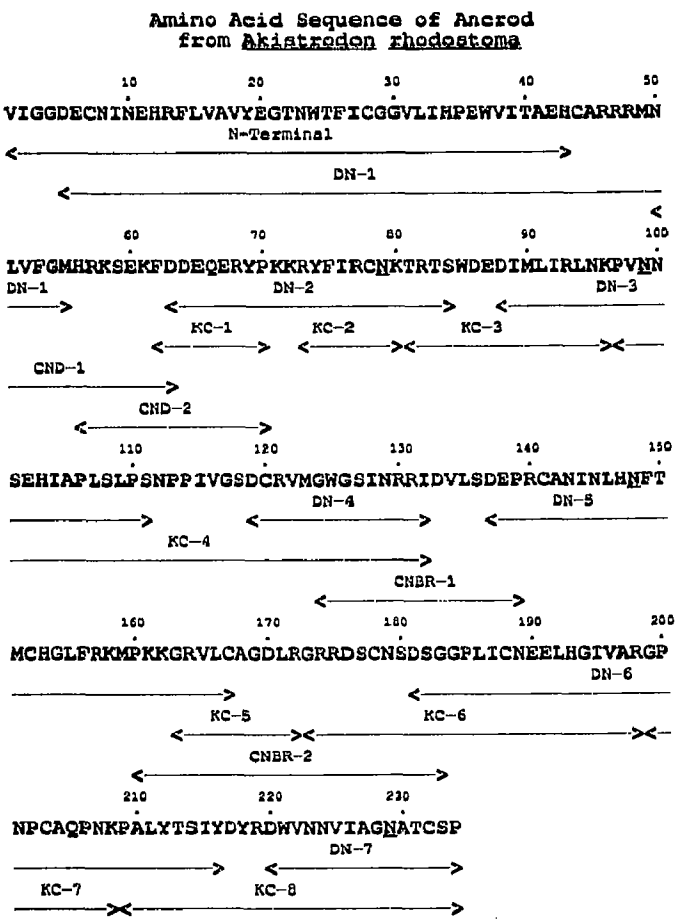

Fig. 2. Amino acid sequence of Ancrod. The overlapping of sequences determined on peptides purified from proteolytic digests and CNBr cleavage of Ancrod is depicted in Fig. 2. Details of the production and isolation of the peptides are presented in Methods. N-terminal = sequence determined on uncleaved Ancrod; DN = endoproteinase Asp-N; KC = endoproteinase Lys-C; CND = CNBr cleavage of DN-1; N = N-linked glycosylation sites (NXS or T).

Fig. 2 presents the strategy for determination of the complete Ancrod amino acid sequence. The sequences of overlapping peptides generated by specific proteolytic digests (at lysine by Lys-C, aspartic acid by Asp-N) and by CNBr cleavage at methionine were aligned with N-terminal sequence of the intact protein to provide the consensus sequence. CND-1 resulted from a partial CNBr cleavage at a M–H bond from DN-1, while CND-2 corresponds to the expected cleavage. Each sequence was redetermined at least twice. Ancrod contains 234 amino acids beginning with the N-terminal valine previously reported [8]. The sequence determined is consistent with the amino acid analysis of the full-

Table I

Amino acid composition of ancrod

Amino acid	Mol/mol Ancrod (found)	Mol/mol (expected)
Asx	30.65	33
Glx	15.77	15
Ser	10.85	13
Gly	18.00	18
His	9.13	8
Arg	22.40	21
Thr	8.03	8
Ala	11.51	11
Pro	15.32	15
Tyr	6.66	5
Val	13.04	15
Met	5.53	6
Cys	ND	ND
Ile	15.28	17
Leu	14.81	15
Phe	7.18	7
Lys	10.54	10

ND = not determined.

SEQUENCE COMPARISON AMONG FIBRINOLYTIC PROTEASES

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                1                   20                  40 *
Ancrod          VIGGDECNINEHRFLVAVYEGTNWTFICGGVLIHPEWVITAEHCARRRMN
Batrox          VIGGDECDINEHPFLAFMYY SPR YFCGMTLINQEWVLTAAHCNRRFMR
Crotalase       VIGGDECNINEHPFLVALYD YWX QXFL
RVV-V           VVGGDECNINEHPFLVALYTSTSSTIHCGGALINREWVLTAAHCDRRNIR
Trypsin I       IVGGYTCPEHSVPYQVSLNSGYH  F CGGSLINDQWVVSAAHCYKS  R
Thrombin        IVEGSNAEIGMSPWQVMLFRKSPQELLCGASLISNRWVLTAAHCLLYPPW

