Tridegin, a new peptidic inhibitor of factor XIIIa, from the blood-sucking leech *Haementeria ghilianii*

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1. Crude salivary gland extract of the giant Amazon leech, *Haementeria ghilianii*, contains an inhibitor of plasma factor XIIIa. 2. The inhibitory agent was purified to homogeneity by anion-exchange, cation-exchange, gel-filtration and reverse-phase chromatography to yield a single band on SDS/PAGE with an apparent molecular mass of 7.3 kDa. It has been named tridegin. 3. Micro-sequencing of proteolytic fragments showed tridegin to be a peptide of 66 amino acids. The sequence is unique with little similarity to other leech-derived proteins. 4. Inhibition of plasma factor XIIIa activity was confirmed by four independent methods: tridegin increased the solubility of fibrin clots in urea, inhibited ammonia produced from the incorporation of ethylamine into casein, inhibited the incorporation of 5'-(biotinamido)pentylamine into casein and prevented γ -

dimer formation in clotting fibrinogen. 5. The IC_{50} of tridegin (approx. 9.2 nM) is very close to the concentration of factor XIIIa used in the assay and in fact depends on its concentration. This is the most potent inhibitor of factor XIIIa yet described. 6. Tridegin also inhibits platelet factor XIIIa (factor XIIIaA) with a similar potency to that of the plasma enzyme. 7. Tridegin also inhibits tissue transglutaminase but with lower potency and independently of the enzyme concentration. 8. Tridegin appears to be specific for transglutaminases, since it has no effect on the coagulation times of human plasma, on thrombin or factor Xa. Moreover it has no effect on other thiol-containing enzymes and has no ability to digest fibrinogen or cleave the isopeptide substrate, L- γ -glutamyl-4-nitroanilide.

INTRODUCTION

Plasma factor XIII is a cysteine transglutaminase which is responsible for the stabilization of fibrin in the final event of blood coagulation by the formation of ϵ -(γ -glutamyl)lysine cross-links between the α -chains and between the γ -chains within the fibrin gel [1]. The cross-links render the gel less soluble in denaturing solvents [2], mechanically more rigid [3] and less susceptible to enzymic lysis [4]. During haemostasis, plasma factor XIII is converted into the activated form, factor XIIIa, through proteolysis by thrombin, followed by a conformational change induced in the enzyme complex by Ca^{2+} ions that reveals a cysteine active centre [5]. The γ -carboxamide of glutamine from the protein substrate reacts with the active-site cysteine, forming a γ -glutamyl thioester and releasing ammonia. The acyl-enzyme intermediate then reacts with an amine such as the ϵ -amino side chain of lysine forming the ϵ -(γ -glutamyl)lysine bond [1]. Low-molecular-mass primary amines such as monodansylcadaverine and putrescine can act as pseudosubstrates and become incorporated into glutamine residues in proteins such as casein. This has permitted the development of a number of assay systems for factor XIIIa [6,7].

Whereas plasma factor XIII comprises two catalytic A subunits and two regulatory B subunits, factor XIII derived from platelets possesses only two catalytic A subunits [8]. The subunits of the platelet proenzyme have the same amino acid sequence as the A subunits of plasma factor XIII. A number of other cellular transglutaminases are known, and one of the better characterized is the tissue transglutaminase from guinea-pig liver [9,10].

As a Ca^{2+} -dependent cysteine transglutaminase, plasma factor XIIIa is susceptible to non-specific inhibition by general alkylating reagents, such as iodoacetamide, or Ca^{2+} -chelating agents, such as EDTA [8]. Very few specific inhibitors of factor XIIIa have been described. These include cerulenin, L-722,

151 (2-1-acetonylthio-3-5-methylthiazolo[2,3-*b*]-1,3,4-thiazolium) [11,12] and two synthetic peptides derived from the sequence of plasma factor XIII [13]. In addition, recent reports [1] describe patients who have developed antibodies to the plasma factor XIII complex after receiving prolonged therapy with certain drugs. For this reason little is known about the pathophysiological role of the enzyme and there is a need for specific and potent inhibitors.

The giant Amazon leech, *Haementeria ghilianii*, feeds solely on blood from a variety of hosts, including mammals, by inserting a long narrow proboscis through the skin and drawing the blood through it. A large adult leech can reach 45 cm in length with a proboscis of up to 7 cm. This leech is known to produce a potent fibrinolytic enzyme, hementin, in the salivary glands [14] which presumably aids feeding by preventing fibrin formation in the narrow lumen of this long proboscis and maintains the ingested blood in a liquid state in the foregut. Hementin has been shown to lyse cross-linked fibrin much more slowly than when cross-links are absent [15], so we hypothesized that *H. ghilianii* may have evolved an inhibitor of fibrin cross-linking which could supplement the effect of the enzyme.

In this paper we describe the identification, characterization and biochemical properties of a new peptidic inhibitor of plasma factor XIIIa from *H. ghilianii* which we name tridegin, a derivative of the Welsh word triarddeg meaning thirteen. Tridegin is the most potent inhibitor of transglutaminases yet described.

EXPERIMENTAL

Materials

All materials were purchased from Sigma–Aldrich Company, Poole, Dorset, U.K. unless otherwise stated.

Abbreviation used: PEU, plasma equivalent unit.

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Standardized plasma was obtained as follows. Plasma from two male and five female healthy volunteers plus a factor XIIIa-deficient plasma (1 ml) was defibrinogenated with bentonite and activated with thrombin, and various concentrations were assayed by the amine-incorporation assay described below. The mean of the slopes of the concentration versus absorbance lines for each plasma was taken as 1.0 plasma equivalent unit (PEU)/ml and the deviation from this measured. The range of the volunteers' plasma was 0.65–1.28 PEU/ml, consistent with literature values [16], and the factor XIII-deficient plasma was found to contain 0.06 PEU/ml. In this way, plasma with a known factor XIII concentration was used to prepare the plasma factor XIIIa used in all the assays described in this paper. If 100% recovery of factor XIIIa from the plasma is assumed, this also permitted the standardization of tridegin where 1 anti-(factor XIIIa) unit was defined as twice the amount required to inhibit 1 PEU of factor XIIIa by 50%.

