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Enzymic Characterization of Rabbit Blastocyst Proteinase with Synthetic Substrates of Trypsin-Like Enzymes

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Summary: Since previous investigations have provided evidence that certain proteinases of the trophoblast play a key role in initiation of implantation of the embryo in the uterus, enzymic characterization of endopeptidase activity of rabbit blastocyst extracts was attempted. Recently developed tripeptide *p*-nitroanilide substrates now allow for the first time quantitative assays of enzyme activities in this material. Substrates containing one amino acid only are found not to be hydrolyzed at any measurable rates.
Relative hydrolysis rates of various tripeptide *p*-nitroanilide substrates indicate a preference of blastocyst proteinase(s) for hydrophobic or thrombin-preferred amino acid residues in position P_2 and/or P_3. The pH optimum is found close to 8.5. Titration experiments with various proteinase inhibitors show a particularly strong inhibition by aprotinin (Trasylol). As judged from both substrate specificity and inhibition experiments, the active site of the trophoblast enzyme(s) is closely related to that of trypsin but substrate specificity is more restricted. The enzymic properties found are consistent with a close similarity of the blastocyst proteinase(s) to kallikreins and/or sperm acrosin. The possibility is thus indicated that enzymes of both types are present in rabbit implantation stage blastocysts. Problems of identification with the previously described gelatinolytic proteinase are discussed.

Enzymes:
Acrosin (EC 3.4.21.10); chymotrypsin (EC 3.4.21.1); elastase (EC 3.4.21.11); kallikrein (EC 3.4.21.8); Factor Xa (EC 3.4.21.6); plasmin (EC 3.4.21.7); thrombin (EC 3.4.21.5); trypsin (EC 3.4.21.4).

Abbreviations:
BSTI–II: boar seminal plasma trypsin-plasmin inhibitor II;
BzArgNHNp: N^{α}-benzoyl-L-arginine-*p*-nitroanilide (L-BAPA);
BzIleGlu(OR)GlyArgNHNp: *N*-benzoyl-L-isoleucyl-L-glutamyl(γ-OR)-glycyl-L-arginine-*p*-nitroanilide (the R represents H and CH_3 in a 50% quantity respectively);
d p.c.: days post coitum;
GlPheNHNp: glutaryl-L-phenylalanine-*p*-nitroanilide;
DPhePipArgNHNp: D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide;
DProPheArgNHNp: D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide;
DValLeuArgNHNp: D-valyl-L-leucyl-L-arginine-*p*-nitroanilide;
DValLeuLysNHNp: D-valyl-L-leucyl-L-lysine-*p*-nitroanilide;
SBTI: soybean trypsin inhibitor (Kunitz);
SucAlaAlaAlaNHNp: *N*-(3-carboxypropionyl)-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide;
TosGlyProArgNHNp: *N*-tosyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide;
ZGlyProArgNHNp: benzyloxycarbonyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide.

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Enzymatische Charakterisierung der Proteinaseaktivität aus Kaninchenblastozysten mittels synthetischer Substrate für trypsinähnliche Enzyme

Zusammenfassung: Nachdem frühere experimentelle Untersuchungen gezeigt haben, daß gewisse Proteinasen des Trophoblasten eine wichtige Rolle bei der Einleitung der Implantation des Embryos im Uterus spielen, gelang nun eine vorläufige enzymatische Charakterisierung der in Extrakten aus Kaninchenblastozysten gefundenen Endopeptidaseaktivität. Neu entwickelte Tripeptid-*p*-nitroanilid-Substrate ermöglichten erstmalig quantitative Bestimmungen der Enzymaktivitäten in diesem Material. Synthetische Substrate mit nur einer Aminosäure werden nicht in messbaren Raten hydrolysiert.

