Analysis of endopeptidase and arylamidase enzymes in uterine fluid of oestrogen-treated rats

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Summary. Fractionation of whole uterine fluid from oestrogen-treated ovariectomized rats on Sepharose 6B showed that endopeptidase activity was distributed over two discrete peaks, with the component of lower molecular weight (II) contributing about 40% of the total activity. Arylamidase activity was likewise found over two peaks, but the lower molecular weight component (II) contributed only 7% to the total activity. Antisera were raised in rabbits to Sepharose column fractions containing endopeptidase I + arylamidase I and endopeptidase II.

Introduction

Certain enzymes of uterine origin and/or enzymes derived from the blastocyst are likely to be important in inducing changes in the uterine environment or in the blastocyst which are necessary for the successful interaction of the mammalian blastocyst with the endometrium (Pinsker, Sacco & Mintz, 1974; Enders & Schlafke, 1974; Sherman & Wudl, 1976; Denker, 1977; Kubo, Spindle & Pedersen, 1981). Proteases which are present at high levels in the uterine epithelial cells and uterine secretions during the pre-attachment stage of pregnancy include an arylamidase which hydrolyses leucine β -naphthylamide (mouse: Bergström, 1972; rabbit: van Hoorn & Denker, 1975; Denker, 1977) and an endopeptidase which acts on the specific substrate *N*-acetyl tetra-L-alanine (rat: Joshi & Murray, 1974; Rosenfeld & Joshi, 1977). The endopeptidase, cathepsin D, has also been shown to be present in high concentrations in uterine epithelial cells during early pregnancy (rat: Elangovan & Moulton, 1980) and a chymotrypsin-like amidase has been reported in uterine flushings from ovariectomized animals receiving oestrogen and progesterone (mouse: Hoversland & Weitlauf, 1978).

Detailed information on the enzymes present in uterine secretions and the raising of antibodies against them should be particularly valuable for studies on the relevance of enzymes of uterine and blastocyst origin in initiating implantation (Denker, 1977). One approach is to administer the antisera intraluminally to the uterine horns of pregnant rats and to determine their effects on implantation (Rosenfeld & Joshi, 1977).

In this study an attempt was made to characterize and purify arylamidase and endopeptidase and to raise antisera against the purified enzymes.

Materials and Methods

Treatment of animals and collection of uterine fluid

Adult female Wistar rats (approximately 250 g body weight) with regular oestrous cycles were ovariectomized through a small incision on each flank. The animals were rested for 2 weeks

after the operation and vaginal smears were taken for 6–8 days to check on constancy of pattern.

The ovariectomized animals were given an injection of oestradiol (0.5 µg/200 g body wt) on each of 3 days and the fluid in the uterine horns was collected by aspiration at laparotomy on the morning of the 4th day, and stored until required. This fluid had a high level of activity of both enzymes and was used to study the elution properties of the enzymes on the gel columns.

Gel chromatography and assay of enzyme activities

The Sephadex G-150 (Pharmacia, particle size 40–120 µm, bed dimensions 58 × 1.6 cm) column was equilibrated with 0.5 M Tris–HCl buffer, pH 7.1 at 2°C. The uterine fluids from 4 rats were pooled (giving approximately 4 mg total protein) and applied to the column. The column was eluted with Tris–HCl buffer in an ascending mode at a flow rate of 0.13 ml/min and fractions (2 ml) of column eluate were collected. These fractions were assayed for endopeptidase and arylamidase activity.

The Sepharose 6B (Pharmacia, bed dimensions 54 × 1.6 cm) column was equilibrated with 0.01 M phosphate–0.3 M-NaCl, pH 7.1 at 2°C. Again each loading to the column consisted of the pooled uterine fluids of 4 rats. The column was eluted with phosphate–NaCl buffer in an ascending manner at 0.1 ml/min and 2 ml fractions were collected. Seven separate runs representing the uterine fluids from 28 rats were carried out.

Endopeptidase activity in the fractions was measured by a method based on that described by Joshi, Yaron & Lindner (1970). The sample (0.1 ml) was treated with an equal volume of the substrate, *N*-acetyl tetra-L-alanine (0.17 mg/ml), in 0.1 M-Tris–HCl buffer, pH 7.1, containing 0.094% (w/v) CaCl₂·2H₂O and 0.036% (w/v) tetra-sodium pyrophosphate. The mixture was incubated at 37°C for 1 h. After cooling, ninhydrin reagent (0.8 ml) was added and after thorough mixing the solution was heated at 90°C for 20 min. It was rapidly cooled and the absorbance measured at 600 nm. The ninhydrin reagent was prepared by mixing equal volumes of ninhydrin (0.4 g/ml) in methyl cellosolve and stannous chloride (1.6 mg/ml) in 0.2 M-citrate buffer, pH 5.0. Sample blanks were prepared using Tris-HCl buffer in place of the substrate solution, and assay blank and reagent blank solutions were also included.