Tissue transglutaminase from guinea-pig liver was 1.8 units/mg, where 1 unit will catalyse the formation of 1 μ mol of hydroxamate/min from *N*- α -carbobenzoxymethyl-L-glutamine and hydroxylamine at pH 6.0 and 37 °C [17].

Crude salivary-gland extract

A colony of *H. ghilianii* was reared and maintained at Biopharm (U.K.) Ltd. from leeches that originated in French Guiana. These were fed on heparinized bovine blood through fresh bovine skin periodically until they reached their third-fed stage, a stage at which they can remain for at least 1 year without the need for further blood meals [18]. Animals were anaesthetized in 8% (v/v) ethanol before dissection. For the initial assay, the proboscis, anterior and posterior salivary glands were removed and homogenized in 10 mM Tris/HCl/0.85% (w/v) NaCl, pH 7.0. The homogenate was centrifuged at 12000 *g* for 2 min at 20 °C and the supernatant used.

Column chromatography

Ion-exchange chromatography and gel filtration were performed using a BioPilot P-6000 System (Pharmacia, Uppsala, Sweden) where protein was detected at 280 nm with a UV-M/1 monitor. Reverse-phase chromatography was performed on a Waters 650 Advanced Protein Purification System (Waters Ltd., Watford, Herts., U.K.); protein was detected at 280 nm.

Protein concentration in samples was determined by the bicinchoninic acid method [19] with BSA as the standard.

Clot-solubility assay

Extracts and column samples were assayed using a microtitre plate method similar to that described by Tymiak et al. [11]. The test sample (0.03 ml) was added to a 10 mg/ml crude bovine fibrinogen preparation, which contained enough contaminating factor XIII for cross-linking to take place (Sigma F8630) in 0.1 M sodium phosphate buffer, pH 7.2 (0.03 ml). Clotting was induced by the addition of 6.25 N.I.H. units/ml of bovine thrombin (EC 3.4.21.5) in 9 mM CaCl₂ (0.04 ml). The plate was incubated for 15 min at 20 °C, then 8 M urea (0.16 ml) was added to the wells and the plate was incubated again for a further 15 min at 20 °C. The attenuation was measured at 405 nm against a water blank.

Defibrinogenation and activation of factor XIII

Plasma was defibrinogenated by the addition of solid bentonite (BDH Chemicals Ltd., Poole, Dorset, U.K.) to a final concentration of 40 mg/ml and incubated at 20 °C for 10 min before

centrifugation at 12000 *g* for 2 min. The supernatant was activated by the addition of bovine thrombin at a final concentration of 100 N.I.H. units/ml in 10 mM CaCl₂ and incubated for 15 min at 37 °C. The reaction was stopped by the addition of hirudin (specific activity 11000 anti-thrombin units/mg) [Biopharm (U.K.) Ltd.] at a final concentration of 200 anti-thrombin units/ml.

Platelet factor XIIIa (Shield Diagnostics, Dundee, Scotland, U.K.) (5 unit vial) was dissolved in water (1 ml), and 0.12 ml was activated with thrombin by adding 150 units/ml bovine thrombin in 15 mM CaCl₂ (0.18 ml) and incubating for 15 min. The activation was stopped by neutralizing the thrombin with 470 anti-thrombin units/ml of hirudin in 0.1 M Tris/HCl, pH 8.5 (0.3 ml). The final concentration of factor XIIIa after this treatment was determined as for the standardized plasma samples.

Ammonia-release assay of plasma factor XIII

Plasma factor XIIIa activity was measured by the method of Muszbek et al. [20] based on the amidation of glutamine residues in β -casein with ethylamine. This releases ammonia, which was continuously monitored at 340 nm by an indicator reaction involving the oxidation of NADH by glutamate dehydrogenase. The reaction cuvette contained 2.5 mM dithiothreitol (0.1 ml), 40 mg/ml dephosphorylated β -casein (0.05 ml), 70 mM ethylamine (0.1 ml), 12 mM sodium 2-oxoglutarate (0.1 ml), 4 mM NADH (0.1 ml), 1.2 mM ADP (0.1 ml), 40 units/ml glutamate dehydrogenase (EC 1.4.1.4) (0.1 ml), test sample or buffer (0.1 ml) and 70 mM Hepes buffer, pH 7.5 (0.05 ml). All components were dissolved in 70 mM Hepes, pH 7.5, where possible. The reaction was started by the addition of activated factor XIII (0.2 ml) and monitored at 340 nm at 20 °C.

Omission of either ethylamine or β -casein resulted in minimal detection of ammonia. Similarly, no ammonia was detected when factor XIII-deficient plasma (Sigma) was used in place of normal plasma confirming the specificity of the method for factor XIIIa.

Amine-incorporation assay for transglutaminases

Transglutaminase activity was determined by the covalent incorporation of 5'-(biotinamido)pentylamine on to *N,N'*-dimethylcasein-coated microtitre plates [21]. Bound biotin was detected by streptavidin-alkaline phosphatase complex and quantified by the cleavage of *p*-nitrophenyl phosphate. *N,N'*-Dimethylcasein was dissolved in 0.1 M Tris/HCl, pH 8.5, at 80 °C for 30 min, then centrifuged at 12000 *g* for 20 min. A concentration of 10–20 mg/ml was used to coat the wells of a microtitre plate by incubation at 37 °C for 1 h. The excess casein was discarded and the wells blocked with 0.5% (w/v) non-fat dry milk (Marvel; Premier Beverages, Adbaston, Staffs., U.K.) in 0.1 M Tris/HCl, pH 8.5, for 30 min. The plate was then washed with 0.35 ml aliquots of 0.1 M Tris/HCl, pH 8.5.

