Decorsin

A POTENT GLYCOPROTEIN IIb-IIIa ANTAGONIST AND PLATELET AGGREGATION INHIBITOR FROM THE LEECH MACROBDELLA DECORA

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The discovery, purification, and characterization of decorsin, a protein isolated from the North American leech Macrobdella decora, are described. Decorsin acts as an antagonist of platelet glycoprotein IIb-IIIa (GPIIb-IIIa), and is a potent inhibitor of platelet aggregation. The protein was purified to apparent homogeneity from crude whole leech extracts by treatment with trifluoroacetic acid followed by GPIIb-IIIa affinity chromatography and C18 reverse-phase high performance liquid chromatography. Decorsin was also isolated from a solution of leech ingestate by treatment with trifluoroacetic acid followed by C18 reverse-phase high performance liquid chromatography. The primary sequence of decorsin indicates that the protein is 39 amino acids long and contains 6 cysteine and 6 proline residues, as well as the sequence Arg-Gly-Asp, (RGD), a proposed recognition site of many adhesion proteins. A molecular mass of 4379 was obtained by fast atom bombardment mass spectrometry and is consistent with the mass calculated from the observed sequence. Evidence for an N-3 isoform, lacking the first 3 amino-terminal residues is also presented. Both decorsin and the N-3 isoform inhibit GPIIb IIIa binding to immobilized fibrinogen with an IC50 of ~1.5 nM. Human platelet aggregation induced by ADP is inhibited by decorsin with an IC50 of ~500 nM; complete inhibition was observed at ~1 µM. Based on overall sequence homology, decorsin does not belong to the family of GPIIb-IIIa protein antagonists that is found in snake venoms (Dennis, M. S., Henzel, W. J., Pitti, R. M., Lipari, M. T., Napier, M. A., Deisher, T. A., Bunting, S., and Lazarus, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2471–2475); however the carboxyl-terminal RGD-containing region from residues 27 to 38 of decorsin is approximately 60% homologous with the corresponding region of the snake venom proteins, suggesting that high affinity binding of these proteins to GPIIb-IIIa is defined by this epitope.

Platelet aggregation plays a fundamental role in hemostasis (1). It is mediated by the interaction of fibrinogen with the platelet membrane glycoprotein IIb-IIIa (GPIIb-IIIa) (2, 3), a member of the integrin family of cell adhesion receptors (4, 5). This interaction appears to be the final common step of aggregation that is induced by all platelet aggregation agonists. Since arterial thrombotic disease is mediated by platelet aggregation, inhibitors of the fibrinogen/GPIIb-IIIa interaction may prove to be very effective agents for therapeutic intervention in thrombotic disease (6).

To date, the most potent inhibitors of platelet aggregation acting via an antagonism of the GPIIb-IIIa receptor which have been reported have been purified or derived from natural sources. These include both the Aa95–97 and Aa572–574 RGD (7, 8) and γ400–411 (9) region peptides derived from the sequence of the γ chains of fibrinogen, respectively (10), anti-GPIIb-IIIa monoclonal antibodies (11–13), and several potent inhibitors recently isolated from snake venoms (14–17). The venom proteins are highly homologous to one another and constitute a family of related proteins that interact directly with GPIIb-IIIa, thereby blocking fibrinogen binding (17).

Leeches have long been known to possess agents that affect hemostasis (18, 19). Several proteins have been isolated from leeches that affect hemostasis by various mechanisms. These include hirudin (20, 21), a potent thrombin inhibitor from Hirudo medicinalis which contains 65 residues, antistasin (22), a factor Xa inhibitor from Haementaria officinalis containing 119 residues as well as a similar factor Xa inhibitor (23) from Haementaria ghilianii, and hementin (24), a fibrinolytic enzyme from Haementaria ghilianii. We hypothesized that hematophagous leeches might also contain antithrombotic agents that act via inhibition of fibrinogen/GPIIb-IIIa binding. After screening a number of crude leech homogenates in a solid-phase ELISA that detects inhibition of fibrinogen binding to GPIIb-IIIa, the North American leech Macrobdella decora was chosen for further characterization.