Die relativen Hydrolyseraten der Tripeptidsubstrate zeigen eine bevorzugte Bindung von Substraten mit hydrophoben oder Thrombin-bevorzugten Aminosäureresten in Position P_2 und/oder P_3 an das aktive Zentrum der Blastozystenproteinase(n). Das pH-Optimum der Spaltung wurde bei 8.5 gefunden. Titrationsexperimente mit Proteinaseinhibitoren ergaben eine besonders starke Hemmung der Spaltungsaktivität durch Aprotinin (Trasylol). Aus Substratspezifität und Hemmspektrum ist zu schließen, daß das aktive Zentrum der Blastozystenproteinase(n) dem des Trypsins eng verwandt ist, jedoch ist die Substratspezifität höher. Die gefundenen enzymatischen Eigenschaften ähneln sowohl denen von Drüsen-Kallikreinen als auch denen des Spermienenzyms Akrosin. Die Möglichkeit, daß beide Enzymtypen im untersuchten Material vorkommen, sowie ihre Beziehung zu der früher beschriebenen gelatinolytischen Proteinase werden diskutiert.

Key words: Blastocyst proteinase, trophoblast proteinase, implantation, trypsin-like enzyme, synthetic substrates.

A peculiar proteinase found in the trophoblast of implanting blastocysts of the rabbit seems to play an important role in initiation of embryo implantation in the uterus, most probably in an action concerted in some way with uterine secretion proteinase(s)[1–4]. As demonstrated by studies on the inhibition of implantation by application of various proteinase inhibitors in vivo, these proteinases are involved in the dissolution of the extracellular blastocyst coverings which is a prerequisite for attachment of the trophoblast onto the uterine epithelium: in uteri into which certain inhibitors such as aprotinin (Trasylol) had been injected intraluminally, dissolution of the blastocyst coverings and implantation of the embryo are prevented[2,3]. A direct participation of these proteinases in the attachment reaction of trophoblast and uterine epithelial cell surfaces has also been envisaged but has not been shown conclusively by the cited investigations.

Rabbit trophoblast proteinase merits special attention for several reasons. First, its physiological significance with respect to implantation initiation seems to be even greater than that of uterine secretion proteinase(s) as demonstrated by several biological experiments[5,6]. Furthermore, inhibition studies revealed both trypsin-like and chymotrypsin-like enzymic properties of rabbit trophoblast proteinase(s)[7]. While previous investigations were hampered by the lack of a suitable substrate which would allow kinetic assays (only a gelatin substrate film test has been available so far), we are now able to present quantitative data obtained with recently developed synthetic oligopeptide substrates, permitting more accurate definition of enzymic properties.

Materials and Methods

Preparation of the blastocyst extracts

Late preimplantation stage blastocysts were obtained by flushing the uteri of mated rabbits (mixed-bred or Alaska strain) with 0.9% (w/v) NaCl solution at 6 2/3 days post coitum (d p.c.) as described previously[2,8]. All further steps of preparation were done at 0–4 °C. The blastocysts were washed twice in 0.9% NaCl solution. Blastocyst tissue and extracellular blastocyst coverings were separated using fine forceps as described before[4]. Blastocyst tissue (predominantly trophoblast) was quickly frozen with dry ice or liquid nitrogen and stored at − 60 to − 30 °C. For the preparation of crude extracts, this material was thawed and sonicated (20 W, Branson

Sonifier B-12 equipped with micro-tip) for 5 s at 0–4 °C in 0.9% NaCl solution or 0.05M Tris/HCl buffer pH 8.5 (volume first adjusted to 25–50 µl per blastocyst), followed by centrifugation for 5 min at 28000 × g. Supernatants were used for the assays.

Determination of enzyme activity and interaction with proteinase inhibitors

Hydrolysis of various *p*-nitroanilide substrates was followed at 38 °C in a Zeiss PMQ III spectrophotometer at 405 nm. Samples of 50 µl of the blastocyst extracts were assayed in 750 µl end volume, 0.05M Tris/HCl buffer or barbital acetate (Michaelis) buffer (pH as indicated) containing the *p*-nitroanilide substrates in the concentrations given below. For comparison of a series of different substrates, the *p*-nitroanilides were first dissolved in *N,N'*-dimethylformamide (50 µl per 750 µl end volume) to overcome problems eventually caused by different solubilities. For routine assays, however, the substrate TosGlyProArgNHNp was dissolved directly in buffer by vigorous shaking, omitting *N,N'*-dimethylformamide because this solvent was found to cause partial decomposition of the substrate yielding inhibitory breakdown products.

