N-α-BENZOYL ARGININE ETHYL ESTER HYDROLYSIS BY PORCINE PLASMIN. A KINETIC STUDY OF DIETHYLENEGLYCOL INFLUENCE ON THIS HYDROLYSIS.

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1. Introduction

Plasmin, apart of its great physiological importance as a fibrinolytic agent, stands along with trypsin, chymotrypsin and other as a serine protease; it was also demonstrated to perform esterolysis through an acyl-enzyme intermediate [1]. On the other hand, for preparation or conservation purposes, plasmin and its zymogen, plasminogen, have often been associated with glycols, v.g. glycerol [2–5], mainly on an empirical basis. The present work aims to examine the influence of a glycol, dietylene glycol, on the hydrolytic activity of porcine plasmin toward benzoyl arginine ethyl ester, and, eventually, help to shed a new light on the nature of this influence.

2. Materials and methods

Porcine plasminogen was prepared by the Deutsch and Mertz method [6], as modified by Liu and Mertz [7] from a euglobin solution. Citrated porcine plasma (obtained at the Institut National de Recherche Agronomique, C.N.R.Z. Jouy-en-Josas) was first defibrinated with a 0.1 volume of $CaCl_2$ 4%, according to Abiko [8]; once fibrin had been removed, euglobins were precipitated in 50% $(NH_4)_2\,SO_4$ final solution with saturated $(NH_4)_2SO_4$, at room temperature. A euglobin solution was made out of the smallest possible volume of 'saline' buffer and pass through a Sepharose-Lysine column ('Saline' buffer is 0.01 M phosphate + 0.15 M NaCl, pH 7.4). Plasminogen, after repeated column washings with 'saline buffer', was rinsed out with ε-ACA 0.2 M, then dialysed overnight against 'saline buffer' to eliminate ε-ACA.

The last step consisted in a separation chromatography on Biogel A 1.5, that gave two peaks (fig.1), respectively 17 and 5.8 S on analytical ultracentrifugation. Peak I could not be activated by urokinase, but peak II showed azocaseinolytic activity when

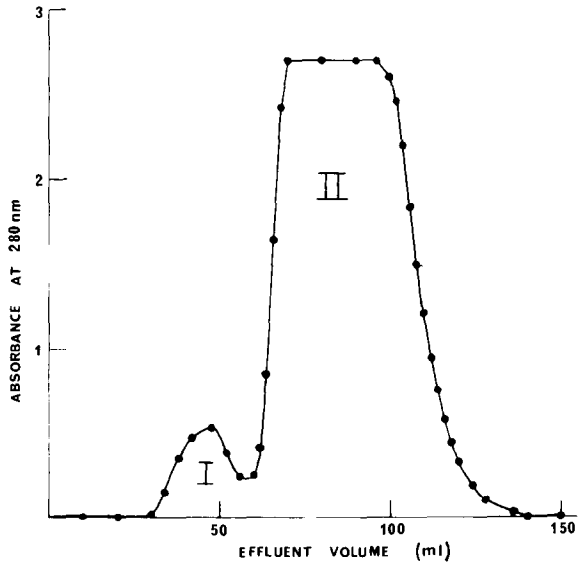

Fig.1. Chromatography of 'crude' plasminogen on Biogel A 1.5. Sample volume: 10 ml; column: 30 × 2.5 cm; flow rate: 25 ml/hr; eluent: 'saline' buffer, pH 7.4; temperature: 4°C.

Abbreviations: BAEE, *N*-α-benzoyl-arginine ethyl ester HCl; DEG, diethylene glycol; NPGB, *p*-nitrophenyl-*p*-guanidinobenzoate HCl; ε-ACA, ε-aminocaproic acid. CTA units: committee on Thrombolytic agents units.

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activated by urokinase. As shown by electrophoresis, at pH 9.1, on polyacrylamide gel [9], peak II would be a plasminogen with multiple molecular forms; similar cases have also been found with plasminogens from other species [10–13]. Peak I was not identified.

Thus prepared, plasminogen (7.5 mg/ml), peak II, was kept frozen. All chromatographies and dialysis were performed at 4°C.

