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Heterogeneity of Polyvalent Proteinase-inhibitors from Certain Snails Demonstrated by Fibrin-agar-electrophoresis

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Saline extracts of the albumin gland of snails from the genus *Helix*, *Cepaea* and *Achatina* show a remarkable capacity for inhibiting several serine-proteinases of animal, plant and bacterial origin. By the use of fibrin-agar-electrophoresis and fibrin-agar-immunelectrophoresis four polyvalent proteinase-iso-inhibitors could be separated, two of them probably directed against the active center of the enzymes. One of the inhibitory proteins may be identical or closely related to the agglutinin anti-A found in the same gland. The importance of these protease-inhibiting and bacterial agglutinating principles for the snails and their eggs is briefly discussed. The possibility of their therapeutic application in man is considered.

Natriumchlorid-Extrakte der Eiweißdrüsen von Schnecken der Gattung *Helix*, *Cepaea* und *Achatina* zeigen eine ausgeprägte Inhibitorwirkung gegen Serinproteinasen tierischer, pflanzlicher und bakterieller Herkunft. Durch Fibrinagarelektrophorese und Fibrinagarimmunoelektrophorese konnten vier polyvalente Proteinase-iso-Inhibitoren nachgewiesen werden. Es wird angenommen, daß zwei von ihnen am aktiven Zentrum des Enzyms angreifen, zwei weitere außerhalb. Ein inhibitor-wirksames Protein ist dem Anti-A_{HP} Agglutinin nahe verwandt oder identisch. Die Schutzfunktion der Inhibitoren und Agglutinine für die Schnecken und ihre Eier wird diskutiert. Es wird auf therapeutische Anwendungsmöglichkeiten beim Menschen hingewiesen.

In the first communication we described a polyvalent proteinaseinhibitor occurring in the albumin gland of *Helix pomatia* (HP) (1). The present report deals with the heterogeneity and specificity of this inhibitor as revealed by fibrin-agar-electrophoresis.

Material and Methods

Snail albumin gland extracts were prepared from the selected albumin glands of *Helix pomatia*, *Cepaea hortensis*, *Achatina granulata* and *Achatina chamaeleon* as described earlier (2). The contents of the eggs from *Helix pomatia* were treated similarly.

Antisera: The antiserum to the crude extract of the albumin gland of *Helix pomatia* was produced in rabbits using complete FREUND's adjuvans (1). The rabbit anti human serum (Kames No. 42) was produced in New Zealand rabbits using complete FREUND's adjuvans and repeated booster injections as described previously (3).

Proteolytic enzymes and inhibitors: trypsin (EC 3.4.4.4) cryst., lyophil. and proteinase K; both from E. Merck, Darmstadt. Pronase B low grade; Calbiochem, Lucerne. Bromelin: Nutritional Biochem. Co., Cleveland, Ohio. Kallikrein (EC 3.4.4.21) and elastase: Bayer Biochemical Laboratories, Wuppertal. Ficin (EC 3.4.4.12) and α -chymotrypsin (EC 3.4.4.5) 3x cryst., salt-free: both from Carl Roth, Karlsruhe. Subtilisin (EC 3.4.4.16), plasmin (EC 3.4.4.14) papain (EC 3.4.4.10) cryst., thermolysin 3x cryst., trypsininhibitor from soybean and trypsin-inhibitor (egg white) B grade: all from Serva, Heidelberg.

Blood group A substance from peptone was obtained by phenol-saline extraction as described earlier (4).

Fibrin-agar-electrophoresis and fibrin-agar-immunelectrophoresis were performed according to HEIMBURGER and SCHWICK (5), partly modified according to SCHEIDEGGER (6) and HERMANN and MIESCHER (7): 9 vol. parts of agarose (No. 11397, Serva, Heidelberg) 0.8% solution in barbital buffer (0.05M, pH 8.2, following the indications of GRABAR and BURTIN (8)), 2 vol. parts of a 0.4% solution of bovine fibrinogen (Behringwerke, Marburg) in

barbital buffer and 1 vol. part 0.1N $\text{Ca}(\text{H}_2\text{PO}_4)_2$ solution were mixed at 53° and immediately distributed on the plates. Electrophoretic separation was performed at room temperature with 70—75 V on the plates and 17.6 mA for each plate for 35—45 minutes. A fibrin-agar-immunelectrophoresis with trypsin as the proteinase, human serum as the antigen-mixture and rabbit-anti-human-immunserum served as the reference for each run. The diffusion time was 12 to 18 h for the immunisera and 5 to 16 h for the proteinases at room temperature. When optimal fibrin digestion and optimal antibody-antigen-reaction were achieved the plates were photographed immediately in transmitted and/or oblique light in a home made apparatus.

Results and Discussion

The fibrin-agar-immunelectrophoresis of the trypsin-inhibitor from *Helix pomatia* is given in Figure 1. As can be seen from the control (1a) the trypsin-inhibitor in the human serum binds the trypsin, so that in the area of the diffusion radius of the trypsin-inhibitor, the fibrin is protected from digestion. Corresponding to the undigested area we find the precipitation-line of the serum trypsin-inhibitor with the specific antiserum. Figure 1b shows the trypsin-inhibitor in the *Helix pomatia* extract: the inhibition area corresponds also to at least two precipitation-lines of the anti-*Helix pomatia* immunserum. Immunelectrophoretic analysis was performed with all the enzymes mentioned below and it confirmed the results of the fibrin-agar-electrophoresis.