For inhibition studies, enzyme preparations were preincubated for 5 min at 38 °C in a total volume of 450 µl of assay buffer containing inhibitor. The enzymic reaction was started by the addition of 300 µl of substrate-buffer solution.

Substrates

SucAlaAlaAlaNHNp, No. 740813 from Protein Research Foundation, Japan; BzArgNHNp · HCl, No. 10754 from E. Merck, Darmstadt; TosGlyProArgNHNp · acetate, No. 206849 and ZGlyProArgNHNp · HCl, No. 200352 from Boehringer, Mannheim; GlPheNHNp, No. 23125 from Serva, Heidelberg. From AB KABI, Mölndal, Sweden were obtained: BzIleGlu(OR)GlyArgNHNp · HCl (S-2222), DPhePipArgNHNp · 2 HCl (S-2238), DProPhe-ArgNHNp · 2 HCl (S-2302), DValLeuArgNHNp · 2 HCl (S-2266), DValLeuLysNHNp · 2 HCl (S-2251).

Inhibitors

Aprotinin (Trasylol) charge SMU 224/9, 6900 KIE/mg corresponding to 3.8 IU/mg if trypsin inhibition is measured with BzArgNHNp as substrate, was obtained from Bayer AG, Elberfeld. Boar seminal plasma trypsin-plasmin inhibitor II (BSTI-II) was isolated as described recently[9]. Soybean trypsin inhibitor (SBTI-Kunitz, No. 24020) was purchased from E. Merck, Darmstadt.

Results and Discussion

Substrate specificity

The relative hydrolysis rates as measured with various *p*-nitroanilide substrates are given in the Table. It is evident from these data that the substrate specificity of the measured enzyme(s) is of the trypsin type although there are a number of remarkable differences compared to the pancreatic enzyme (see below). There is no measurable hydrolysis of chymotrypsin substrate X and elastase substrate IX.

Table. Relative hydrolysis rates of various *p*-nitroanilide substrates as caused by rabbit blastocyst extracts.

	Substrate[a] (notation: P_3 P_2 P_1)	Relative hydrolysis rate (I = 100)	Preferably hydrolyzed by
I:	TosGlyProArgNHNp	100.0	thrombin
II:	DValLeuArgNHNp	130.8	glandular kallikreins
III:	DProPheArgNHNp	94.4	plasma kallikrein
IV:	DPhePipArgNHNp	68.7	thrombin
V:	ZGlyProArgNHNp	51.7	thrombin
VI:	BzIleGlu(OR)GlyArgNHNp	16.0	factor Xa
VII:	DValLeuLysNHNp	5.6	plasmin
VIII:	BzArgNHNp	(2.7)[b]	trypsin
IX:	SucAlaAlaAlaNHNp	(2.4)[b]	elastase
X:	GlPheNHNp	(1.2)[b]	chymotrypsin

[a] Substrate concentration: 0.5mM in 0.05M Tris/HCl buffer, pH 8.0. 38 °C. Note that under these conditions substrate saturation might not be achieved, at least in some cases, cf. the text.
[b] At the limit of detectability.

Regarding the trypsin-like character of the blastocyst proteinase its strong preference for arginyl bonds is remarkable while lysyl bonds are much less rapidly hydrolyzed, cf. substrates II and VII. In the group of arginyl-*p*-nitroanilides elongation of the peptide chain increases the sensitivity dramatically, cf. substrate VIII with I-VI; BzArgNHNp (VIII) is a very poor substrate for this enzyme. On the other hand, the high degree of substrate specificity of the blastocyst proteinase is illustrated by the relative resistance to hydrolysis of substrate VI which is hydrolyzed quite rapidly by factor Xa and trypsin as well[10].