In this paper, we report on the discovery, purification and characterization of decorsin, a potent GPIIb-IIIa protein antagonist isolated from M. decora. The protein has been purified from crude whole leech homogenate, as well as from a solution of leech ingestate, reported to contain platelet aggregation inhibitors (25). We describe both the primary sequence of the protein as well as the effectiveness of decorsin as an inhibitor of platelet aggregation. To our knowledge, decorsin is the first GPIIb-IIIa antagonist isolated from leeches.

EXPERIMENTAL PROCEDURES

Materials—Live M. decora leeches were obtained from St. Croix Biological Supply (Stillwater, MN) and maintained in nonchlorinated mineral water at room temperature prior to use. Human fibrinogen from Kabi (Uppsala, Sweden) was further purified by the method of

The Abbreviations used are: GPIIb-IIIa, glycoprotein IIb-IIIa; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; FAB-MS, fast atom bombardment mass spectrometry; ELISA, enzyme-linked immunosorbant assay; PRP, platelet-rich plasma.

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Prior to use. AP3, a murine monoclonal to human GPIIIa (28) was provided by Dr. P. Newman (Blood Center at Southeastern Wisconsin, Milwaukee). Goat anti-mouse IgG conjugated to horseradish peroxidase was purchased from Tago (Burlingame, CA). O Phenyldiamine dihydrochloride was from Sigma. TWEEN 20, and 120 mM NaCl prior to use. The neutralized extract was spun at 23,000 X g for 45 min; with 10 column volumes of 40 mM added to the EDTA-containing fractions and the column was washed with 100 ml of 50 mM Tris, pH 7.5. The homogenate was spun at 11,000 g for 20 min using a Sorvall refrigerated centrifuge. The supernatant was saved, and the pellets were extracted again with 225 ml of TACTS buffer in a Tissuemizer (Tekmar, Cincinnati, OH) tissue homogenizer. This homogenate was spun as before, and the supernatants from both extractions were pooled to form the crude extract.

Extraction and Trifluoroacetic Acid Precipitation—500 g of leeches were homogenized in 400 ml of TACTS buffer in a Waring blender. The homogenate was spun at 11,000 x g for 20 min using a Sorvall RC5B refrigerated centrifuge. The supernatant was saved, and the pellets were extracted again with 225 ml of TACTS buffer in a Tissuemizer (Tekmar, Cincinnati, OH) tissue homogenizer. This homogenate was spun as before, and the supernatants from both extractions were pooled to form the crude extract.

The crude extract was stirred magnetically at room temperature while 50% trifluoroacetic acid was added dropwise to a final concentration of 1%. Following the addition of trifluoroacetic acid, the mixture was stirred at room temperature for 5 min and then spun at 23,000 x g for 20 min. The pellets were discarded, and the supernatant was neutralized to pH 7.6 by the dropwise addition of ammonium hydroxide. The neutralized extract was spun at 23,000 x g for 45 min; the supernatant was then used directly or stored frozen at -4°C prior to use.

GPIIb-IIIa Affinity Chromatography—The neutralized trifluoroacetic acid supernatant pool containing 2 mM CaCl2 was loaded onto a 0.5 x 7 cm GPIIb-IIIa affinity column prepared as described above. The column was equilibrated in 50 mM Tris, pH 7.5, containing 2 mM CaCl2. The sample was loaded at a flow rate of 0.5 ml/min, and 25-ml fractions were collected. The column was washed with equilibration buffer and then eluted with 4 column volumes of 4 mM EDTA, 50 mM Tris, pH 7.5. Immediately following elution, 8 mM CaCl2 was added to the EDTA-containing fractions and the column was washed with washes of phosphate-buffered saline containing 0.01% Tween 20 between each step. Elution was performed and developing reagent buffer (0.67 mg/ml 6-n HCl vapor in the Millipore Piratog system for 20 h at 110°C. The hydrolysates were vacuum evaporated in a Savant speed vacuum concentrator, and analyzed on a Beckman model 6300 amino acid analyzer equipped with a ninhydrin detector.

