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Partial Purification and Characterization of a Trypsin-Like Proteinase from Rabbit Preimplantation Uterine Fluid

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Summary: Previous investigations have proved that proteinases are involved in implantation of the rabbit embryo into the uterine tissues. This study describes a trypsin-like enzyme found in the blastocyst fluid and uterine flushings at the time of implantation. The proteinase isolated from uterine flushings has a molecular mass of about 50 kDa and exists in two differently charged forms of pI 4.0 and 4.5. Tests with low molecular mass 4-nitroanilide substrates proved a marked cleavage selectivity of the enzyme for arginyl bonds. The catalytic activity is not affected by $Ca^{2\oplus}$ and EDTA but inhibited by aprotinin and a high concentration (10^{-6} M) of lima bean trypsin inhibitor.

Partielle Reinigung und Charakterisierung einer Trypsin-ähnlichen Proteinase aus dem Uterussekret vom Kaninchen

Zusammenfassung: In histochemischen und biochemischen Versuchen an Kaninchen konnte gezeigt werden, daß Proteinasen eine wichtige Rolle bei der Embryo-Implantation spielen. In dieser Arbeit wird ein Trypsin-ähnliches Enzym beschrieben, das wenige Stunden vor der Implantation in der Blastozystenflüssigkeit und in der Uterus-Spülflüssigkeit auftritt, aus der es isoliert werden kann. Die Proteinase hat eine Molekülmasse von ca. 50 kDa. In der Isoelektrofokussierung findet man je einen Aktivitätsgipfel bei pH 4.0 und 4.5. Untersuchungen mit verschiedenen Peptid-4-nitroanilid-Substraten ergaben, daß das Enzym vorzugsweise Arginylbindungen spaltet. Die katalytische Aktivität wird durch $Ca^{2\oplus}$ und EDTA nicht beeinflußt aber durch Aprotinin und hohe Konzentrationen (10^{-6} M) von Limabohnen-Trypsin-Inhibitor gehemmt.

Key words: Trypsin-like proteinase, implantation, rabbit uterine flushings, blastocyst fluid.

Implantation of the mammalian embryo in the uterus involves, in species like the human, rodents and the rabbit, invasion of the trophoblast through the uterine epithelium into the stromal tissue and into blood vessels of the endometrium (haemo-chorial placentation)[1]. These processes are accompanied not only by gross protein degradation but seem to involve also limited proteolysis of extracellular matrix materials, both requiring the action of proteinases. The existence of endopeptidases at implantation sites in the mouse[2] and the rabbit[3,4] is well documented although information on their biochemical and enzymic characteristics is very limited. Isolation

Enzymes:
Chymotrypsin (EC 3.4.21.1); leukocyte elastase (EC 3.4.21.37); trypsin (EC 3.4.21.4).

Abbreviations:
Amino acids and peptides are abbreviated according to the Recommendations (1983) of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem.* **138**, 9–37; Bz, *N*-benzoyl; Nan, 4-nitroanilide; Suc, *N*-(3-carboxypropionyl); Tos, tosyl. *Example:* Bz-Ile-Glu(OR)-Gly-Arg-Nan = *N*-benzoyl-L-isoleucyl-L-glutamyl-(γ-OR)-glycyl-L-arginine 4-nitroanilide (R represents H and CH_3 in a 1 : 1 ratio).

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of the proteinases is difficult to perform since only nanogram quantities can be obtained from each rabbit uterus. On the other hand knowledge of the biochemical and catalytic properties of the enzymes is of interest since studies in vivo with proteinase inhibitors suggest that the endopeptidases are essential for implantation[3].

Materials and Methods

Materials

Suc-Ala_3-Nan, Suc-Ala_2-Pro-Phe-Nan and Suc-Ala_2-Pro-Leu-Nan were obtained from Bachem (CH-4416 Bubendorf), Tos-Gly-Pro-Arg-Nan from Boehringer (D-6800 Mannheim) and H-D-Val-Leu-Arg-Nan and Bz-Ile-Glu(OR)-Gly-Arg-Nan from Kabi Vitrum (S-11287 Stockholm). Sephadex G-100, DEAE-Sephadex A-50 and PD 10 columns were purchased from Pharmacia (S-75104 Uppsala). Servalyt precotes pH 3–6 were from Serva (D-6900 Heidelberg). Lima bean trypsin inhibitor (2829) was obtained from Worthington Diagnostic Systems Inc. (Freehold, NJ 07728) and aprotinin (Trasylol) was a gift from Bayer AG (D-5090 Leverkusen).

