

## Description

The present invention concerns a novel anticoagulant factor from the saliva of the medicinal leech *Hirudo medicinalis* and pharmaceutical compositions for modifying blood coagulation containing the same.

More particularly, the present invention concerns a novel polypeptidic anticoagulant factor isolated from the saliva of the medicinal leech, *Hirudo medicinalis*, said factor specifically inhibiting bovine Factor Xa factor but not thrombin and having a molecular weight in the range of 10-30 kd. It is different from hirudin, the main anticoagulant factor in the said saliva, which inhibits thrombin, but as became apparent, does not inhibit bovine F.Xa.

Another important characterizing property of the novel fraction subject of the present application is, that it is a mild inhibitor, in contrast to hirudin, and also in contrast to heparin, which accelerates the action of antithrombin and is presently therapeutically used. This property of the isolated factor may be termed a modulating action, opening up the possibility of a therapeutic use in cases where a more controllable or milder inhibition of blood coagulation is desirable. An indication that the novel factor is a mild inhibitor, or a modulator, of blood coagulation is that it is capable of slowing down the inactivation of the bovine F.Xa induced by heparin and antithrombin.

The proof that the novel factor is in principle different from hirudin is that the leech saliva treated with an excess of bovine thrombin inhibits the action of bovine F.Xa on a suitable substrate, and as a complementary check, saliva treated with an excess of bovine F.Xa inhibits the action of bovine thrombin, the action of hirudin being specific to thrombin.

The isolation of the novel factor is based on the same principle, that is, adsorption of the same on bovine F.Xa, suitably immobilized, and subsequent elution. More particularly, a suitable adsorbent is Affi-Gel, equilibrated with F.Xa, and, as eluent, weak acids at pH of about 2.9 are suitable. The substance may be eluted by hydrochloric acid but is rapidly destroyed by the high acidity. Acetic acid is more suitable. The molecular weight of the anticoagulant factor subject of the present invention is between 10 and 30 kD, as it passes an ultrafilter with a cut-off point of 30kD and is essentially retained on an ultrafilter with a cut-off point of 10 kD.

Regarding the methods, synthetic substrates having a chromogenic end group such as pNA (para-nitroanilide) are preferably used, enabling easy monitoring of the progress of the coagulation by spectrophotometer methods. The substrates used were, in particular, N-methoxycarbonyl-D-cyclohexylGly-Gly-L-Arg-pNa (CHG), (Pentapharm Ltd., Switzerland and Boehringer Mannheim GmbH, F.R.G.), Tos-Gly-L-Pro-L-Arg-pNA (Chromozym TH, resp. CZTH), (Pentapharm Ltd., Switzerland and Boehringer Mannheim GMBH, F.R.G.) and D-Ile-L-Pro-L-Arg-pNA (S-2288, Kabi Peptide Research, Sweden) (pNA designates p-nitroalanine). The reactions were monitored at 405 nm in a spectrophotometer connected to a computer and the kinetic parameters were calculated from the appropriate extinction coefficients by a Jackson-Labanowski linear regression program. The measurements were carried out in disposable polystyrene cuvettes at pH of 7.8 and a temperature of 25 °C. Known values (Lottenberg, R. et al, Biochem-Biophys. Acta 874 (1986) pp. 326-335; Lottenberg, R. et al, ibid 742 (1983) pp. 539-557 (bovine thrombin)) of the parameters  $K_m$  and  $k_{cat}$  were used for the determination of F.Xa, thrombin and their inhibition.

An important characteristic of the factor subject of the present invention, which may differentiate the same from other known anticoagulants such as heparin and also hirudin, is its dose-dependent mode of action. When incremental amounts of saliva, both native and more particularly treated with thrombin, were added to bovine Xa, the activity of the same at first dropped sharply, but afterwards tended to level off asymptotically at 50 to 60%. This effect is essentially the same with thrombin-treated saliva. This result is illustrated graphically in appended Figure 1. An important characteristic of the factor subject of the present application, already referred to above, is the slowing down of the rate of inactivation of bovine F.Xa by heparin-antithrombin. Fig. 2 illustrates this by a comparative plot of the inactivation of B.Xa by heparin-antithrombin, in the absence and presence of saliva. The possible importance of this phenomenon is already discussed above.