Fibrinogen/GPIIb-IIIa ELISA—Activity was monitored throughout the purification using a fibrinogen/GPIIb-IIIa ELISA. This assay is based on a modification of the method of Nachman and Leung (32). The assay was performed according to the following protocol, with washes of phosphate-buffered saline containing 0.1% Tween 20 between each step. Microtiter plates were coated with purified human fibrinogen (10 μg/well), washed, and blocked with TACTS, 0.5% bovine serum albumin. The plates were washed, and samples to be evaluated were added, followed immediately by addition of purified GPIIb-IIIa (40 μg/ml) and goat anti-mouse IgG conjugated to horseradish peroxidase at 1 μg/ml. After a 1-h incubation, the plate was washed and AP3 (1 μg/ml) was added. Following a 1-h incubation and another wash, goat anti-mouse IgG conjugated to horseradish peroxidase was added. A final wash was performed and developing reagent buffer (0.67 mg/ml α-phenylendiamine dihydrochloride, 0.012% H2O2, 22 mM sodium citrate, 50 mM sodium phosphate, pH 5.0) added; plates were incubated until color developed, typically about 10 min. The reaction was stopped with 2× HLG, and the absorbance at 492 nm measured. A four parameter fit (33) was used to estimate the half-maximal inhibition concentration.

Platelet Aggregation Assay—Platelet aggregation assays were performed in human platelet-rich plasma (PRP) as follows: 50 μl of whole human blood (9 parts) was drawn on 3.8% sodium citrate (1 part) from a donor who had not taken any aspirin or related medication for at least 2 weeks. Blood was centrifuged at 160 x g for 10 min at 22°C, allowed to stand for 5 min, and the PRP was decanted. Platelet poor plasma was prepared from the remaining blood by centrifugation at 2,000 x g for 25 min at 22°C. PRP platelet count was measured on a Baker 9000 hematology analyzer and diluted to 300,000 platelets/μl using platelet poor plasma. PRP (225 μl) plus 25 μl of a solution of decorin of known concentration in phosphate buffered saline or phosphate buffered saline alone was incubated for 5 min in a Chrono-log Aggregometer at 25°C. Light transmittance was recorded and ADP (8 μM) was added to initiate platelet aggregation. The inhibition of platelet aggregation was measured at the maximum aggregation response.
Decorsin: a Potent GP IIb-IIIa Antagonist from Leech

RESULTS AND DISCUSSION

Purification—Decorsin was purified to apparent homogeneity from a crude homogenate extract of whole _M. decora_ leeches by treatment with trifluoroacetic acid followed by GPIIb-IIIa affinity chromatography, and C<sub>18</sub> reverse-phase HPLC. The activity observed in the crude homogenate was found to be soluble in 1% trifluoroacetic acid. Decorsin bound to the GPIIb-IIIa affinity resin and was eluted by treatment with EDTA, which is known to disrupt the calcium dependent GPIIb-IIIa complex (34). The affinity resin could be regenerated by treatment with Ca<sup>2+</sup>, however, the column was reusable only two to three times, losing binding capacity with each use. Presumably, this loss of binding ability is due to irreversible dissociation of the GPIIb-IIIa complex, although the presence of a GPIIb-IIIa-binding molecule in the trifluoroacetic acid supernatants that is not eluted by EDTA cannot be excluded as a possibility. The final purification of decorsin was carried out by _C<sub>18</sub>_ reverse-phase HPLC using pooled affinity eluates. HPLC fractions containing the activity were rechromatographed using a 0.3%/min acetonitrile gradient (Fig. 1); a second peak of activity eluted earlier than _decorsin_ and is discussed below. The final recovery of _decorsin_ was ~10 μg; this represents less than 10% of the _decorsin_ loaded onto the affinity column. The remainder of the _decorsin_ was found in the flow through.