Arylamidase activity in the fractions was determined by a method based on the fluorescence of β-naphthylamine. The sample (0.1 ml) was treated with an equal volume of substrate, L-leucyl-β-naphthylamide (0.2 mg/ml) in 0.1 M-phosphate buffer, pH 7.1. The mixture was incubated at 37°C for 1 h. After cooling, 2 N hydrochloric acid (0.1 ml) was added and the solution thoroughly mixed. An aliquot (0.05 ml) was diluted with distilled water (1.55 ml) and the fluorescence emitted at 404 nm was measured at an excitation wavelength of 276 nm.

Raising of antisera and testing by immunodiffusion technique

Fractions from the Sepharose 6B column containing endopeptidase activity ± arylamidase activity (depending on the separation achieved above) were pooled and diafiltered using a UM-2 membrane (Amicon Corp., Lexington, U.S.A.) at 172.5 kPa. The diafiltered samples were freeze-dried and this material was dissolved in sterile 0.154 M-NaCl to give a concentration of 2 mg/ml. A sample (0.5 ml) of this solution was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly into an adult male rabbit (Day 1 of immunization schedule). The same material was injected on Day 8. On Days 14, 15 and 16, 0.2 ml injections of alum-precipitated protein in 0.066 M-phosphate buffer, pH 6.8 (prepared from a solution of 2 mg freeze-dried material/ml distilled water) were given (Peplow, Breed & Eckstein, 1974). Beginning on Day 29 the above sequence of injections was repeated and blood (approximately 20 ml) was collected from a peripheral ear vein on Day 49 (Hafez, 1970). The sera were stored frozen until required.

Sera were tested for immunological reactivity by the double immunodiffusion method (Clausen, 1971). The outer wells cut in the agarose gel (1% w/v in barbitone 0.05 M, pH 8.4) were filled with rabbit sera (5 μ l) while the centre well was filled with undiluted uterine fluid or reconstituted fractions (25 μ l). The samples were left at room temperature for 6 days and the gels subsequently stained with Amido black.

Results

Fractionation of uterine fluid enzymes

As shown in Text-fig. 1, arylamidase activity was contained within a single peak over the fractions 28–46 from the Sephadex column, while over the same range of fractions endopeptidase activity was present in two peaks which were not well separated.

The fractionation pattern on Sepharose 6B (Text-fig. 2) was strikingly different.

Text-fig. 1. Fractionation of rat uterine fluid on Sephadex G-150. The sample consisted of the pooled fluids from 4 ovariectomized rats treated with oestradiol. The sample blanks in the endopeptidase assay provided a measure of total protein.

Text-fig. 2. Fractionation of rat uterine fluid on Sepharose 6B. Each loading to the column consisted of the pooled fluids from 4 ovariectomized rats treated with oestradiol. EI and EII are peaks of endopeptidase activity, while AI and AII are peaks of arylamidase. The vertical arrow shows the position of peak maximum for blue dextran marker D. The hatched areas represent the fractions which were pooled for the raising of antisera.

Table 1. Endopeptidase and arylamidase activities in rat uterine fluid fractionated on Sepharose 6B

Sample no.*	Endopeptidase†			Arylamidase†		
	EI	EII	EII	AI	AII	AII
			(EI + EII)			(AI + AII)
1	1.37	0.84	0.38	1.04	0.08	0.07
2	1.09	0.58	0.35	1.48	0.11	0.07
3	0.52	0.42	0.45	0.62	0.07	0.10
4	0.99	0.57	0.37	0.75	0.06	0.07
5	0.80	0.70	0.47	0.66	0.04	0.06

* Each sample applied to the column consisted of the pooled uterine fluids from 4 ovariectomized rats given oestradiol.

† A measure of activity is provided by determining areas of peaks identified by assaying the fractions (see Text-fig. 2).

Endopeptidase activity was present in two well defined peaks (EI, fractions 20–29; EII, fractions 32–46) and arylamidase was also located within two distinct peaks (AI, fractions 20–28; AII, fractions 38–45). The peak maxima for EI and AI were delayed in comparison to the peak maximum of a blue dextran marker, and both of these enzymes have therefore been considered not to be excluded by the gel. The same separation of the endopeptidase and arylamidase enzymes on Sepharose 6B was found for 7 different samples of pooled undiluted uterine fluid. The percentage of total endopeptidase activity found in peak EII was constant at 40%, while peak AII contained 7% of the total arylamidase activity (Table 1).