Microtitre wells containing 5 mM CaCl₂, 10 mM dithiothreitol, 0.5 mM 5'-(biotinamido)pentylamine (Pierce and Warriner, Chester, U.K.), test samples and either factor XIIIa or tissue transglutaminase in 0.1 M Tris/HCl, pH 8.5, with a final volume of 0.2 ml were incubated for 45 min at 37 °C. After incubation for 45 min, the liquid was discarded and the reaction stopped by two washes in 0.2 M EDTA (0.35 ml) followed by two washes with 0.1 M Tris/HCl, pH 8.5.

Streptavidin-alkaline phosphatase (0.25 mg/ml) was diluted 1:150 with 0.5% (w/v) non-fat dry milk in 0.1 M Tris/HCl, pH 8.5, and 0.25 ml was added to each well and incubated for 1 h

Figure 1 Purification of tridegin from salivary glands

The supernatant from a homogenate of the posterior salivary glands from 50 leeches in 20 mM Tris/HCl, pH 8.0 (50 ml) was applied to a Q-Sepharose column (6 cm $\times$ 10 cm) equilibrated in the same buffer and eluted with a linear gradient from equilibration buffer to 100% buffer

at 20 °C. The plate was washed once with 0.01 % (w/v) Triton (0.35 ml) followed by three washes with 0.35 ml of 0.1 M Tris/HCl, pH 8.5. Bound alkaline phosphatase was measured by the addition of 1 mg/ml 4-nitrophenyl phosphate, 5 mM MgCl₂ in 0.1 M Tris/HCl, pH 8.5 (0.05 ml) and Tris buffer (0.2 ml), and A_{405} measured after 30 min.

Omission of activated factor XIIIa plasma, CaCl₂ or 5'-(biotinamido)pentylamine, resulted in production of minimal amounts of 4-nitrophenol. Similarly the inclusion of factor XIII-deficient plasma in place of normal factor XIIIa plasma also resulted in minimal absorbance.

Formation of fibrin cross-links by plasma factor XIIIa

Bovine fibrinogen containing contaminant amounts of factor XIII (Sigma F8630) was used to investigate the effect on fibrin cross-linking. Fibrinogen was dialysed against 50 mM sodium acetate, pH 7.4, before use. A solution containing 20 mg/ml fibrinogen, 0.2 M NaCl, 15 mM dithiothreitol (0.1 ml) and either 31 μ g/ml tridegin or water (0.04 ml) was clotted by the addition of 50 N.I.H. units/ml bovine thrombin in 0.135 M KCl/0.18 M CaCl₂ (0.01 ml). For non-cross-linked clots, 20 mg/ml dialysed bovine fibrinogen, 0.2 M NaCl, 15 mM dithiothreitol and 0.153 M EGTA (total volume 0.14 ml) was clotted with 50 N.I.H. units/ml thrombin in the absence of CaCl₂. The clots were incubated for 6 h at 37 °C before the addition of denaturing buffer [25 mM NaH₂PO₄, 5.7 M urea, 1.9 % (w/v) SDS and 1.9 % (w/v) dithiothreitol; final concentrations] and then incubated overnight at 37 °C. Samples were boiled in a water bath for 10 min before centrifugation at 12000 *g* at 20 °C for 3 min; the supernatants were examined by SDS/PAGE.

Amino acid sequence

For sequencing work any minor contaminants in the preparation were removed by reverse-phase HPLC (see Figure 1). The N-terminal sequence of 22 amino acid residues was determined by automated Edman degradation of the reduced *S*- β -pyridylethylated native peptide by Alta Bioscience, University of Birmingham, U.K. using an Applied Biosystems 473A protein sequencer. Since a number of the amino acids could not be recognized from the N-terminal sequencing process, the possibility that some residues may be glycosylated was investigated using a commercial GlycoTrack carbohydrate detection kit K-050 (Oxford Glycosystems Ltd., Abingdon, Oxfordshire, U.K.). Biotinylated tridegin was prepared by the manufacturer's recommended method and compared with biotinylated ovalbumin prepared in the same way. The degree of glycosylation was estimated to be less than one-fifth of a residue per molecule, so it was concluded that tridegin is not significantly glycosylated.

A at a flow rate of 20 ml/min, where buffer A was 20 mM Tris/HCl/0.1 M NaCl, pH 8.0. The active fractions were pooled, adjusted to pH 4.0 with formic acid and applied to an SP-Sepharose column (5 cm $\times$ 12 cm) equilibrated in 20 mM sodium formate buffer, pH 3.5. The column was eluted with a linear gradient from equilibration buffer to 100% buffer B at a flow rate of 10 ml/min, where buffer B was 20 mM sodium formate/1.0 M NaCl, pH 3.5. The active fractions were dialysed against water, lyophilized and redissolved in water (2.4 ml). This was applied to a Superdex-75 gel-filtration column (1.6 cm $\times$ 60 cm) equilibrated in PBS, pH 7.4, at a flow rate of 1 ml/min. A sample of the active fractions from the Superdex-75 column (0.3 ml) was applied to a C8 ProRPC reverse-phase HPLC column (0.5 cm $\times$ 10 cm) equilibrated with 0.1 % (v/v) trifluoroacetic acid in distilled water and eluted with a linear gradient from the equilibration buffer to 100% solvent C at a flow rate of 0.3 ml/min, where solvent C was 0.1 % (v/v) trifluoroacetic acid in acetonitrile. The fractions from the HPLC column were lyophilized and reconstituted in water (0.1 ml) for the assays. —, A_{280} ; ····, conductivity. The solid bars indicate the location of the active fractions as measured in the fibrin clot-solubility assay.

In order to deduce the full sequence, samples of the native purified peptide were first denatured, reduced and then amidocarboxymethylated with iodoacetamide before enzymic digestion with either Asp-N endopeptidase (Boehringer-Mannheim U.K. Ltd., Lewes, East Sussex, U.K.) or trypsin (Boehringer-Mannheim) [22]. The peptide fragments were separated by reverse-phase chromatography using a C8 ProRPC column (0.5 cm $\times$ 10 cm; Pharmacia) equilibrated with 0.06% (v/v) trifluoroacetic acid. The column was eluted at 0.1 ml/min with sequential linear gradients from 2 to 38% solvent B (over 60 min) followed by 38 to 75% solvent B (over 30 min) followed by 75 to 98% solvent B (over 15 min) where solvent B was 0.0675% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile. Separation was performed on a Waters 650 Advanced Protein Purification System. Peptides were detected at 210 nm and sequenced as above.