We hypothesized that _decorsin_ may prevent blood from clotting during either feeding and/or storage of ingested blood. Inhibition of platelet aggregation has been previously observed from the dilute saliva of _H. medicinalis_ (25). Therefore, in addition to the purification of _decorsin_ from crude leech homogenate, we also isolated the protein from a solution of leech ingestate, the ingested arginine-saline solution was treated with trifluoroacetic acid followed by _C<sub>18</sub>_ reverse-phase HPLC. The relative level of contaminating proteins in the ingestate was far less, based on SDS-polyacrylamide gel electrophoresis (data not shown), than in whole leech homogenate. This allowed the elimination of the affinity step, which, although quite useful during the purification of _decorsin_ from crude homogenate, requires relatively large amounts of GPIIb-IIIa. Approximately 10 μg of _decorsin_ was isolated from 45 ml of leech ingestate. Protein isolated in this manner was identified as _decorsin_ purified from whole leech homogenate based on molecular weight (FAB-MS), amino acid composition, and specific activity in the fibrinogen/GPIIb-IIIa ELISA (see below).

**Amino Acid Sequence, Purity, and Molecular Weight**—The entire amino acid sequence of _decorsin_ was determined from replicate runs of the reduced protein by automated sequential Edman degradation. The protein consists of 39 amino acids (Table I) and contains the RGD sequence, a well-known recognition sequence in many adhesion proteins (3), near its C terminus. In addition _decorsin_ is very rich in cysteine and proline (6 each). The calculated molecular weight of the reduced protein is 4384; the calculated pI is 4.45 (35).

During the sequencing of purified _decorsin_, two minor additional sequences were observed (data not shown); these sequences were identical to _decorsin_ except that they lacked the first 2 or 3 amino-terminal residues. The separation of _decorsin_ and the N-3 isoform is shown in Fig. 1. Amino acid composition of peak B was consistent with N-3 _decorsin_. These isoforms may represent native structures or more likely, result from proteolytic cleavage of _decorsin_. A variety of _leech_ species have been investigated for the presence of endogenous proteases, revealing similar exopeptidase activities.

**Table I**

Sequence analysis of _decorsin_

| Cycle | Amino acid | Yield | Cycle | Amino acid | Yield |
|-------|------------|-------|-------|------------|-------|
|       |            | pmol  |       |            | pmol  |
| 1     | Ala        | 169   | 1     | Glu        | 21    |
| 2     | Pro        | 90.5  | 2     | Cys        | 6     |
| 3     | Leu        | 50.2  | 3     | Pro        | 23    |
| 4     | Glu        | 82.6  | 4     | Pro        | 24    |
| 5     | Pro        | 48.2  | 5     | Gly        | 25    |
| 6     | Gln        | 62.7  | 6     | Cys        | 27    |
| 7     | Gly        | 48.8  | 7     | Arg        | 28    |
| 9     | Glu        | 32.8  | 9     | Phe        | 29    |
| 10    | Asp        | 24.5  | 10    | Lys        | 30    |
| 11    | Asp        | 38.4  | 11    | Asp        | 31    |
| 12    | Gln        | 25.1  | 12    | Gln        | 32    |
| 13    | Pro        | 18.5  | 13    | Asp        | 33    |
| 14    | Lys        | 17.3  | 14    | Ala        | 34    |
| 15    | Cys        | 4.8   | 15    | Cys        | 35    |
| 16    | Leu        | 16.5  | 16    | Arg        | 36    |
| 17    | Cys        | 16.7  | 17    | Lys        | 37    |
| 18    | Asn        | 10.1  | 18    | Cys        | 38    |
| 19    | Lys        | 14.5  | 19    | Lys        | 39    |
| 20    | Asp        | 6.5   | 20    | Glu        | 4.8   |

* Cysteine was identified by the presence of a dehydratating peak and absence of a serine peak during this cycle.