                                                    *
Ancrod          LVFGMHRHSE  KFDDEQERYPKKRYFIRCNKTRTSWDEDIMLIRLNKP
Batrox          ILGKLGKHAGSVANYDEVVRYPKEKFICPNKKKNVITDKDIMLIRLDRP
Crotalase                                                 LIRLNKP
RVV-V           IKLGMHSKNIRNEDEQIRVPRGKYFCLNTKFPN  GLDKDIMLIRLRRP
Trypsin I       IQVRLGEHNINVLEGDEQFINAAKIIKHPNYSSWT LNNDIMLIKLSSP
Thrombin        NKIGKHSRTRYERNIEK ISMLEKIYIHPRYNWRENLDRDIALMKLKKP

Ancrod          VNNSEHIAPLSLPSNPPIVGSDCRVMGWGSINRRIDVLS DEPRCANINL
Batrox          VKNSEHIAPLSLPSNPPSVGSVCRIMGWGAITTS EDTYPDVPHCANINL
Crotalase       VSYSEHIAPLSLPSSPPIVGSVCRAMGWGQTTSP QETLPDVPHCANINL
RVV-V           VTYSTHIAPVSLPSRSRGVGSRCRIMGWGKISTT EDTYPDVPHCTNIFI
Trypsin I       VKLNARVAPVALPSACAPAGTQCLISGWGNTLSN GVNNPDLLQCVDAPV
Thrombin        VAFSDYIHPVCLP NRETAAS   LLGAGYKGRVTGYGN  LKSTVTADV

                                                  *
Ancrod          HNFTMCHGLFRKMPKKGRVLCAGDLRGRRDSCNSDSGGPLICNEELHGIV
Batrox          FNNTVCREAYNGLPAKT  LCAGVLQGGDTCG GDSGGPLICNGQFQGIL
Crotalase       LDYEVC
RVV-V           VKHKWCEPLYPWVPADSRTLCAGILKGGRDTCHGDSGGPLICNGQIQGIV
Trypsin I       LSQADCEAAYPGEITSSM ICVGFLEGGKDSCQGDSGGPVVCNGQLQGIV
Thrombin        GKGQVCKDSTRIRITDN MFCAGYPDEG DACEGDSGGPFVMNRWYQGIV

Ancrod          ARGPNPCAQPNKPALYTSIYNYRDWVNNVIAGNATCSP
Batrox          SWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAGNKTATCP
Crotalase
RVV-V           AGGSEPCGQHLKPAVYTKVFDYNNWIQNIIAGNRTVTCPP
Trypsin I       SWGYG CALPDNPGVYTKVCNFVGWIQDTIAAN
Thrombin        SWGE GCDRDGKYGFYTHVFRLKKWIQKVIDQFGE
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Fig. 3. Alignment of Ancrod sequences with other thrombin-like serine proteases. Sequences of the proteases were aligned by eye. * = putative catalytic triad residues H^{43}, D^{88}, and S^{182}. Batrox = batroxabin; RVV-V = Russell's viper venom Factor V cleaving esterase.

length protein (Table I). N-Terminal sequence analysis of the commercial Ancrod (Knoll, Sigma) was identical to that purified from venom. The C-terminal Lys-C (KC-8) and Asp-N (DN-7) fragments and several other peptides were confirmed by Fourier transform mass spectroscopy and tandem mass spectroscopy. Glycosylation sites were inferred from loss of the asparagine corresponding to the five consensus N-linked glycosylation sites, (NXS,T) noted in Fig. 2. All other amino acid residues could be accounted for, suggesting that Ancrod contains only N-linked carbohydrate. The modified residues were directly identified by mass spectroscopy of the *N*-glycanase-treated peptides, supporting the tentative Edman degradation interpretation.

Previous studies (summarized in [9]) had shown that Ancrod was a serine esterase. Comparison of the sequence to those of several other known thrombin-like serine esterases revealed fairly obvious similarities. The

positioning of many of the 12 cysteine residues within the sequence is analogous to trypsin as is the catalytic triad (H^{43}, D^{88}, and S^{182}). Other active site regions found in trypsin can readily be identified in Ancrod. Fig. 3 shows an alignment of Ancrod with trypsin [14], and bovine thrombin [14] as well as with Crotalase (*Crotalus admanteus*) [14] and batroxobin (*Bothrops atrox*) [14]. The latter two enzymes are reptilian serine esterases with a thrombin-like specificity for fibrinogen for which significant amino acid sequence is available. A Factor V-cleaning serine esterase from Russell's viper venom has also been sequenced [15] and shares considerable sequence homology with Ancrod.

The availability of both the amino acid sequence of Ancrod as well as monoclonal antibodies which will recognize the polypeptide backbone in distinction to carbohydrate or native conformational epitopes should facilitate its cloning from a venom gland cDNA library. Expression systems are available for both prokaryotic and eukaryotic sources. The number of sulfhydryls in Ancrod suggest that renaturation from *E. coli* inclusion bodies will be challenging. Although trypsin has been successfully expressed in an active form in the periplasmic space [16], significant quantities of such sulfhydryl-rich proteins may only be available from eukaryotic cell systems.

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