Clotting times

Clotting times were measured by standard methods in human plasma, to which tridegin or PBS had been added, in a Sysmex C 500 Coagulometer.

Fibrinolysis

Bovine fibrinogen (2 mg/ml) was incubated with the test sample in the presence of 20 mM Hepes buffer, pH 7.5, containing 10 mM CaCl_2 and 0.1% Brij 35 at 37 °C for 60 min [15]. The remaining fibrinogen was assayed by the addition of bovine thrombin to a final concentration of 9 units/ml, to cause clotting. The clot was measured after 30 min by its attenuation at 405 nm on a microtitre plate reader.

RESULTS

Whole salivary-gland extract

Using a clot-solubility assay, it was established that fibrin clots formed in the presence of crude salivary-gland extract were more soluble in urea than those formed in the presence of buffer alone. This effect was clearly visible by eye and was confirmed by the attenuation as a reduction, for example from 0.735 to 0.292, caused by salivary-gland extract (representative of five separate experiments). The increased solubility of a fibrin clot under denaturing conditions is a property of non-cross-linked fibrin and is probably caused by an inhibitor of plasma factor XIIIa, the precursor of which, factor XIII, is a contaminant of the fibrinogen sample used. Pilot experiments indicated that the inhibitory activity was highest in the posterior salivary glands, and, since the activity was lower in animals that had recently fed, starved leeches were used for the purification described below.

Protein purification

The clot-stabilization inhibitor was purified from the posterior salivary glands from 50 starved third-fed-stage leeches to near-homogeneity by a combination of ion-exchange chromatography on Q-Sepharose followed by SP-Sepharose and gel filtration. The inhibitory activity was detected by the clot-solubility assay, which, although essentially non-quantitative, was simple, reliable and could be performed very rapidly. Activity was eluted from the Q-Sepharose column at approx. 0.09 M NaCl (Figure 1), which enabled it to be applied, after adjustment to pH 4.0, directly to the SP-Sepharose column, from which it was eluted at approx. 0.62 M NaCl (Figure 1). The material was further purified by gel filtration on Superdex-75 (Figure 1). SDS/PAGE of the pooled active fractions showed a major band of 7.3 kDa

Figure 2 SDS/PAGE of column fractions showing the purification of tridegin

SDS/PAGE was carried out in high-density homogeneous 20% cross-linked gels containing 0.112 M acetate, 0.112 M Tris, pH 6.4, and 30% (w/v) 1,2-dihydroxyethane and run in 0.2 M Tricine/0.2 M Tris/0.55% (w/v) SDS, pH 8.1. Protein samples were solubilized in 5% (w/v) SDS/10% (w/v) dithiothreitol and boiled for 10 min. The protein bands were visualized with silver stain. Lane 1, peptide marker standards (Pharmacia) with added aprotinin (6.5 kDa); lane 2, active fractions from a typical C8 ProRPC column showing one major band of 7.3 kDa; lane 3, active fractions from a typical Superdex-75 column; lane 4, supernatant from homogenate of posterior salivary glands; lane 5, low-molecular-mass standards (Pharmacia) with added aprotinin.

and a minor one of 17.9 kDa. The major band was estimated to be approx. 90% of the total and this preparation was used for further work. For sequencing work only, a final step of reverse-phase HPLC was used to remove any remaining contaminants. A single major peak was eluted at about 40% acetonitrile and contained the activity (Figure 1). SDS/PAGE showed that the pooled active fractions contained a single 7.3 kDa protein (Figure 2).

Amino acid sequence

The N-terminal sequence of tridegin was determined by automated Edman degradation of the reduced *S*- β -pyridylethylated native peptide. The preparation contained only one sequence as determined from the first 22 amino acids. The complete sequence was deduced from the digestion of the denatured, reduced and amidocarboxymethylated native protein by either Asp-N endoprotease or trypsin. The resultant peptides were separated by reverse-phase chromatography and micro-sequenced. Three Asp-N and five trypsin peptides provided the necessary overlaps to deduce the complete peptide sequence (Figure 3). Amidocarboxymethylcysteine and glutamate could not be separated adequately by the sequencer and were eluted in the same position; these residues are denoted Cys/Glu in Figure 3. Tridegin consists of 66 amino acids with an estimated molecular mass of 7.6 kDa, and, assuming each of the unidentified residues to be about 120 Da, this confirms the value of 7.3 kDa deduced from PAGE of the denatured reduced peptide (Figure 2). Three residues remain unidentified, as no obvious peaks were eluted from the sequencer in these positions. These are probably natural amino acids that have been modified post-translationally but are not glycosylated, since no glycosyl groups could be detected using a commercial kit.

Figure 3 Amino acid sequence of tridegin

The fragments were obtained by enzymic cleavage of native tridegin with Asp-N endoprotease or trypsin as indicated. — indicates fragments where all the amino acids were identified. ---- indicates fragments where some residues were not identified but which could be fitted unambiguously into the sequence. Residues denoted Cys/Glu could be either Cys or Glu as amidocarboxymethylcysteine did not separate adequately from Glu for unambiguous assignment in the sequencer used.

Figure 4 Inhibition of factor XIIIa in an ammonia-release assay

The reaction cuvette contained 2.5 mM dithiothreitol (0.1 ml), 40 mg/ml dephosphorylated β -casein (0.05 ml), 70 mM ethylamine (0.1 ml), 12 mM sodium 2-oxoglutarate (0.1 ml), 4 mM NADH (0.1 ml), 1.2 mM ADP (0.1 ml), 40 units/ml glutamate dehydrogenase (EC 1.4.1.4) (0.1 ml), test sample or buffer (0.1 ml) and 70 mM Hepes buffer, pH 7.5 (0.05 ml). All components were dissolved in 70 mM Hepes buffer, pH 7.5, where possible. The reaction was started by the addition of activated standardized plasma containing 0.76 PEU/ml (0.2 ml) and monitored at 340 nm at 20 °C. Data points were fitted by a non-linear regression equation and the IC_{50} determined to be 0.046 μ g/ml.