Standard plasmin was kindly supplied by the American National Red Cross (National Heart Institute); it contains 10 CTA units/ml in glycerol 50% (list No. 03711, lot 8F897). Azocasein was a Sigma Chemical Corporation product, lot 71c-2240; a stock solution was prepared by dissolving 500 mg azocasein in 10 ml $NaHCO_3$ 1%, at 60°C, under agitation, and diluted with water up to 20 ml to yield a 2.5% final solution. Azocasein stock solution was kept frozen.

Human urokinase was purchased from Leo Pharmaceutical Products, Denmark (Urokinase Reagent. 10 000 Ploug units/ampul, arbitrary units as described by Ploug and Kjeldgaard) [14]. *N*-α-benzoyl-L-arginine ethyl ester HCl is a Fluka (Puriss) product, *p*-nitrophenyl-*p*-guanidinobenzoate HCl, a Nutritional Biochemicals Corp. product, diethylene glycol a E. Merck product.

2.1. *Plasminogen activation procedure*

Before pH-stat measurements, 0.1 ml plasminogen (7.5 mg/ml), 0.1 ml urokinase (2000 Ploug units/ml) and 0.8 ml NaCl 0.15 M are mixed together and incubated at 37°C pH 7.4 for 60 min, and afterwards kept on ice. At the end of the 60 min incubation time, plasmin concentration is not varying significantly as respect to time [15].

2.2. *Enzyme titration*

Active plasmin content of incubation mixture was measured on a Cary 15 spectrophotometer by the NPGB 'burst' assay, following Chase and Shaw [16]. A mol. wt of 75 400 and a $E\frac{\%}{cm}_{1}$ of 17.0 were assumed for plasmin [17].

In order to compare our plasmin preparation with international standards, actually to CTA units, a standard curve was prepared from the proteolysis of azocasein by standard plasmin. Our plasmin preparation was afterwards compared to that standard curve and its specific activity was expressed in CTA units/mg. The Charney and Tomarelli method was followed for the proteolysis of azocasein [18].

2.3. *pH-Stat measurements*

Activity titrations were carried out on a Radiometer Titrator. For the kinetic parameters determinations, the following experimental conditions were adopted: under nitrogen atmosphere, at pH 8.0, 37°C, a constant volume of plasmin (2.0×10^{-8} M plasmin active sites final concentration, as measured by the NPGB 'burst' assay, or 0.3 CTA units as referred to plasmin standard curve) is mixed with variable volumes of a 8.0×10^{-2} M BAEE stock solution (final BAEE concentration ranging from 0.32 to 1.6×10^{-2} M). Reaction mixture is completed to 5.0 ml with NaCl 0.15 M. BAEE stock solution was prepared in NaCl 0.15 M. In assays with DEG, the glycol was added before final volume adjustment. Blanks and corrections were made for BAEE spontaneous hydrolysis with and without DEG; urokinase did not exhibit esterolytic activity toward BAEE in these experimental conditions.

Every experimental point on figures is the mean value of two or more measurements. All kinetic parameters were obtained by applying an unweighted least-square treatment to the data.

3. Results

3.1. *Kinetic parameters*

For substrate concentrations ranging from 0.32 to 1.6×10^{-2} M. the double reciprocal plot gave the following results: $K_{M_{app.}} = 1.97 \times 10^{-2}$ M and $k_{cat_{app.}}$ $(= V_{max}/E) = 18\ min^{-1}$ (fig.2a). These values are liable to some imprecision because they were obtained at substrate concentrations lower than K_M. At higher substrate concentrations, the Lineweaver-Burk plot shows inhibition that could be attributed to an excess of substrate (fig.2b).

3.2. *Hydrolysis modification by DEG*

At given BAEE and plasmin concentration hydrolysis initial rates were measured in presence of variable DEG concentrations. The glycol proved to enhance hydrolysis initial rates. Table 1 illustrates this enhancement at different apparent pHs; it seems to get more important toward acid pH.