Especially striking is the observation that the kallikrein substrates II and III and thrombin substrate I are cleaved most rapidly by the blastocyst proteinase. Thrombin substrates IV and V are also cleaved with appreciable velocity. This indicates that either a hydrophobic (substrates II and III) or a thrombin-preferred (Pro or Pip in substrates I, V and IV, respectively) residue in position P_2 strongly facilitates substrate binding. The blastocyst enzyme may in fact be related to kallikreins, especially to glandular kallikreins, cf. substrate II. This aspect is underlined further by the finding of a particularly strong inhibition with aprotinin; see below. On the other hand, a specialized trypsin-like enzyme might also exhibit strong kallikrein like activity as is known, for example, for sperm acrosin.

Previous investigations had already shown that the short chain substrates benzoylarginine ethyl ester and benzoylarginine-β-naphthylamide as well as the protein substrates casein and fibrin are not hydrolyzed at measurable rates by rabbit trophoblast proteinase(s)[2,4,7]. However, gelatin substrate films are split easily[1,7,11–13], and this assay formed the basis of all previous investigations before the synthetic substrates listed in the Table became available. Micro disc as well as agar gel electrophoresis experiments have shown that only a single gelatinolytic proteinase fraction could be demonstrated in rabbit trophoblast material[2,4]. The question whether this gelatinolytic activity is due also to the trypsin-like enzyme(s) measurable with synthetic substrates deserves further study. Since no purification was done with the enzyme material used in the present investigations, we should still be aware of the possibility that different enzymes are responsible for gelatinolysis and for cleavage of the applied synthetic substrates, respectively. This problem will be subject to further investigations involving purification experiments.

Since DValLeuArgNHNp was not available yet in sufficient quantities for routine assays, all further tests were done with TosGlyProArgNHNp. We would like to emphasize also that, due to the limited amount of enzyme material available, the relative hydrolysis rates given in the Table were measured without verifying substrate saturation, at least in some cases. Minor changes of the ratios between the hydrolysis rates to be expected if measured under optimal conditions will be without influence, however, on the principal conclusions drawn above.

pH Optimum

The pH dependence of TosGlyProArgNHNp hydrolysis is indicated in Fig. 1. There is a broad cleavage rate optimum around pH 8.5. Also with other proteinases pH curves are usually not very steep with this type of substrate[14]. The pH optimum found corresponds well with observations made earlier using the gelatin substrate film test where maximum lytic reaction was obtained around pH 8.5[2].

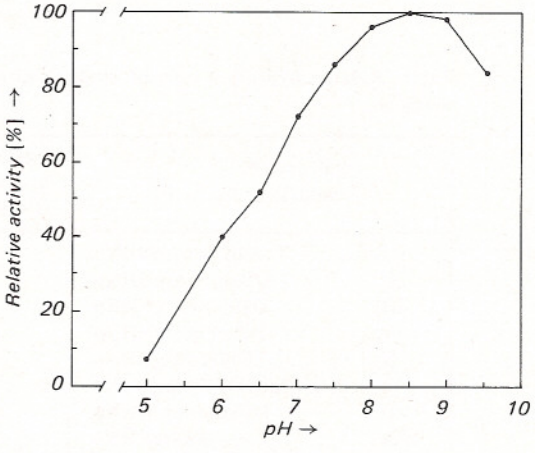

Fig. 1. pH dependence of rabbit blastocyst proteinase activity.

Substrate TosGlyProArgNHNp (0.5mM), barbital acetate (Michaelis) buffer, 38 °C. For further expt. details, see Materials and Methods.

Buffers

With TosGlyProArgNHNp (0.5mM), the hydrolysis rate measured at pH 8.5 or 8.0 in barbital acetate (Michaelis) buffer was 70% of the rate obtained under the same conditions in Tris/HCl buffer (0.05M). Triethanolamine buffer proved unsuitable because cloudy precipitates were formed.