Ammonia-release assay of plasma factor XIIIa

Plasma factor XIIIa activity was estimated by the rate of ammonia release during the formation of covalent cross-links between ethylamine and β -casein by plasma transglutaminase. Figure 4 shows that the rate of ammonia production was reduced in the presence of tridegin in a concentration-dependent manner to approx. 20% of the control; this is similar to that observed when factor XIII-deficient plasma was used in place of the

Figure 5 Inhibition of fibrin cross-linking

Crude bovine fibrinogen (13 mg/ml) containing contaminating factor XIII (Sigma F8630) was incubated with bovine thrombin (3 units/ml) in the presence of the test substances for 6 h at 37 °C to allow full cross-linking to take place. The cross-linked products were then analysed by SDS/PAGE on homogeneous 7.5% cross-linked gels and stained with Coomassie Brilliant Blue followed by silver stain. Lane 1, fibrin formed in the presence of tridegin (8 μ g/ml) showing the absence of γ - γ and α -polymers; lane 2, fully cross-linked fibrin showing the γ - and β -bands (52 kDa), which overlap, the α -band (59 kDa) and the cross-linked proteins, γ - γ (117 kDa) and α -polymers (209 kDa and greater), the majority of which are too large to enter the gel; lane 3, fibrinogen incubated without thrombin showing the positions of the γ - (52 kDa), B β - (58 kDa) and A α - (65 kDa) bands; lane 4, low-molecular-mass markers (Pharmacia). The positions of the fibrinogen and fibrin chains were identified from the molecular-mass markers and are indicated. The appearance of a γ - γ band and α -polymer bands in lane 3 is presumably caused by the presence of some factor XIIIa as well as the factor XIII in the crude fibrinogen used in this work.

standard plasma (16% of the control). Ammonia release was also inhibited to 8.6% by 83 μ M iodoacetamide, a non-specific inhibitor which reacts with the active-site thiol of factor XIIIa, and to 0.6% of control by 83 mM EDTA, which removes Ca^{2+} ions essential for activity. As tridegin had no direct effect on the glutamate dehydrogenase detection reaction, the reduction in the amount of ammonia detected can only be attributed to the inhibition of plasma transglutaminase, factor XIIIa. The IC_{50} was calculated from the non-linear regression line to be 0.046 ± 0.0034 μ g/ml.

Formation of fibrin–fibrin cross-links by plasma factor XIIIa

In the absence of any inhibitors, γ -chain dimers were formed by the reciprocal cross-linking of the γ -chains between adjacent fibrin molecules by plasma factor XIIIa, and were visualized by SDS/PAGE in a position equivalent to double the molecular mass of the individual γ -chains (Figure 5, lane 2). The α -chains were also cross-linked, but several links were formed resulting in the formation of α -polymers with a high molecular mass (Figure 5, lane 2). When clots were formed in the presence of tridegin, a visible clot was observed in the tube, indicating that tridegin had not affected the polymerization of fibrin; however, when the clots were visualized by SDS/PAGE, only single γ -chains and α -chains were observed with no staining in the positions where γ -

Figure 6 Inhibition of factor XIIIa in an amine-incorporation assay

The incorporation of 5'-(biotinamido)pentylamine (0.5 mM) on to casein-coated microtitre plates was measured at 37 °C in the presence of activated standardized plasma containing 0.76 PEU/ml factor XIIIa (0.05 ml), 5 mM CaCl_2 , 10 mM dithiothreitol in 0.1 M Tris/HCl, pH 8.5 (total volume 0.2 ml) at various concentrations of tridegin. After washing, the incorporated biotin was complexed with streptavidin-alkaline phosphatase and visualized by cleavage of 4-nitrophenyl phosphate measured at 405 nm (see the Experimental section). The data points were fitted by non-linear regression and the IC_{50} was calculated to be $0.07 \pm 0.003 \mu\text{g/ml}$.

Figure 7 Inhibition of factor XIIIa-catalysed amine incorporation into casein at different enzyme concentrations

The incorporation of 5'-(biotinamido)pentylamine (0.5 mM) on to casein-coated microtitre plates was measured at 37 °C in the presence of activated standardized plasma containing 1.02 PEU/ml [0 ml, (●), 0.02 ml (△), 0.05 ml (▲), 0.075 ml (□) or 0.1 ml (■)], 5 mM CaCl_2 and 10 mM dithiothreitol in 0.1 M Tris/HCl, pH 8.5, at various concentrations of tridegin. The total volume of the assay was maintained at 0.2 ml with 0.1 M Tris/HCl, pH 8.5. After washing, the incorporated biotin was complexed with streptavidin-alkaline phosphatase and visualized by cleavage of 4-nitrophenyl phosphate measured at 405 nm (see the Experimental section). The data points were fitted by non-linear regression and the variation of IC_{50} with the concentration of factor XIIIa in PEU plotted (inset).

dimer or α -polymers run. This confirms the activity of tridegin by demonstrating the inhibitory effect on the cross-linking of both the α - and the γ -chains of fibrin (Figure 5, lane 1).