Fibrin-agar-electrophoresis was performed with all the proteolytic enzymes listed in Table 1. Trypsin-inhibitor from egg white and soybean served as control experiments. The electrophoresis revealed four inhibitory

Fig. 1

Fibrin-agar-immunelectrophoresis

1a: upper trough trypsin, hole human serum, lower trough rabbit anti human immunserum

1b: upper trough trypsin, hole *Helix pomatia* extract, lower trough anti-HP

Tab. 1

Inhibition of proteinases by different inhibitory proteins from the albumin gland of *Helix pomatia* (HP), as revealed by fibrin-agar-electrophoresis

inhibitory protein electrophoretic mobility	HP ₁ γ	HP ₂ α ₂	HP ₃ α ₁	HP ₄ ρ
trypsin	∅	+	+	∅
kallikrein	∅	+	+	∅
α-chymotrypsin	∅	+	+	+
pronase B	∅	+	+	+
thermolysin	∅	+	+	+
papain	∅	+	+	+
subtilisin	+	+	+	+
proteinase K	+	+	+	+
plasmin	+	+	+	+

proteins, which we designate, according to their source, as HP₁, HP₂, HP₃ and HP₄. Their electrophoretic mobility lies, compared to the serum proteins, for HP₁ in the γ, for HP₂ in the α₂, for HP₃ in the α₁ and for HP₄ in the ρ field (Fig. 2). The inhibitory capacity of these four proteins changes with the different proteinases (Tab. 1). They represent iso-inhibitors, reacting in various constellations. We may distinguish three types:

1. Trypsin-type: inhibition by HP₂ and HP₃. Kallikrein belongs to this group.
2. α-chymotrypsin-type: inhibition by HP₂, HP₃ and HP₄. Pronase B, thermolysin and papain react in a similar way.
3. Subtilisin-type: inhibition by HP₁, HP₂, HP₃ and HP₄. Other enzymes of this type are proteinase K and plasmin.

Ficin, bromelin and elastase, which are inhibited by the *Helix pomatia* extract (1), do not digest fibrinogen. Subsequently they cannot be characterized by fibrin-agar-electrophoresis. The method developed by Fossum (9) using casein-agar as substrate may be of further value in elucidating the heterogeneity of the inhibitor active proteins against these proteases.

In a previous paper we reported the fractionation of the crude *Helix pomatia* extract into an anti-A agglutinin (anti-A_{HP}) positive inhibitor-free fraction II

Fig. 2

Fibrin-agar-electrophoresis

2a: upper and lower trough trypsin, hole *Helix pomatia* extract
2b: upper and lower trough pronase B, hole *Helix pomatia* extract
2c: upper and lower trough proteinase K, hole *Helix pomatia* extract

Fig. 3

3a: Immunelectrophoresis (usual agar gel)
upper hole fraction II, middle hole *Helix pomatia* extract, lower hole fraction I, upper and lower trough anti-HP
3b: Fibrin-agar-immunelectrophoresis
upper trough proteinase K, lower trough blood group A substance, hole *Helix pomatia* extract

and an inhibitor active, agglutinin-free fraction I (1). Their immunelectrophoresis with anti-HP immunserum is shown in Figure 3a. Fraction II has only one precipitation-line, the one for the agglutinin anti-A_{HP}. Fraction I has at least four precipitation-lines. By fibrin-agar-electrophoresis we obtained evidence of only three inhibitory proteins in this fraction; the HP₁ protein was missing and it was found in the agglutinin positive fraction II. As can be seen from Figure 3b, the electrophoretic mobility of the anti-A_{HP} agglutinin and the HP₁ protein is almost the same. The question whether they are identical still awaits additional investigations.

The crude extracts of the snails *Cepaea hortensis* (CH) *Achatina chamaeleon* and *Achatina granulata* also possess inhibitory activity, as could be shown by the Azocoll test (1) and by fibrin-agar-electrophoresis. The same activity was found in the eggs of these snails. The *Cepaea hortensis* extract produced, in fibrin-agar-electrophoresis, four similar inhibition areas, which we called, corresponding to those from *Helix pomatia*, CH₁, CH₂, CH₃ and CH₄. The occurrence of proteinase-inhibitors in all these snails indicates an inhibitor polymorphism.

Our experiments show that we are dealing with a heterogeneity of at least four polyvalent proteinase-inhibitors. They occur as iso-inhibitors in three possible compositions: trypsin-type, α -chymotrypsin-type and subtilisin-type. The proteins HP₂ and HP₃ inhibit all the proteinases tested, especially the broad range of serine-proteinases from different sources. This led us to the assumption, that they represent competitive inhibitors reacting with the active center of these proteinases. The inhibitor active proteins HP₁ and HP₄ do not react with the whole proteinase spectrum. HP₄ does not interfere with trypsin and kallikrein, the HP₁ protein inhibits only plasmin, subtilisin and proteinase K. This indicates that the HP₁ and HP₄ proteins,

which inactivate some serine-proteinases but not others, may be directed — by an allosteric effect — towards a protein region topographically not related to the active center of the enzyme.

The occurrence of polyvalent proteinase-inhibitors and antibacterial agglutinins in the albumin gland of snails and in their eggs underlines again the protective role of these substances. The problem of whether the observed growth-stimulating effect of dead snail eggs on the surviving ones (10) is connected with this inhibitor, has still to be investigated.

As it has been shown in monkeys (11), that parenteral application of the gland extract of *Helix pomatia* does no harm to the recipient, it may be possible to use the further purified inhibitor substances or their subunits for therapeutical purpose in man. The anticomplementary effect of *Helix pomatia* extracts (12) may also be due to the serine-protease-inhibitor property (C₁-esterase-inhibition) and must be taken into account in such in vivo experiments.

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