The inhibitor/modulator (henceforth designated XaM) subject of the present invention is a mixed type inhibitor, which means that either the enzyme-inhibitor complex (EI) may react with the substrate, or the enzyme-substrate complex (ES) may react with the inhibitor (I). This view is strengthened by the fact, that the inhibitor/modulator, bound to bovine F.Xa immobilized on a suitable column cannot be eluted by D-Phe-Pipecolyl-L-Arg-pNa(S2238-Kabi Peptide which has a high affinity ( $K_m$ , 46,9  $\mu$ M) and a low catalytic rate constant ( $k_{cat}$  0.681  $s^{-1}$ ) for F.xa.

The inhibitor/modulator of the present invention may be isolated from the saliva by adsorption on an Affi-Gel column on which bovine F.Xa is fixed and subsequent elution with acetic acid. Hirudin was not adsorbed on said gel. Affi-Gel 10 is used, which is a derivatized cross-linked agarose containing a 10-atom

spacer arm esterified to N-hydroxysuccinimide. The capacity of this gel is 10 micromoles/ml.

An example of isolating the inhibitor/modulator factor of the present invention comprises the following steps:

1. Coupling. To 1ml washed Affi-Gel 10 in a mini-column were added 2ml of a dialyzed BXA solution (40 nmoles) in 0.05 M Hepes/0.1 M NaCl buffer pH 7.5. The column was shaken for 2 h at room temp. The supernatant was then run off the column, which was not allowed to dry. Ninety four percent of the BXA load was bound to the gel.

2. Neutralization of remaining N-hydroxysuccinimide groups. One ml of a mixture of 4 parts ethanolamine pH 8 and 6 parts 0.01M Tris/0.01M Hepes/0.1M NaCl/0.1% polyethylene glycol buffer pH 7.8 were added. Shaking for 1 h at room temperature was followed by three washes with same buffer.

3. Affinity binding of XaM. Leech saliva (40 ml, 305 pmoles XaM) was adjusted to pH 7.9 by adding 1% v/v 1M Tris pH 8 and was passed through the column. Ninety two percent of the XaM was bound to the gel. Hirudin was not bound.

4. Elution of XaM. The column was shaken with successive 1 ml volumes of 0.1M acetic acid (pH 2.9) which were neutralized on elution with 1 M  $\text{NH}_4\text{OH}$ . Recovery of bound XaM was 72.5 percent (203 pmoles).

The inhibitor/modulator factor of the present invention is useful in the treatment of thromboembolic diseases owing to its action of inhibiting the coagulation enzymes and at the same time slowing down the rate of inactivation of other anticoagulants, maintaining these two in balance. The advantages over heparin, the most commonly presently used anticoagulant are that heparin increases the risk of hemorrhage and interferes with the functioning of platelets. The present Xa modulator bears a strong resemblance to antistasin, the antimetastatic inhibitor which Tuszynski, Gasic and Gasic (The Journal of Biological Chemistry 262/20 1987, 9718-23) isolated from the Mexican leech, *Haementeria officinalis*. Both proteins are similar in molecular weight, in their specificity towards Xa and in the fact that the dose-response curve flattens out at about 50% remaining BXA activity. However, these two leech species belong to different orders in the classification scheme. The relationship between the genera *Haementeria* and *Hirudo* is best given by the following scheme (Roy T. Sawyer, "Leech Biology and Behaviour, Vol. II", Oxford Science Publications, 1986):

|         |                                               |                                                                      |
|---------|-----------------------------------------------|----------------------------------------------------------------------|
| Class:  | <u>Hirudinea</u>                              |                                                                      |
| Order:  | <u>Rynchobdellida</u> (protrusible proboscis) | <u>Arynchobdellida</u> (pharynx, no proboscis, with or without jaws) |
| Family: | <u>Glossiphoniidae</u>                        | <u>Hirudinidae</u> (jawed)                                           |
| Genus:  | <u>Haementeria</u>                            | <u>Hirudo</u>                                                        |

Proteins derived from leeches belonging to said different orders may be dissimilar in both identity and properties.

The invention is also concerned with pharmaceutical compositions comprising as active ingredient the anticoagulant/modulator factor of the present invention with a suitable pharmaceutically acceptable carrier or diluent. More particularly the pharmaceutical compositions of the present invention are intended for modifying blood coagulation. Still more particularly the pharmaceutical compositions of the present invention are intended for modifying blood coagulation associated with thromboembolic diseases.