Preparation of the uterine flushings and blastocyst fluid

Late preimplantation stage uterine fluid and blastocysts were obtained by flushing each uterus of pregnant rabbits 6 $^2/_3$ days post coitum with 5 m*l* 0.9% (w/v) NaCl solution as described previously[3]. All blastocysts from each rabbit were transferred to 2 m*l* 50mM Tris/HCl buffer pH 8.0 and crushed in the buffer with forceps so that the blastocyst fluid leaked out. After removal of the tissue the Tris/HCl buffer with the blastocyst fluid is referred to as diluted blastocyst fluid.

Determination of enzyme activity

The hydrolysis rate of different peptide 4-nitroanilide substrates was followed photometrically at 405 nm. Samples of 50–200 μl uterine flushings or diluted blastocyst fluid were incubated at 37 °C in a final volume of 400 μl of 50mM Tris/HCl pH 8.0 containing the substrate in a final concentration of 250μM for Suc-Ala_3-Nan, Suc-Ala_2-Pro-Phe-Nan, Suc-Ala_2-Pro-Leu-Nan, Tos-Gly-Pro-Arg-Nan, and 100μM for Val-Leu-Arg-Nan and Bz-Ile-Glu(OR)-Gly-Arg-Nan. EDTA, $Ca^{2\oplus}$ or inhibitors were included in some tests in concentrations as mentioned in the Results.

Isolation of the enzyme

Uterine flushings from 5 rabbits were concentrated 50 times on an Aminco ultrafiltration unit, cut-off 10 kDa and then used for gel filtration on Sephadex G-100 (70 × 1 cm) equilibrated with 50mM Tris/HCl pH 8.0, 0.1% Tween-20. The fractions able to hydrolyse Val-Leu-Arg-Nan were pooled and applied to 5 m*l* DEAE-Sephadex equilibrated with 5mM Tris/HCl, 10mM NaCl, 0.1% Tween-20, pH 7.4. Elution was performed with a linear gradient from 10 to 550mM NaCl in the same buffer. The fractions found to hydrolyse Val-Leu-Arg-Nan were pooled, desalted on Sephadex PD-10 columns and lyophilized.

Isoelectric focusing

Servalyt precotes pH 3–6 were used. After the focusing they were cut in 0.5-cm pieces. Proteins were eluted with 1 m*l* of 50mM Tris/HCl buffer pH 8.0 and assayed for enzyme activity by hydrolysis of Val-Leu-Arg-Nan.

Results and Discussion

A trypsin-like enzyme was isolated from uterine flushings by gel filtration (Fig. 1) and DEAE-Sephadex chromatography (Fig. 2). This simple procedure was sufficient to separate the enzyme completely from the other endopeptidases as detectable with the applied substrates. Suc-Ala_3-Nan, Suc-Ala_2-Pro-Leu-Nan and Suc-Ala_2-Pro-Phe-Nan, typical substrates for elastase and chymotrypsin, were cleaved by crude uterine flushings but not by the isolated enzyme.

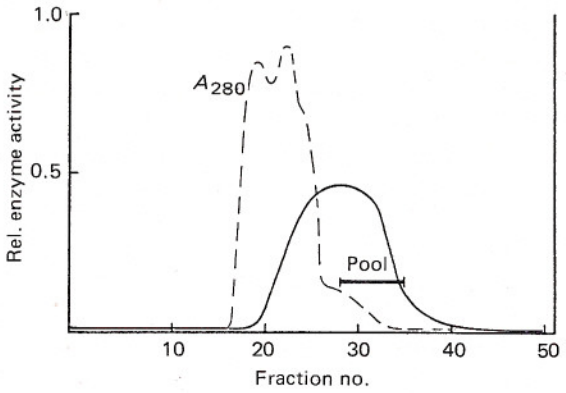

Fig. 1. Gel filtration of uterine flushings on Sephadex G-100.

The column was equilibrated and eluted with 50mM Tris/HCl pH 8.0 and 0.1% Tween-20. – – – – A_{280}, —— relative enzyme activity detected by hydrolysis of Val-Leu-Arg-Nan.