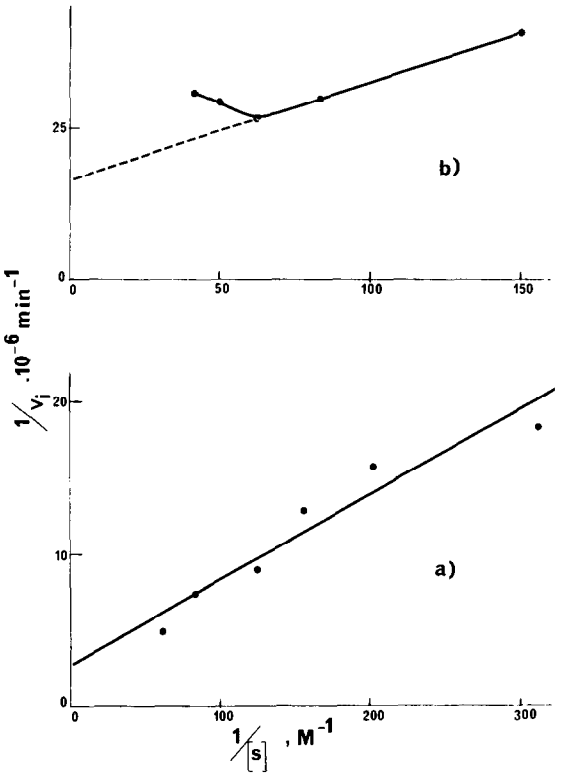

Fig.2. Double-reciprocal (Lineweaver-Burk) plots for BAEE hydrolysis by plasmin, obtained by an unweighted least-square fit, at pH 8.0 and T° = 37°C. (a) Plasmin is 2.0 × 10^{-8} M or 0.3 CTA units, BAEE concentration ranges from 0.32 to 1.6 × 10^{-2} M. Correlation coefficient (R) = 0.96 and slope error (S_b) = 0.79. (b) Plasmin is 1.0 × 10^{-8} M or 0.15 CTA units, BAEE concentration ranges from 0.8 to 2.4 × 10^{-2} M; dotted lines are hypothetical for no inhibition.

Table 1
% increase of initial rates of BAEE hydrolysis by plasmin, at different pHs apparent and DEG concentrations

$$\text{where \% increase} = \frac{(v_{i_{\%DEG}} - v_{i_{0\%DEG}}) \times 100}{v_{i_{0\%DEG}}}$$

DEG % (v/v)	% increase of initial rates pH 6	pH 7	pH 8
4	44	38	33
10	206	100	56
20	415	179	100

3.3. *Kinetic parameters modifications by DEG*

On fig.3, K_M and k_{cat} and k_{cat}/K_M ratio are plotted versus DEG concentration. At low DEG concentration (below 2%) occurs a phase where k_{cat} decreases. But over 2% DEG concentration, k_{cat} seems to be increasing. K_M drops abruptly as a function of DEG concentration, and afterwards tends to level off. $k_{cat}/K_{M_{app.}}$ increases linearly.

4. Discussion

Keeping in mind the possible restrictions affecting the results precision (BAEE concentration range), we attempt to comment their main features.

Since DEG influence could be suspected of being nucleophilic, Bender's simplest equation was examined [19]:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES'$$

$$+ P_1 \quad \begin{matrix} \xrightarrow{k_3 W} E + P_2 \\ \xrightarrow{k_4 N} E + P_3 \end{matrix}$$

where E is free enzymes, ES, Michaelis complex (enzyme–substrate complex), ES',acyl-enzyme, P_1, first released product (alcohol moiety), P_2, acyl-enzyme hydrolysis product (acid moiety), and P_3, deacylation product through the nucleophilic agent.

In this scheme, we should assist to modifications of K_M and k_{cat}, when the nucleophile concentration increases; but k_M is never expected to be lowered when there is nucleophilic attack [20–22]. In our experiments, K_M does decrease as much as 4 to 5 times from its initial value. This lowering and the linear increase of k_{cat}/K_M were compelling to reject a simple nucleophilic competition, and to suggest that DEG intervention could be located elsewhere in the mechanistic pathway, possibly prior to the acylation step (k_2).