Substrate concentration and Michaelis constant

Hydrolysis rates as a function of varying substrate concentrations are shown for TosGlyProArgNHNp in Fig. 2. The Michaelis constant as determined by plotting [S]/v versus [S] (Fig. 2b) is approximately K_m = 0.18mM (0.05M Tris/HCl buffer pH 8.0, 38 °C) which is in the range of K_m values determined for some other enzymes such as factor Xa and plasmin with this type of substrate[10].

Interaction with proteinase inhibitors

Titration experiments with the inhibitors aprotinin (Trasylol), the boar seminal inhibitor BSTI-II and the Kunitz soybean inhibitor SBTI are illustrated in Figs. 3a–c. By far the strongest inhibition is obtained with aprotinin, the basic kallikrein-trypsin inhibitor from bovine organs, cf. Fig. 3a. The dissociation constant K_i of the complex of this inhibitor with the blastocyst proteinase is found to be below 10pM if estimated graphically according to Green and Work[15]. It seems interesting, in this context, that aprotinin had previously been shown to be the most effective of those inhibitors which were found to block dissolution of blastocyst coverings and implantation in the rabbit in vivo[2].

A clear characterization of the enzymic nature of the blastocyst proteinase is complicated by the fact that inhibition is not only observed with aprotinin, an inhibitor of plasma and glandular kallikreins as well, but also with the trypsin-plasmin inhibitor from boar seminal plasma (Fig. 3b) and the Kunitz soybean inhibitor (Fig. 3c), which exhibits high affinity to trypsin, plasmin and plasma kallikrein. It might be concluded, therefore, that the blastocyst proteinase estimated in these studies is either a specialized trypsin-like enzyme with enzymic kallikrein-like properties as well, like e.g. sperm acrosin, or represents a mixture of such different types of

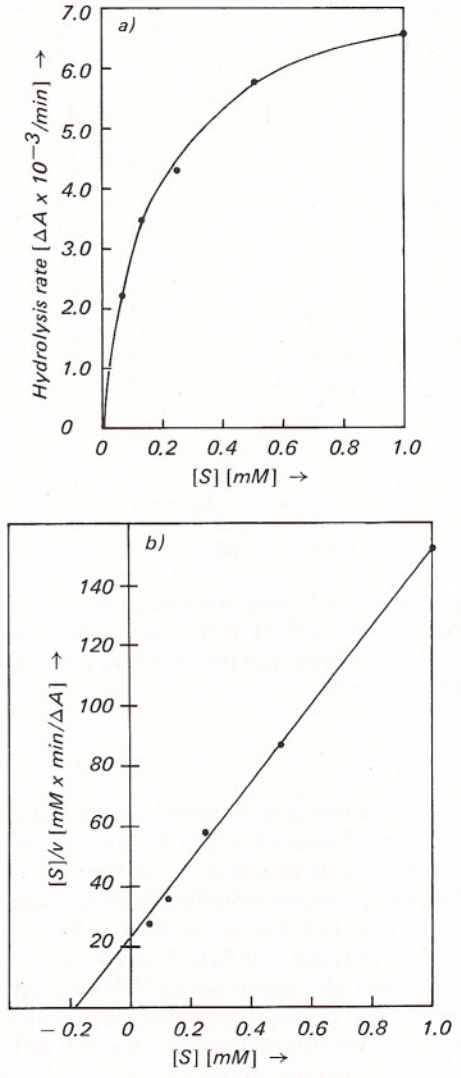

Fig. 2. TosGlyProArgNHNp hydrolysis by rabbit blastocyst proteinase as a function of substrate concentration. Conditions: 0.05M Tris/HCl buffer pH 8.0, 38 °C. In the [S]/v versus [S] plot (b), a K_m value of approximately 0.18mM is obtained. For further expt. details, see Materials and Methods.

enzymes. The same conclusion may be drawn from the substrate specificity studies discussed above.