The preparations according to the present invention may be administered orally or parenterally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as desired. The term parenteral as used herein includes subcutaneous, intravenous, intra-articular and intratechal injection and infusion techniques. Dosage unit preparations may contain daily required amounts of the purified compositions of matter of the present invention or submultiples thereof to make up the desired dose. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the activity material employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, etc.

As used herein, the term "pharmaceutically acceptable carrier" means an inert, non-toxic solid or liquid filler, diluent or encapsulating material, not reacting with the active ingredients according to the invention. These carriers are known to the man versed in the art. Wetting agents and emulsifiers, as well as release agents, coating agents and preservatives can also be present in the preparations of the invention. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary, depending upon the patient treated and the particular mode of administration.



## Claims

1. A polypeptide isolated from the saliva of the medicinal leech *Hirudo medicinalis*, which specifically inhibits bovine Xa factor but does not inhibit bovine thrombin and has a molecular weight in the range of 10-30 kd.
2. The polypeptide of claim 1 being an inhibitor of the mixed type, not eluted by a substance having a high affinity ( $K_m$ ) and low  $K_{cat}$  for factor Xa, said polypeptide being absorbed on a suitable column comprising immobilised bovine factor Xa.
3. The polypeptide of claim 2, wherein the  $K_m$  of the elution substance is  $46.9 \mu M$  and the  $K_{cat}$  is  $0.681 s^{-1}$ .
4. The polypeptide of claim 2 or claim 3 being an inhibitor of the mixed type not eluted by D-phe-pipecolyl-L-Arg-pNA when adsorbed on a suitable column comprising immobilized factor Xa.
5. A mixed type leech factor Xa inhibitor polypeptide which specifically inhibits bovine factor Xa but does not inhibit bovine thrombin, has a molecular weight in the range of 10-30 kd, and is not eluted from a column comprising immobilised factor Xa by D-phe-pipecolyl-L-Arg-pNA.
6. A pharmaceutical composition comprising as active ingredient the polypeptide of any of the preceding claims and a pharmaceutically acceptable carrier.
7. A pharmaceutical composition for modifying blood coagulation comprising as active ingredient the polypeptide of any of the claims 1 to 5 and a pharmaceutically acceptable carrier.
8. A pharmaceutical composition for modifying blood coagulation in the treatment of thromboembolic diseases comprising as active ingredient the polypeptide of any of the claims 1 to 5 and a pharmaceutically acceptable carrier.
9. A method for producing the polypeptide of any of the claims 1 to 5 comprising adsorbing said factor on suitably immobilised bovine factor Xa and subsequent elution with acid, and neutralizing the eluate.
10. The method according to claim 9 wherein the bovine factor Xa is immobilised by being coupled to an insoluble solid support.
11. The method of claim 10 wherein said solid support is an Affi-Gel 10 column and the polypeptide is eluted by acetic acid.

## Patentansprüche

1. Polypeptid, isoliert aus dem Speichel des heilsamen Blutegels *Hirudo medicinalis*, das spezifisch Rinder-Faktor Xa hemmt, nicht jedoch Rinder-Thrombin hemmt, und ein Molekulargewicht im Bereich von 10-30 kd hat.
2. Polypeptid nach Anspruch 1, das ein Inhibitor des gemischten Typs ist, das nicht durch eine Substanz mit einer hohen Affinität ( $K_m$ -Wert) und niedrigem  $k_{cat}$ -Wert für Faktor Xa eluiert wird, wobei das Polypeptid an eine geeignete, einen immobilisierten Rinder-Faktor Xa umfassende Säule adsorbiert ist.
3. Polypeptid nach Anspruch 2, worin der  $K_m$ -Wert der Elutionssubstanz  $46,9 \mu M$  ist und der  $K_{cat}$ -Wert  $0,681 s^{-1}$  ist.
4. Polypeptid nach Anspruch 2 oder 3, das ein Inhibitor des gemischten Typs ist, das nicht durch D-Phe-Pipecolyl-L-Arg-pNA eluiert wird, wenn es an eine geeignete, einen immobilisierten Faktor Xa umfassende Säule adsorbiert ist.
5. Blutegel Faktor Xa - Inhibitor-Polypeptid des gemischten Typs, das spezifisch Rinder-Faktor Xa inhibiert, nicht jedoch Rinder-Thrombin inhibiert, ein Molekulargewicht im Bereich von 10-30 kd hat und



nicht von einer einen immobilisierten Faktor Xa umfassenden Säule durch D-Phe-Pipécolyl-L-Arg-pNA eluiert wird.