Amine-incorporation assay of plasma factor XIIIa

Plasma factor XIIIa activity was also estimated by the incorporation of 5'-(biotinamido)pentylamine into N,N' -dimethylcasein induced by plasma transglutaminase. Tridegin inhibited in a concentration-dependent manner by up to 91 %

Figure 8 Inhibitory effect on purified platelet factor XIIIa

Activated platelet factor XIIIa (0.05 ml) was assayed as in Figure 6. The IC_{50} for tridegin was calculated from the non-linear regression curve to be $0.026 \pm 0.015 \mu\text{g/ml}$.

with an IC_{50} of $0.07 \pm 0.003 \mu\text{g/ml}$ under these conditions (Figure 6). The relative molecular mass of tridegin being approx. 7.6 kDa, this equates to approx. 9.2 nM. In the absence of any standards for factor XIIIa activity, the concentration of the enzyme was estimated from the average factor XIII concentration for plasma from healthy volunteers, that is $0.07 \mu\text{M}$ [23], with two active sites and the assumption that the enzyme is quantitatively recovered and fully activated to factor XIIIa in our procedure using our standardized plasma samples. A concentration of factor XIIIa in the assay used in Figure 6 of 21 nM was estimated in this way. The molar ratio was therefore found to be 0.43 mol of tridegin to inhibit approx. 1 mol of factor XIIIa by 50 %, a stoichiometry close to 1. The dependence of the inhibitory effect on the enzyme concentration was therefore investigated in the same assay. There was a clear dependence on the enzyme concentration, which appeared linear over the range used (Figure 7). By similar calculations to those described above, a molar ratio of 0.41 mol of tridegin to inhibit 1 mol of plasma factor XIIIa was estimated to inhibit by 50 %.

Platelet factor XIIIa

A commercial preparation of purified platelet factor XIIIa was activated by thrombin and assayed by the amine-incorporation method by comparison with one of the standardized plasmas described above. A concentration of 0.06 PEU/ml (8.4 nM) was used in the assay. Tridegin inhibited this enzyme with an IC_{50} of $0.026 \pm 0.015 \mu\text{g/ml}$ (approx. $3.42 \pm 2.0 \text{ nM}$) (Figure 8). The ratio can be calculated to be 0.40 mol of tridegin for 50 % inhibition of approx. 1 mol of enzyme, similar to that for inhibition of the plasma enzyme and once again with a stoichiometry close to 1.

Tissue transglutaminase

Tridegin also inhibited guinea-pig liver tissue transglutaminase. Although the IC_{50} was low, it was independent of the enzyme concentration used in the assay over the range 0.3–0.6 $\mu\text{g/ml}$. The IC_{50} was measured as 1.83, 1.67 and 1.61 $\mu\text{g/ml}$ at 0.48, 0.80 and 0.96 m-units/ml tissue transglutaminase respectively (Figure 9).

Plasma clotting times

There was no difference between the coagulation times for normal plasma and plasma containing tridegin (4.6 $\mu\text{g/ml}$), indicating that tridegin has no effect on the clotting time of

Figure 9 Inhibition of tissue transglutaminase

Tissue transglutaminase was assayed as in Figure 6 at different concentrations of enzyme. The tissue transglutaminase concentrations in the wells were 0 (●), 0.48 (△), 0.8 (▲) and 0.96 (□) m-units/ml.

Table 1 Comparison of the effects of tridegin on various enzymes and substrates

Chromogenic substrate assays were carried out at 20 °C monitoring the release of 4-nitroaniline at 405 nm. Bovine thrombin (final concentration 0.075 unit/ml) was assayed in 50 mM Tris/HCl, pH 8.3, by cleavage of 0.1 mM *H*-D-Phe-Pip-Arg-4-nitroanilide (S2238, Quadratech, Epsom, Surrey, U.K.). Human factor Xa (Quadratech) was assayed at a final concentration of 0.32 nkat/ml in 100 mM Tris/HCl, pH 8.3, with 0.1 mM *N*-α-Z-D-Arg-Gly-Arg-4-nitroanilide (S2765, Quadratech). Bromelain (5.6 units/ml) was assayed with 2 mM *N*-α-benzoyl-L-arginyl-4-nitroanilide in 10 mM dithiothreitol/50 mM sodium acetate buffer, pH 4.5. Papain (0.63 unit/ml) was assayed with 2 mM *N*-α-benzoyl-L-arginyl-4-nitroanilide in 10 mM dithiothreitol/50 mM Tris/HCl, pH 6.2. Cathepsin C (1.0 unit/ml) was assayed with 1 mM Gly-Phe-4-nitroanilide in 10 mM dithiothreitol/50 mM Tris/HCl, pH 6.8. Destabilase was assayed with 1.9 mM L-γ-glutamyl-4-nitroanilide in 50 mM Tris/HCl/10 mM CaCl₂, pH 8.0, in the presence of the test sample [20]. *Hirudo medicinalis* extract was prepared by homogenization of the anterior one-third of a single specimen of the leech [Biopharm (U.K.) Ltd.], in 50 mM Tris/HCl, pH 8.0 (1 ml) and centrifugation at 12000 *g* for 2 min. The supernatant was used. Results are means ± S.E.M. from three determinations. rTAP = recombinant tick anti-coagulant peptide.

Enzyme	Rates of reaction (milli-absorbance units/min)			Reference used
	+ PBS	+ Tridegin (2.2 μg/ml)	+ Reference	
Thrombin	43.4 ± 1.8	40.4 ± 2.4	4.8 ± 0.2	Hirudin (0.044 μg/ml)
Factor Xa	68.1 ± 2.1	66.4 ± 1.4	1.7 ± 0.3	rTAP (0.044 μg/ml)
Bromelain	9.3 ± 0.3	9.6 ± 0.4	7.0 ± 0.2	Iodoacetamide (50 μM)
Papain	34.0 ± 0.6	33.5 ± 2.3	9.6 ± 1.2	Iodoacetamide (50 μM)
Cathepsin C	20.8 ± 1.4	18.8 ± 0.4	11.8 ± 1.4	Iodoacetamide (50 μM)
Destabilase activity	-0.2 ± 0.2	0.0 ± 0.0	4.2 ± 0.4	<i>Hir. medicinalis</i> extract (0.1 ml/ml)

normal plasma. It seems unlikely that tridegin has any effect on any of the coagulation enzymes other than factor XIIIa as they are all involved in pathways activated during clotting measured by these methods. Nevertheless we measured the effect on the two most abundant, thrombin and factor Xa.