**Table II**

Amino acid composition of _decorsin_ and N-3 _decorsin_

| Amino acid | _Decorsin_ | N-3 _Decorsin_ |
|------------|------------|----------------|
| Asp        | 6.43 (6)   | 6.33 (6)       |
| Ser        | 0.16 (4)   | 0.46 (4)       |
| Glu        | 6.31 (7)   | 6.25 (7)       |
| Gly        | 3.07 (3)   | 3.21 (3)       |
| Ala        | 1.71 (2)   | 1.09 (1)       |
| Cys        | 4.09 (6)   | 2.54 (6)       |
| Ile        | 0.04 (0)   | 0.08 (0)       |
| Leu        | 1.96 (2)   | 1.95 (2)       |
| Tyr        | 1.01 (1)   | 1.00 (1)       |
| Phe        | 1.00 (1)   | 1.04 (1)       |
| Lys        | 2.03 (2)   | 1.99 (2)       |
| Arg        | 2.62 (3)   | 2.19 (2)       |
| Pro        | 5.57 (5)   | 4.82 (5)       |

* The numbers in parentheses represent the number of residues determined by amino acid sequence analysis.

Since both the size and affinity of _decorsin_ for GPIIb-IIIa were unknown initially, the entire trifluoroacetic acid supernatant was applied to the affinity column. Based on a molecular weight of 4379 for _decorsin_ (vide infra) and assuming a 1:1 stoichiometry, ~145 μg represents the theoretical maximum attainable from the 8 mg of GPIIb-IIIa on the resin, assuming 100% correct orientation of the receptor.
Decorsin: a Potent GPIIb-IIIa Antagonist from Leech

Amino Acid Composition—The amino acid composition of both decorin and N-3 decorin was determined on native protein acid hydrolysates. The data matches the composition predicted by amino-terminal sequencing, with the exception of cysteines, which were partially destroyed during hydrolysis (Table II).

GPIIb-IIIa Antagonist/Platelet Aggregation Inhibition Activity—Evidence that decorin binds to GPIIb-IIIa is based on experiments with immobilized GPIIb-IIIa affinity resin. After binding, decorin is eluted from the resin by treatment with EDTA, which dissociates the calcium-dependent heterodimeric structure of GPIIb-IIIa (34). In addition the interaction of GPIIb-IIIa with immobilized decorin as measured by the solid-phase fibrinogen/GPIIb-IIIa ELISA is directly inhibited by decorin. The specific activity (IC50) of both decorin and the N-3 isoform was determined in the ELISA based on the dose-dependent response that is observed (Fig. 3); an IC50 of ~1.5 nM was calculated for each protein. This is considerably more potent than the pentapeptide GRGDV which has an IC50 of ~40 nM in this assay.

The dose-dependent inhibition of human platelet aggregation by decorin was measured by inhibition of ADP-induced platelet aggregation in PRP. Complete inhibition was observed with 1 μM decorin; an IC50 of ~500 nM was calculated based on the titration curve. In comparison, the pentapeptide GRGDV has an IC50 of ~75 μM in the platelet aggregation assay.

The apparent 100-fold increase in potency of decorin in the fibrinogen/GPIIb-IIIa ELISA relative to the platelet aggregation assay is similar to activity differences noted with the snake venom GPIIb-IIIa antagonists (17). This difference in apparent potency may reflect the combined effects of fibrinogen immobilization and low GPIIb-IIIa concentration in the ELISA, as well as a relatively high concentration of fibrinogen in the platelet aggregation assay; in addition, there may be differences in the affinity of purified GPIIb-IIIa relative to that of the intact platelet. These differences are more pronounced with the pentapeptide relative to either decorin or the snake venom GPIIb-IIIa antagonists (17) and will be discussed elsewhere.