In previous experiments the effects of a series of inhibitors with different inhibition specificities

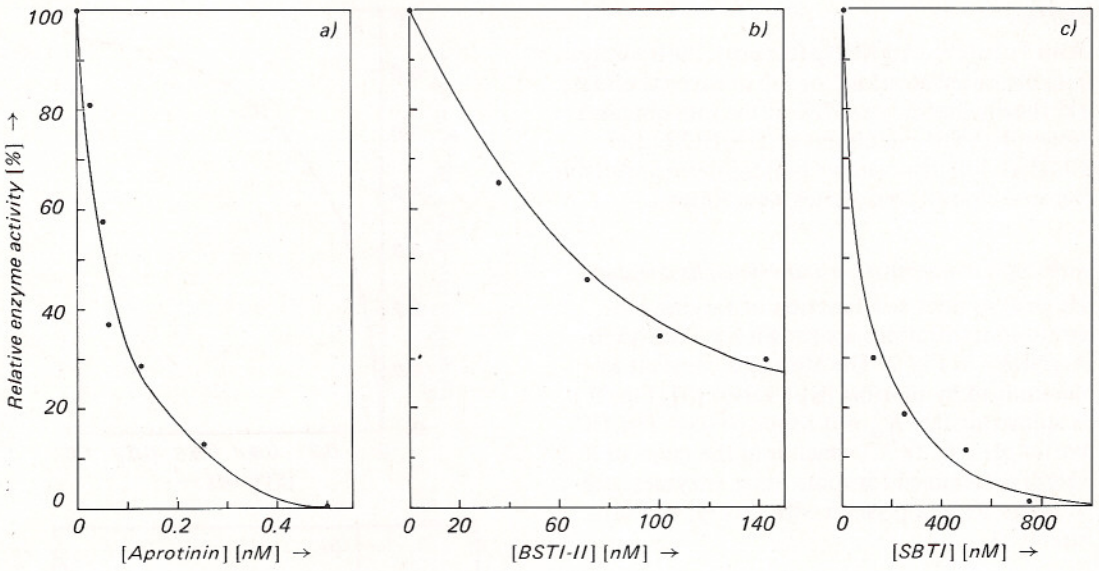

Fig. 3. Titration of rabbit trophoblast proteinase with proteinase inhibitors: a) aprotinin (Trasylol), b) boar seminal plasma inhibitor BSTI-II, and c) Kunitz soybean inhibitor SBTI.

Substrate TosGlyProArgNHNp (0.5mM), 0.05M Tris/HCl buffer, pH 8.5, 38 °C. For further expt. details, see Materials and Methods.

on rabbit trophoplast-dependent proteinase had been assayed qualitatively in the histochemical gelatin substrate film test[2,7]. In this case not only typical trypsin inhibitors including highly specific ones like the pancreatic secretory trypsin inhibitor of Kazal type (which was shown recently to inhibit also sperm acrosin[16]) were effective, but also chymotrypsin-elastase inhibitors[2]. This indicates also that proteinases with different enzymic properties should be present in blastocysts.

Since physiological experiments as cited in the introduction give strong evidence for an important role of blastocyst (trophoblast) proteinase(s) in initiation of embryo implantation in the rabbit, the peculiar enzymic properties of the proteinase activity found in blastocyst extracts seem to deserve more detailed investigation. As discussed above, it cannot be excluded that more than one endopeptidase may be present in this material. In any case, the described data on substrate specificity suggest that the enzyme(s) hydrolyze(s) only a few peptide bonds in the physiological substrates, and that the biological function lies in *limited proteolysis*, i.e. in weakening rather than complete dissolution of blastocyst coverings[2] or in changing cell adhesion via limited degradation of cell surface glycoproteins. In addition, on the basis of the observed enzymic properties which resemble, in some points, those of glandular kallikreins, we have to envisage the possibility of a physiological function of blastocyst proteinase(s) in liberation of kinins, particularly because plasma proteins probably including kininogens are known to appear in the uterine lumen of rabbits at the implantation phase.

The fact that only very small quantities of enzyme can be obtained from mammalian embryos has forced us so far to postpone purification work until sensitive quantitative assays as described here have been developed. Based on these data, attempts at purification and further characterization of the blastocyst proteinase(s) as well as studies on its (their) physiological regulation by uterine inhibitors are in progress.

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