Inhibition of human sperm acrosin by synthetic agents

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Summary. Twenty-two synthetic proteinase inhibitors were tested for their inhibitory properties towards human acrosin. p-Nitrophenyl-p'-guanidino benzoate (NPGb) was the most effective (K_i value of 1.5×10^{-8} M), producing a non-competitive type of inhibition in contrast to all other inhibitors which showed a competitive type of inhibition. The Michaelis constant for human acrosin on BAEE at pH 8.1 was calculated to be 4.25×10^{-5} M.

The neutral acrosomal proteinase, acrosin, has been found in the spermatozoa from many species (see reviews by McRorie & Williams, 1974; Zaneveld, 1975). Acrosin appears to be biochemically and immunologically unique. The addition of naturally occurring and synthetic inhibitors of acrosin to capacitated spermatozoa prevents fertilization (Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Robertson & Williams, 1970; Zaneveld, Robertson, Kessler & Williams, 1971; Newell, Polakoski & Williams, 1972; Suominen, Kaufman & Setchell, 1973; Miyamoto & Chang, 1973). Synthetic acrosin inhibitors also prevent the fertilizing capacity of ejaculated (vaginal) spermatozoa in contrast to the natural inhibitors, and the synthetic inhibitors may therefore have practical applications as non-hormonal contraceptive agents.

The few synthetic proteinase inhibitors that have been tested on human acrosin include tosyl-lysine chloromethyl ketone (TLCK), tosylamide phenylethyl chloromethyl ketone (TPCK) and diisopropyl fluorophosphate (DFP) (Zaneveld, Dragoje & Schumacher, 1972; Fritz *et al.*, 1972; Gilboa, Elbana & Rigbi, 1973). In the present study, therefore, several synthetic agents were evaluated and compared for their inhibitory properties towards human acrosin.

Human acrosin was partly purified from spermatozoa as described by Zaneveld *et al.* (1972). The enzyme was stored at pH 3.0 in 0.001 M-HCl at 4°C unless used immediately. Acrosin activity was measured using benzoyl arginine ethyl ester (BAEE) as substrate in 0.05 M-borate buffer, pH 8.1, containing 0.05 M-CaCl₂ (Schwert & Takenake, 1955). The change in optical density was recorded at 253 nm with a Gilford spectrophotometer, model 240, at 23°C. The concentration of BAEE was varied from 0.0125 mM to 0.6 mM although the standard concentration was 0.5 mM. The final volume was always 3.0 ml. The activity of the enzyme was expressed as μ mol BAEE hydrolysed per min (mi.u.).

Compounds for analysis were obtained by contacting several companies and requesting all available synthetic proteinase inhibitors. Inhibitor assays were performed by incubating 50 μ l of approximately 20 mi.u. acrosin with 50 μ l inhibitor at various concentrations in 0.9 ml of the borate buffer for 20 min before addition of the mixture to the substrate. For assay of the speed of interaction between enzyme and inhibitor, shorter incubation periods were occasionally used. Although the enzyme was initially at pH 3.0, the addition of inhibitor solution and buffer immediately raised the pH of the incubation mixture to 8.0. Two controls were routinely made: (1) the activity of the acrosin incubated with buffer was measured, and (2) the inhibitor solutions were added to BAEE since a slight change in optical density was sometimes caused by the inhibitor alone.

Table 1. Effect of synthetic agents on the hydrolysis of BAEE by human acrosin

Compound	Source	Conc. giving 50% inhibition (M)
p-Nitrophenyl-p ¹ -guanidino benzoate (NPGB)	Nutr. Biochem. (Cleveland, Ohio, U.S.A.)	1.2×10^{-8}
3,8-Di(m-aminophenyl diazoamino)-5-ethyl-6-phenyl-phenanthridinium dihydrochloride hydrate (M & B 4596)	May & Baker (Rhodia Inc., New York, U.S.A.)	2.3×10^{-6}
p-Aminobenzamidine (ABD)	Sigma (St Louis, Missouri, U.S.A.)	2.0×10^{-5}
p-[m-(m-Fluorosulphonyl phenyl ureido) phenoxy ethoxy] benzamidine (FPB)	Cyclo (Los Angeles, California, U.S.A.)	2.5×10^{-5}
N- α -p-tosyl-L-lysine chloromethyl ketone (TLCK)	Sigma	3.5×10^{-5}
p-Amidino phenyl pyruvic acid hydrochloride, hemihydrate (APPA)	Cyclo	3.6×10^{-5}
2-2'-Dibromopropamidine isethionate	May & Baker	4.0×10^{-5}
Pentamidine isethionate	May & Baker	6.0×10^{-5}
Propamidine isethionate	May & Baker	7.5×10^{-5}
Cholic acid (Na-salt)	Sigma	6.3×10^{-4}
Benzamidine hydrochloride (BH)	Fisher (Chicago, Illinois, U.S.A.)	6.7×10^{-4}
2-Hydroxy stilbamidine isethionate	May & Baker	7.5×10^{-4}
Thiocarbamide	Aldrich (Milwaukee, Wisconsin, U.S.A.)	8.0×10^{-4}
1,10-Phenanthroline	Aldrich	8.2×10^{-4}
N-aminobenzamidine	Eastman (Rochester, New York, U.S.A.)	8.6×10^{-4}
Benzamidine HCl, hydrate	Aldrich	9.0×10^{-4}
6-Acetamido hexanoic acid	Aldrich	2.3×10^{-3}
6-Amino caproic acid	Aldrich	3.0×10^{-3}
1,4-Diaminobenzene dihydrochloride	Cyclo	5.5×10^{-3}
Amicarbalide isethionate	May & Baker	5.7×10^{-3}
Stilbamidine isethionate	May & Baker	9.0×10^{-3}
Urea	Fisher	1.0×10^{-2}