Comparison with Other Proteins—Decorin is similar to the snake venom family of GPIIb-IIIa protein antagonists in that they all contain the RGD sequence, have ~16% of their amino acids present as cysteines and have very similar IC50 values in both the solid-phase fibrinogen/GPIIb-IIIa ELISA and platelet aggregation assays (14–17). A comparison of the primary sequences of decorin with some of the snake venom proteins is presented in Fig. 4. Based on primary sequence analysis, decorin does not appear to belong to the snake venom family of inhibitors; the only significant region of homology is in the region of the RGD sequence from residues 27–38 of the decorin sequence. In this area 8 out of 13 of the residues can be aligned, with the addition of a gap inserted in the decorin sequence to maximize homology. Although we have no direct evidence, we propose that it is the presentation of this epitope that is important for the high affinity of both GPIIb-IIIa antagonists.

Fig. 3. Inhibition of GPIIIb-IIIa binding to immobilized fibrinogen by decorin and N-3 decorin. Inhibition of fibrinogen/GPIIIb-IIIa binding was measured by a solid-phase ELISA assay as described under “Experimental Procedures.” Samples (decorsin (●—●), N-3 decorin (○—○), and GRGDV (■—■)) were serially diluted 1:3 with TACTS buffer and added to the fibrinogen-coated microtiter wells immediately preceding the addition of GPIIIb-IIIa. Protein concentrations of the undiluted samples were determined by amino acid analysis. The concentration is plotted on a log scale and the absorbance is expressed as percent absorbance relative to wells containing only TACTS buffer and GPIIIb-IIIa.

Fig. 4. Amino acid sequence homology of decorin with snake venom GPIIIb-IIIa antagonists. The sequence of decorin is compared with echistatin (15), trigramin (14), applaggin (16), and kistrin (17), members of the snake venom family of GPIIIb-IIIa antagonists (17). Areas of ≥50% identity within the snake venom family and decorin are shaded; the homologous RGD-containing region is boxed. The two positions (26 and 44) in the snake venom family where identity is 50% for each amino acid are unshaded. A gap was inserted into the decorin and applaggin sequences to maximize homology.

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified decorin. Purified decorin (lane B) and molecular mass standards (lane A, molecular mass indicated in kilodaltons) were electrophoresed on a 8–25% SDS-polyacrylamide PhastGel (Pharmacia). Proteins were reduced with dithiothreitol prior to electrophoresis and were visualized after electrophoresis by silver staining.

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decorsin and the snake venom proteins for GPIIb-IIIa. It is known that simple RGD containing peptides are able to inhibit platelet aggregation, presumably via their interaction with GPIIb-IIIa (7, 8); however these peptides are considerably less potent than decorin or the venom proteins (17). The higher affinity observed for all of these proteins relative to RGD peptides is likely due to a specific conformation of the RGD epitope and/or to other binding interactions of the protein with the receptor. The lack of sequence homology outside the RGD containing region argues against a second remote linear epitope playing a major role in binding affinity.

Decorin is similar in both size and cysteine content to a number of serine protease inhibitor proteins that have been isolated from leeches. These include the hirudins (21), which are potent thrombin inhibitors, and the bdellins (37), which are inhibitors of trypsin and plasmin. However, there is no apparent primary sequence homology between these proteins. The high proline and cysteine content, assuming that all of the cysteines are present in disulfide bonds, consistent with the FAB-MS data, suggests that decorin has a very rigid structure. A search of the Dayhoff protein sequence database failed to identify any proteins with substantial homology to decorin.

The function of decorin in M. decora is not known, although it is likely that decorin serves to keep the host blood flowing, or possibly to keep ingested blood from coagulating. This second putative function is important because leeches store ingested blood for long periods of time, digesting it slowly as needed (38). Although we cannot specifically define whether decorin is present in saliva or secreted from cells in the digestive tract, its presence implies that it may have a primary role in hematophagous leech biology. If this is true then it is likely that there are proteins homologous to decorin in other hematophagous leech species. In fact, a recent report has indicated that prolonged bleeding occurs after a leech bite from H. medicinalis in the apparent absence of hirudin (39); future work will determine whether a GPIIb-IIIa antagonist homologous to decorin might be responsible for this phenomenon.

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