Effect on thrombin

The effect of tridegin on thrombin was determined by the effect on thrombin-mediated release of *p*-nitroanilide from the chromogenic substrate, S2238. The effect of tridegin was compared with that of hirudin [24]. Table 1 shows that tridegin (2.2 μg/ml) had no effect on the cleavage of chromogenic substrate by thrombin.

As a positive control, hirudin at a molar concentration approx. 50 times lower than that of tridegin inhibited thrombin activity by 88.9%. Tridegin cannot therefore be considered a thrombin inhibitor.

Effect on human factor Xa

Since the salivary glands of *H. ghilianii* also possess an inhibitor of factor Xa, ghilanten [25], the effect of tridegin on factor Xa-mediated release of *p*-nitroanilide from the chromogenic substrate S2765 was evaluated and compared with the inhibitory effect of recombinant tick anti-coagulant peptide [26]. Tridegin (2.2 μg/ml) had no effect on the amidolytic release of *p*-nitroanilide by factor Xa in contrast with the effect of recombinant tick anti-coagulant peptide, which inhibited factor Xa activity by 97.5% at approx. 50-fold lower molar concentration (Table 1). Tridegin is therefore not an inhibitor of factor Xa and as such is not similar to ghilanten.

Thiol-containing proteases

Tridegin (2.2 μg/ml) had no effect on the thiol proteases, bromelain, papain or cathepsin C, whereas they were inhibited by the general thiol-blocking reagent, iodoacetamide confirming that tridegin is unlikely to be a non-specific thiol-reactive reagent (Table 1). Note that the assays were carried out in the presence of 10 mM dithiothreitol, which reacts with the iodoacetamide thus reducing its effective concentration and resulting in less than the expected inhibitory effect.

Fibrinogenolytic effect

Since this species of leech possesses a potent fibrinolytic enzyme, hementin, and this could have affected some of our assays, we investigated whether tridegin had any ability to lyse fibrinogen. Fibrinogenolytic activity was evaluated by assessing, by a turbidity method [15], the clottability of fibrinogen after incubation with the purified protein. Tridegin (35 μg/ml) had no effect on the attenuation, indicating that it had little or no proteolytic action on fibrinogen, whereas a preparation of hementin [Biopharm (U.K.) Ltd.] completely destroyed the fibrinogen, rendering it unclottable.

Effect on γ-glutamyl-*p*-nitroanilide

An enzyme called destabilase, which cleaves the γ-glutamyl-ε-lysyl amide bond formed in cross-linked fibrin, has been demonstrated in the leech, *Hirudo medicinalis* [27]. If tridegin shared this effect, then it could also have affected some of the assays we have used. Destabilase was assayed by the release of *p*-nitroanilide from the chromogenic substrate L-γ-glutamyl-4-nitroanilide [27]. The present study confirmed the presence of destabilase in an extract of *Hir. medicinalis*, but tridegin (2.2 μg/ml) did not mediate the release of *p*-nitroanilide at all and therefore probably cleaves the γ-glutamyl-ε-lysyl amide bond in cross-linked fibrin once it has formed (Table 1). Clearly, tridegin does not share the enzymic activity of destabilase.

DISCUSSION

During the final event of blood coagulation, fibrin clots are stabilized by the formation of intermolecular ε-(γ-glutamyl)lysine cross-links between the α-chains and between the γ-chains of the fibrin gel [1]. These covalent cross-links render the gel less soluble in denaturing solvents [2], mechanically more rigid [3] and less susceptible to enzymic lysis [4,28].

A simple assay based on the extent of clot dissolution by denaturants can be utilized and reflects the number of stabilizing cross-links present in a clot [11]. In the present study, clots formed in the presence of the crude salivary-gland extract of *H. ghilianii* showed a greater degree of solubility under denaturing conditions than clots formed in the presence of buffer alone, indicating the presence of a clot-stabilization inhibitor within the crude extract. Purification of the clot-stabilization inhibitor from the salivary glands of *H. ghilianii* was accomplished by a combination of anion-exchange chromatography, cation-exchange chromatography and subsequently gel filtration. Removal of minor contaminants was achieved by reverse-phase HPLC. The inhibitor preparation thus obtained was a single peak on reverse-phase chromatography and homogeneous according to SDS/PAGE. The inhibitor was named tridegin derived from the Welsh word triarddeg meaning thirteen.

Amino acid sequencing of both the entire peptide and Asp-N endoprotease and tryptic fragments, showed tridegin to have a single sequence of 66 amino acids. This included nine basic residues and one histidine plus four aspartic acids and three to six glutamic acids, indicating that it is a neutral to basic peptide, which is consistent with its behaviour on ion-exchange resins, where it binds more tightly to a cation exchanger than to an anion exchanger. The estimated molecular mass based on the sequence was approx. 7.6 kDa. After a search on the data bank of SwissProt and PIR (protein identification resource), tridegin showed greatest similarity to two isoforms of ornatin, an antagonist of the glycoprotein IIb/IIIa receptor on platelets that has been isolated from the leech *Placobdella ornata*, a leech in the order Rhynchohdellida like *H. ghilianii* [29]. The spacing of the cysteine residues in leech anti-haemostatic proteins is highly conserved [29]. By aligning tridegin with ornatin C and E after an insertion of one gap, tridegin²⁻¹⁹ shares eight identical amino acids (42 % identity) with these isoforms in the region of ornatin¹⁰⁻²⁸; in addition, three amino acids have been conservatively replaced (61 % similarity). It is important to note that this alignment does not include the putative active site, Arg-Gly-Asp sequence which resides at ornatin⁴⁷⁻⁴⁹. Little similarity was detected to any other ornatin isoform and there was no significant alignment in any other parts of the two molecules. Tridegin²⁻¹⁹ also shares six identical (33 % identity) and one similar amino acid with ghilanten¹⁰⁻²⁸ (38 % similarity). Ghilanten is a factor Xa inhibitor from the same species of leech, *H. ghilianii*, as tridegin [25]. It is notable that tridegin inhibits neither platelet aggregation (S. Finney, L. Seale, R. T. Sawyer and R. B. Wallis, unpublished work), like ornatin C, nor factor Xa, like ghilanten.