Raising and testing of antisera to fractions of uterine fluid

Fractions 20–24 and 32–37 from the Sepharose column were pooled and used for production of antisera in rabbits. Rabbit 1 was immunized with fractions 20–24 of uterine fluid, Rabbit 2 with fractions 32–37, while Rabbit 3 was not immunized and served as a control.

Testing of the undiluted uterine fluid against the serum obtained from Rabbit 1 revealed 3 immunoprecipitin lines (one strong and two weak lines), while against the serum from Rabbit 2 one intense line was observed (Text-fig. 3a). No reaction was found when tested against the serum of Rabbit 3. A spur was seen where one of the strong lines met the pair of weak ones (presumably brought about by partial absorption of the weak precipitin lines). Reacting the isolated fractions against the rabbit sera produced the patterns shown in Text-figs 3b and 3c. Fractions 20–24 produced one weak line against Rabbit 2 serum and gave one intense line plus two weak lines, close together, against the serum from Rabbit 1. Fractions 32–37 produced one intense line against the serum from Rabbit 2 and 2 weak lines against the serum of Rabbit 1. In

Text-fig. 3. Immunodiffusion patterns of rat uterine fluid in agarose gel. In all gels, wells 1 and 2 were filled with serum from Rabbit 2, wells 3 and 4 with serum from Rabbit 1, and wells 5 and 6 with serum from Rabbit 3. The centre well contains (a) whole uterine fluid, (b) fractions 20–24 from Sepharose 6B chromatography, and (c) fractions 32–37 from Sepharose 6B chromatography. The thicker precipitin lines indicate an intense reaction and the thinner ones a weak reaction.

both patterns a spur was present where the precipitin line against Rabbit 2 serum met the two weak lines formed against Rabbit 1 serum.

The antisera raised in Rabbits 1 and 2 caused inhibition of endopeptidase activity in whole uterine fluid when incubated for 30 min at room temperature and tested against the substrate. The controls included reaction tests of the sera from all 3 rabbits and uterine fluid separately with the substrate.

Discussion

Joshi *et al.* (1970) found only a single endopeptidase component in whole rat uterine fluid, but the present study, using a higher resolution column, revealed two different sized components for endopeptidase and for arylamidase although the contribution of the smaller sized component to the total arylamidase activity was very small. The endopeptidase activity in the sample was determined against the same substrate as used originally by Joshi *et al.* (1970), who reported on the basis of testing against a wide range of different substrates that the enzyme splits internally between adjacent alanyl residues and that no hydrolysis of substrates used for measuring carboxypeptidase activity occurred. With regard to the arylamidase no attempt was made here to classify the enzyme as being type A or B, although this could have been performed using different substrates or testing in the presence of known inhibitors.

The fractionation on Sepharose 6B permitted choice of fractions to prepare an anti-(EI + AI) serum (using fractions 20–24), and also an antiserum to EII with little or no contaminating arylamidase (using fractions 32–37). The amount of freeze-dried material obtained was sufficient only to immunize one rabbit against the earlier fractions and one rabbit against the later ones. The immunization schedule used with the male rabbits was shown to be successful when the bleedings were tested by an immunodiffusion technique against the samples used to inject the rabbits. The immunopatterns showed that fractions 20–24 contained three components. Since lines closest to the centre well represent components with the greatest molecular weight, the intense reaction line probably corresponded to the larger sized arylamidase and endopeptidase. Reaction against the heterologous antiserum showed that fractions 32–37 contained two components, and a monospecific antiserum was probably raised in the rabbit immunized against these fractions. The partial absorption of immunoprecipitin lines leading to spurs suggests partial identity of the antigens used for preparing the antisera. Further interpretation of the patterns will require more information on the behaviour of the components of the fractions in other chromatographic systems and in immuno-electrophoresis.

Although in this study the high molecular weight endopeptidase EI has not been obtained free of arylamidase AI, it has been possible to obtain the smaller molecular weight endopeptidase EII (free of AI) by choosing the appropriate fractions from the Sepharose column. The lack of low molecular weight proteins in these fractions (20–24 and 32–37) was confirmed by disc electrophoresis in polyacrylamide gel (7% w/w) (Davis, 1964), when only one intense band was found and this was situated in the sample gel close to its junction with the spacer gel. The separation found for the enzymes could be significant in view of the involvement of high molecular weight proteins in blastocyst development and implantation (Dunbar & Daniel, 1979). Joshi & Murray (1974) raised an antiserum against rat uterine endopeptidase by using an anti-(whole uterine fluid) serum absorbed with serum protein. Although it will be necessary to attempt separation of endopeptidase I and arylamidase I before further study of the involvement of these enzymes in blastocyst-endometrial relations, the present results show that antisera can be raised successfully against fractions of rat uterine fluid.

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