- 5 6. Pharmazeutische Zusammensetzung, umfassend als Wirkstoff das Polypeptid nach einem der vorhergehenden Ansprüche und einen pharmazeutisch verträglichen Träger.
7. Pharmazeutische Zusammensetzung zur Modifizierung der Blutgerinnung, umfassend als Wirkstoff das Polypeptid nach einem der Ansprüche 1 bis 5 und einen pharmazeutisch verträglichen Träger.
- 10 8. Pharmazeutische Zusammensetzung zur Modifizierung der Blutgerinnung in der Behandlung von thromboembolischen Erkrankungen, umfassend als Wirkstoff das Polypeptid nach einem der Ansprüche 1 bis 5 und einen pharmazeutisch verträglichen Träger.
- 15 9. Verfahren zur Herstellung des Polypeptids nach einem der Ansprüche 1 bis 5, bei dem der Faktor an einen geeignet immobilisierten Rinder-Faktor Xa adsorbiert und nachfolgend mit Säure eluiert wird und das Eluat neutralisiert wird.
10. Verfahren nach Anspruch 9, worin der Rinder-Faktor Xa immobilisiert wird, indem er an einen unlöslichen festen Träger gebunden wird.
- 20 11. Verfahren nach Anspruch 10, worin der feste Träger eine Affi-Gel 10-Säule ist und das Polypeptid durch Essigsäure eluiert wird.

#### Revendications

- 25 1. Polypeptide isolé à partir de la salive de la sangsue médicinale d'*Hirudo medicinalis*, qui inhibe spécifiquement le facteur Xa bovin mais n'inhibe pas la trypsine bovine et possède un poids moléculaire dans la gamme de 10 à 30 kd.
- 30 2. Polypeptide selon la revendication 1, qui est un inhibiteur du type mixte, non élué par une substance ayant une grande affinité ( $K_m$ ) et une faible constante d'activité  $K_{cat}$  pour le facteur Xa, ledit polypeptide étant absorbé sur une colonne convenable comprenant du facteur Xa bovin immobilisé.
- 35 3. Polypeptide selon la revendication 2, dans lequel le coefficient  $K_m$  de la substance d'éluion vaut 46,9  $\mu M$  et la constante  $K_{cat}$  vaut  $0,681 s^{-1}$ .
- 40 4. Polypeptide selon la revendication 2 ou la revendication 3, qui est un inhibiteur du type mixte non élué par D-phé-pipécolyl-L-Arg-pNA lorsqu'il est adsorbé sur une colonne convenable comprenant du facteur Xa immobilisé.
- 45 5. Polypeptide de type mixte inhibiteur du facteur Xa, qui inhibe spécifiquement le facteur bovin Xa mais n'inhibe pas la trypsine bovine, a un poids moléculaire dans la gamme de 10 à 30 kd et n'est pas élué, par D-phé-pipécolyl-L-Arg-pNa, d'une colonne comprenant du facteur Xa immobilisé.
- 50 6. Composition pharmaceutique, comprenant à titre d'ingrédient actif le polypeptide de l'une quelconque des revendications précédentes et un véhicule ou support ou excipient pharmaceutiquement acceptable.
- 55 7. Composition pharmaceutique pour modifier la coagulation du sang, cette composition comprenant à titre d'ingrédient actif le polypeptide selon l'une quelconque des revendications 1 à 5 et un véhicule ou excipient pharmaceutiquement acceptable.
8. Composition pharmaceutique pour modifier la coagulation du sang lors du traitement de maladies thromboemboliques, cette composition comprenant à titre d'ingrédient actif le polypeptide selon l'une quelconque des revendications 1 à 5 et un excipient ou véhicule pharmaceutiquement acceptable.
9. Procédé de production du polypeptide selon l'une quelconque des revendications 1 à 5, comprenant l'adsorption de ce facteur sur un facteur Xa bovin immobilisé de façon appropriée et une éluion



subséquente par de l'acide, et une neutralisation de l'éluat.

10. Procédé selon la revendication 9, dans lequel le facteur Xa bovin est immobilisé du fait qu'il est couplé à un support solide insoluble.

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11. Procédé selon la revendication 10, dans lequel ledit support solide est une colonne de "Affi-Gel 10" et le polypeptide est élué par l'acide acétique.

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