Actually, Fink and Bender [23] have observed in the papain-catalysed hydrolysis of nitrophenyl esters that increasing pentanol concentration reduced K_M and k_{cat}, and increased k_{cat}/K_M. They supposed

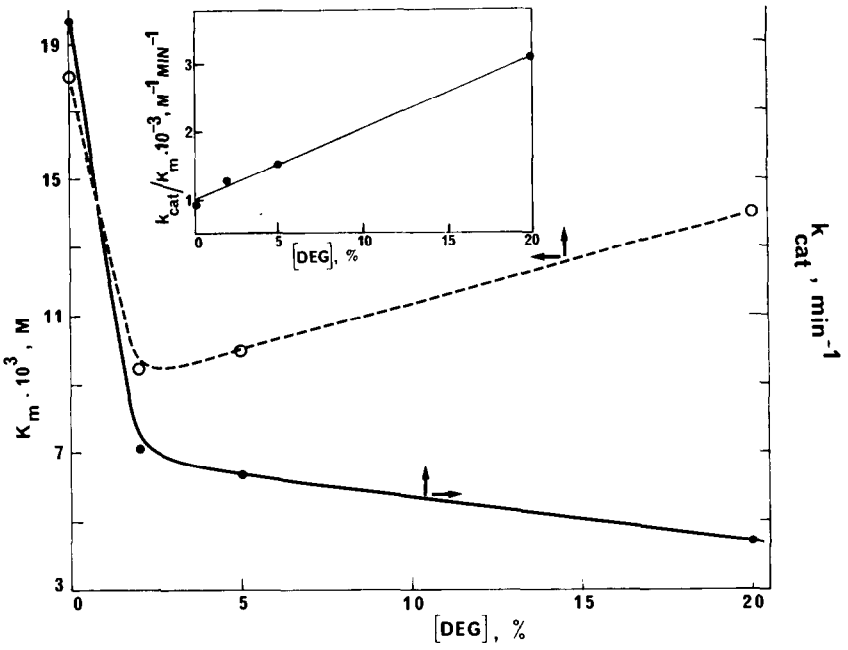

Fig.3. Variations of K_m, k_{cat} and k_{cat}/K_M ratio, as a function of diethylene glycol concentration. Unweighted least-square treatment of k_{cat}/K_M ratio vs DEG concentration yields a correlation coefficient = 0.99 and slope error = 0.06.

that because of its long hydrocarbon backbone (longer than methanol), pentanol could act not only as a nucleophilicage (like methanol), but also would bind to the Michaelis complex, or even to free enzymes to form a ternary complex. At low DEG concentrations, two effects could be considered: one where the glycol competes with water for the nucleophilic site of acyl plasmin, and the other, where it binds to ES or E, thus modifying k_2 and/or K_s, as reflected by K_M lowering. At higher glycol concentrations, the binding effect would outgrow the other one, hence the direction reversal of k_{cat}. In terms of kinetic mechanism, K_M levelling off as well as the reversing of sign of k_{cat} could indicate a change between the acylation and the deacylation steps, as the rate-limiting step, a possibility which is likely to occur when those steps are of comparable magnitude ($k_2 \sim k_3$) [24]. On the other hand, DEG binding to the protein may induce conformational changes [25] finally increasing the rate of catalysis.

Another phenomenon which could be implicated is a non-productive binding of the substrate to the enzyme. Such a binding was reported in papain/BAEE system [26] and papain or ficin/nitrophenyl esters systems [27]. As a matter of fact we have observed inhibition by excess of BAEE in our experiments; BAEE might be susceptible also of non-productive binding to plasmin. If DEG binds in the vicinity of the active center, it could perturb the substrate position; moreover, if it does have a similar or a greater affinity than BAEE for the non-productive binding site, it would then favor a catalytically efficient orientation of the substrate, by eliminating probabilities of BAEE non-productive binding.

Even while retaining the thesis of DEG binding to plasmin, there could be additional factors such as solvent effects. Just to mention them: dielectric constant changes might reduce plasmin tendency to polymerize; they could also be related to the entropy changes of the ES complex (Laidler and Ethier) [28]; finally, a more complex solvent effect on plasmin conformation is possible, as shown with β-lactoglobulin in hydro-alcoholic solutions [29].

Through kinetic experiments results, we have been led to make a few comments about the possible nature of DEG influence on plasmin-catalysed hydrolysis of

BAEE. They were intended to help the purpose of a better understanding of the empirical use of glycols for plasmin preparation and conservation.

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