The inhibitors tested were effective at concentrations ranging from 10^{-2} to 10^{-8} M (Table 1). The most active compound was NPGB. The compounds M & B 4596, p-amino benzamidine, FPB, TLCK, APPA, 2-2'-dibromopropamidine isethionate, pentamidine isethionate and propamidine isethionate were also effective inhibitors of human acrosin, but were at least 200–3000 times less so than NPGB which inhibited human acrosin instantaneously (i.e. without requiring preincubation) even at concentrations as low as 1×10^{-7} M. The high affinity of human acrosin for NPGB suggests that the enzyme has a serine group at its active site (Shaw, 1974).

Double reciprocal (Lineweaver–Burk) plots of initial reaction rates against the varied concentrations of BAEE showed that the intersection of the TLCK-acrosin and the acrosin control curves was at the ordinate whereas the NPGB-acrosin and the acrosin control curves intersected at the abscissa (Text-fig. 1), indicating that TLCK behaves as a competitive inhibitor while NPGB shows a non-competitive type of inhibition. The other inhibitors tested also bound competitively with human acrosin, except perhaps FPB at low substrate concentrations. Competitive interaction of rabbit acrosin with TLCK was previously reported by Connors, Greenslade & Davanzo (1973) and with p-aminobenzamidine by Stambaugh & Buckley (1972). Although many of the synthetic inhibitors bind irreversibly to acrosin, presumably a useful property for contraception for avoiding removal in the female genital tract, non-competitiveness is probably additionally advantageous since the formation of a complex with the enzyme would be less susceptible to interference from surrounding molecules.

The K_i values for the interaction of acrosin with NPGB and TLCK were determined from Dixon plots by varying the concentration of inhibitor. The K_i for NPGB was 1.5×10^{-8} M and that for TLCK was 3.1×10^{-4} M. The latter value is very similar to the K_i of 5×10^{-4} reported for TLCK with

Text-fig. 1. Double reciprocal (Lineweaver-Burk) plot for the inhibition of human acrosin by NPGB and TLCK using BAE concentrations from 0.0125 mM to 0.4 mM. ○, No inhibitor; Δ, NPGB (5×10^{-8} M); ●, TLCK (5×10^{-4} M). Initial velocities were determined spectrophotometrically at 253 nm as described in the text. Inset: plot of the acrosin activity against the substrate concentration. Note the decrease in reaction rate at high substrate levels.

rabbit acrosin by Connors *et al.* (1973). The K_i for NPGB was the lowest of the inhibitors tested, further indicating the highly active complex formation between NPGB and acrosin.

The apparent Michaelis constant for human acrosin, using BAE concentrations ranging from 0.0125 mM to 0.4 mM, was 4.25×10^{-5} M (Text-fig. 1). This K_m value for human acrosin is in good agreement with that of purified boar acrosin ($K_m = 4.8 \times 10^{-5}$ M: Polakoski & McRorie, 1973) and rabbit acrosin preparations ($K_m = 6.4 \times 10^{-5}$ M: Connors *et al.*, 1973). In contrast to these observations, a value of 5.2×10^{-6} was obtained for rabbit acrosin by Stambaugh & Buckley (1972). In a Lineweaver-Burk plot, human acrosin showed a linear curve over a wide range of substrate concentrations (Text-fig. 1). A decrease in reaction rate at concentrations above 0.4 mM occurred due to substrate inhibition. This is further illustrated by the inset of Text-fig. 1; the reaction rate of acrosin with substrate increased rapidly, levelled off and finally decreased at high substrate concentrations.

The present results indicate that NPGB has a greater potential as an antifertility agent than any of the other inhibitors tested so far, and it is ultimately hoped that highly specific inhibitors can be found. Experiments are in progress to determine the specificity of NPGB and other inhibitors towards human acrosin as compared with different human proteinases, such as pancreatic trypsin, which are known to be inhibited by such agents.

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