Factor XIIIa catalyses the transglutamination reaction usually between protein chains, resulting in the formation of an amide bond between the γ -carboxamide group of a peptidic glutamine residue and the ϵ -amino group of a peptidic lysine:

The reaction can be assayed by either the physical properties of the resulting cross-linked protein, for example by its solubility in denaturing solvents, or by identification of cross-linked complexes by SDS/PAGE. Alternatively transglutaminase activity can be assayed by measurement of end products, such as the rate of release of ammonia, or the rate of incorporation of primary amines into the protein substrate. The clot-solubilization inhibitor described in this paper is confirmed as an inhibitor of plasma factor XIIIa since it: (i) renders a fibrin clot more soluble in urea; (ii) inhibits ammonia release; (iii) inhibits fibrin-fibrin cross-linking; (iv) inhibits the incorporation of a primary amine into a protein substrate.

Factor XIIIa is not the only enzyme capable of forming the isopeptide ϵ -(γ -glutamyl)lysyl bond. Transglutaminases are found also in liver, the so-called tissue transglutaminase [9,10], in the epidermis and in the keratinocytes themselves [10]. Moreover, the catalytic A subunits of factor XIII are also found in platelets [8]. The ability of tridegin to inhibit factor XIIIa from platelets and tissue transglutaminase from guinea-pig liver as well as plasma factor XIIIa indicates that it may be a general inhibitor of this type of enzyme.

Our estimations of the IC₅₀ vary from 0.046 μ g/ml in the ammonia-release assay to 0.07 μ g/ml in the amine-incorporation assay. These differences are close to proportional to the differences in the enzyme concentrations used in the individual assays. For this reason we determined whether the IC₅₀ varied with the enzyme concentration in the amine-incorporation assay and confirmed that it was always close to 50 % of the enzyme concentration. Estimations of the potency of tridegin have necessitated a number of assumptions, which may have some influence on the result. The dependence of the inhibitory effect of tridegin on the concentration of factor XIIIa indicates that the K_i is lower than the enzyme concentration in the assay. This is in fact a common finding for enzyme inhibitors from leeches. For example, hirudin and eglin from *Hir. medicinalis* and antistasin from *Haementeria officinalis* have a K_i in the range 0.02–800 pM [30–32]. In our estimations of the stoichiometry of inhibition we have made use of measurements of factor XIII concentration in plasma reported by other research groups. These in fact vary quite widely, so we have used that most accepted in the literature [1,23]. Our estimations also depend on quantitative recovery of factor XIII after removal of fibrinogen from the plasma with bentonite, which is the most commonly used method, and on the full activation by the concentration of thrombin that we used. If recovery of factor XIIIa from the plasma was less than quantitative, then the IC₅₀ that we calculate would be proportionately higher. In the absence of a suitable standard, we have standardized plasma factor XIIIa by measuring the activity of factor XIIIa after activation of plasma from seven apparently healthy volunteers. We assumed the mean to be 1.0 PEU/ml. Our range was 0.65–1.28 PEU/ml which is consistent with the literature [16]. Since the IC₅₀ of tridegin varies with factor XIIIa concentration, an IC₅₀ of 0.44 μ g/ml can be calculated for tridegin if activated plasma is the milieu.

The potency of tridegin as an inhibitor of tissue transglutaminase is clearly less than that for inhibition of factor XIIIa, since inhibition of the tissue enzyme does not depend on the enzyme concentration, and the IC₅₀ was found to be 1.7 μ g/ml.

From the foregoing experiments, a number of possible mechanisms for the action of tridegin on factor XIIIa can be eliminated. Tridegin is not a substrate that competes with the normal substrates, since we have shown previously that ammonia produced from the incorporation of ethylamine into casein is inhibited rather than unchanged by the addition of tridegin. Moreover it is unlikely that tridegin destroys or interacts in some other way with either of the substrates since both have been varied quite diversely. Tridegin is not a general thiol-blocking reagent since a high concentration of dithiothreitol was included in the assays and in fact it is no more potent in its absence (results not shown). Moreover even high concentrations of tridegin have no effect on other thiol-dependent enzymes such as papain, bromelain or cathepsin C.

In the case of tissue transglutaminase, the inhibitory effect of tridegin differs in that it is Ca²⁺-dependent (S. Finney, L. Seale, R. T. Sawyer and R. B. Wallis, unpublished work), whereas the effect on factor XIIIa is independent of Ca²⁺. The Ca²⁺-de-

pendence of the inhibitory effect on tissue transglutaminase is not the same as that for activation of the enzyme, as it happens only at much higher concentrations of Ca^{2+} . It seems unlikely on evolutionary grounds that the mechanism of action on the two transglutaminases differs dramatically, but further investigations are necessary.

Tridegin is not a general anti-coagulant and seems only to inhibit the cross-linking phase. It has no effect on thrombin in either a chromogenic or a clotting assay or on factor Xa and has no effect on the clotting times, prothrombin time, which measures the extrinsic pathway, or kaolin cephalin clotting time, which measures the intrinsic pathway. Moreover it does not digest fibrinogen or catalyse the cleavage of the ϵ -(γ -glutamyl)lysine bonds like the previously described leech proteins, hementin [14] and destabilase [27] respectively.

In conclusion, we have isolated an extremely potent peptidic inhibitor of plasma factor XIIIa from the leech *H. ghilianii*. The peptide has 66 amino acids with a molecular mass of approx. 7.6 kDa. Tridegin is a new protein, with only very low similarity to part of ghilanten and a small region of two isoforms of the leech-derived protein, ornatin. Tridegin is demonstrated to be an inhibitor of plasma factor XIIIa by four different assays and is the most potent inhibitor of this enzyme described to date. It is also the first such inhibitor with this specificity from blood-sucking animals.

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