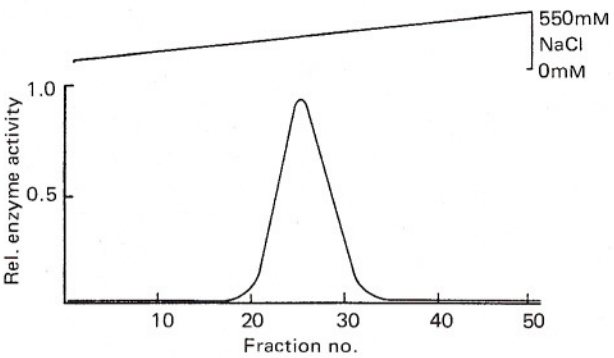

Fig. 2. Ion-exchange chromatography.

The pooled fractions from gel filtration were applied on a column of DEAE-Sephadex and eluted with a linear gradient from 10mM to 550mM in 5mM Tris/HCl pH 7.4 and 0.1% Tween-20. —— relative enzyme activity detected by the hydrolysis of Val-Leu-Arg-Nan.

Table. Relative hydrolysis rates with different peptide nitroanilide substrates.

Substrate	Uterine flushings (crude)	Diluted blastocyst fluid (crude)	Isolated enzyme
Val-Leu-Arg-Nan	100	100	100
Tos-Gly-Pro-Arg-Nan	101	88	87
Bz-Ile-Glu(OR)-Gly--Arg-Nan	6	*	*
Suc-Ala_3-Nan	12	*	*
Suc-Ala_2-Pro-Phe-Nan	14	*	*
Suc-Ala_2-Pro-Leu-Nan	26	*	*

* Below detection limit.

In our assay series of synthetic substrates the purified enzyme had a strong preference for some substrates with arginine in the P_1 position (see table). The highest cleavage rate was observed between pH 8 and 9. The endopeptidase was inactive and unstable in the acid range but remarkably stable at neutral or slightly alkaline pH. Fifty or 100μM $Ca^{2\oplus}$, 100μM EDTA or 0.03μM lima bean trypsin inhibitor did not affect the hydrolysis rate of Val-Leu-Arg-Nan. The enzyme activity was completely inhibited by 0.015μM aprotinin and 1μM lima bean trypsin inhibitor. The molecular mass of the proteinase was estimated by gel filtration as approximately 50 kDa. The preparation contained two differently charged forms of the enzyme since isoelectric focusing yielded a peak of activity at pH 4.0 and one at pH 4.5 (Fig. 3).

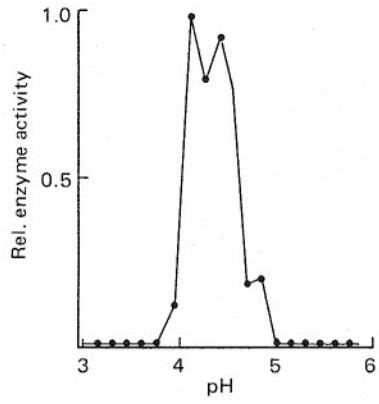

Fig. 3. Isoelectric focusing on Servalyt precotes pH 3–6. After focusing, the proteins were eluted from the precotes with 50mM Tris/HCl pH 8.0 and assayed for enzyme activity by hydrolysis of Val-Leu-Arg-Nan.

We observed that the blastocysts became very fragile and broke easily during the flushing procedure as soon as the proteinase was detectable. It was not possible to obtain sufficient material for an enzyme isolation from unruptured blastocysts. Therefore, uterine flushings were used for the enzyme purification. The observed correlation between fragility of the blastocysts and detectability of the enzyme supports the suggested involvement of the proteinase in the lysis of the blastocyst coverings since these latter structures convey mechanical strength to the blastocysts.

The isolated enzyme is most probably identical with the main trypsin-like endopeptidase found previously in crude blastocyst tissue homogenates[4]. Although it has been proposed that this enzyme may be identical with the gelatinolytic proteinase which has been studied extensively with the histochemical substrate film test[3], further investigations are necessary in order to fully understand the biological function of the various proteinases present at implantation sites. The rabbit is considered a particularly useful model system for the investigation of implantation-associated proteinases since it is the largest laboratory animal with haemo-chorial placentation while the pig, which other groups have used for proteinase/inhibitor studies[5], represents a non-invasive (epithelio-chorial) implantation type and is therefore not comparable to the human.

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