PREPARATION AND PROPERTIES OF ORGANOPHILIC TRYPSIN MACRO-INHIBITORS: DIAMIDINO-α,ω-DIPHENYLCARBAMYL-POLY(ETHYLENE GLYCOL)

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1. Introduction

Aqueous two-phase systems are largely used to purify biological macromolecules [1]. One of the most frequently used systems is obtained by mixing aqueous solutions of poly (ethylene glycol)* (PEG) and dextran. Better results were obtained in this system upon introduction of poly (ethylene glycol) carrying covalently bound charged groups [2]. Conditions for obtaining optimal separation of glycolytic enzymes of yeast lysate by this procedure have been investigated by Johansson et al. [3]. However, good separation requires the use of counter-current distribution.

It would be of interest to investigate how specific ligands bound to PEG, affect the partition of proteins. For this purpose, p-aminobenzamidine PAB, a strong inhibitor of trypsin, was attached to PEG of different molecular weights. The preparation and some of the properties of these new trypsin inhibitors are described here.

2. Materials and methods

PEG 200 was obtained from Koch-Light. PEG 2000 and PEG 9000 were obtained from Fluka. Trypsin, PAB and DL-benzoylarginine *p*-nitroanilide (BAPNA) were supplied by Sigma.

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PEG 200, 2000 and 9000-α,ω-dichloroformiates were prepared at room temperature by reaction of phosgene with PEG in toluene [4].

Absorption spectra were recorded with a Unicam SP 800 spectrophotometer. Extinction coefficients were determined using a Zeiss PMQ II spectrophotometer. Infrared spectra were recorded with a Perkin–Elmer model 257 spectrometer. Melting points were determined on a Leitz apparatus and were not corrected. Refractive indexes of chromatography column effluent were determined with a Zeiss refractometer. Purity of compounds was tested by gel filtration using columns of Sephadex G 25 or G 75.

Trypsin activity was determined according to Erlanger et al. [5] and Mares-Guia and Shaw [6] using DL-BAPNA as substrate. After addition of the enzyme to the sample cuvette, the absorbance changes were recorded at 410 nm for 3–4 min; the slopes of the straight lines obtained gave the initial rates of reaction.

K_i values were determined according to Dixon [7].

2.1. *Preparation of diamidino-α,ω-diphenylcarbamyl-poly (ethylene glycol) 200, 2000 and 9000 (PEGPAB)*

p-Aminobenzamidine hydrochloride (350 mg; 2 mM) was dissolved in 20 ml of 1 M $NaHCO_3$ solution. PEG 200-α,ω-dichloroformiate (320 mg; 1 mM) was added in 5 fractions in 30 min.

After 20 hr of continuous stirring at room temperature, the mixture was adjusted to pH 3 by addition of 5 N HCl and then evaporated to dryness in vacuo. The residue was extracted with anhydrous

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methanol and the extract was concentrated to a small volume. Upon addition of ether, the product was precipitated (yield : 300 mg; 45%). The compound turned brownish without melting when heated above 280°C.

IR(KBr)cm^{-1} : 2900 (alkane), 1735 (amide I), 1680 and 1610 (amidine), 1525 (amide II), 1150 – 1050 (ether). UV : $\lambda_{max}^{H_2O}$: 270 nm: $E_{1\,cm}^{1\%} = 380$.

PEG 2000 PAB and PEG 9000 PAB were obtained in a similar way but the solution was stirred for 48 hr and the purification was carried out in the following way. The methanolic extract was concentrated to dryness and there sidue taken up in anhydrous tetrahydrofuran. Ether was added to the filtrate and the solution allowed to stand at –10°C for 2 hr. The white material obtained was collected by filtration, washed with anhydrous ether and dried in vacuo.

PEG 2000 PAB: yield : 68%; m.p. 51°C

UV: $\lambda_{max}^{H_2O} = 270$ nm; $E_{1\,cm}^{1\%} = 82$.

PEG 9000 PAB: yield : 80%; m.p. 61°C

UV: $\lambda_{max}^{H_2O} = 270$ nm; $E_{1\,cm}^{1\%} = 16.5$.

3. Results and discussion

3.1. *Inhibitor characterization*

The structure of the prepared compounds was proved by infrared spectroscopy, UV-absorption, gel chromatography and determination of inhibition constants.

First, it appears from IR and UV spectra that PAB has been covalently bound to PEG by its primary amino group. IR spectra show an absorption at 1735 and 1525 cm^{-1} assignable to > CO of a carbamate group. They also exhibit a strong absorption at 1680 and 1610 cm^{-1} assignable to the amidine function. No absorption can be detected in the 1330–1340 cm^{-1} range, showing the absence of primary aromatic amino group. UV spectra of the compounds in water show an absorption maximum at 270 nm whereas the UV spectrum of PAB exhibits a strong absorption at 290 nm (fig. 1). This blue shift is related to the substitution of the amino group of PAB.

Secondly, from the gel filtration and the melting points of the compounds, it may be concluded that the molecular weights of PEGPAB and of original

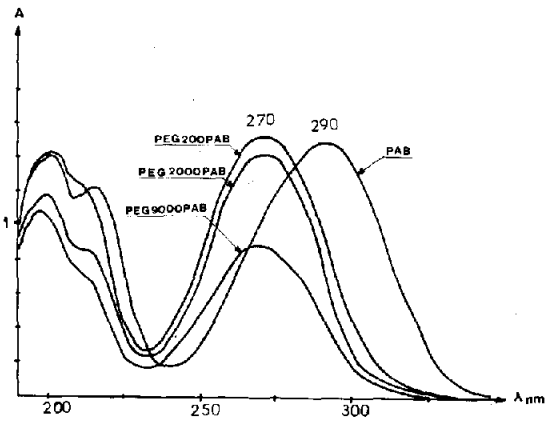

Fig. 1. Absorption spectra of PAB (17 μg/ml), PEG200PAB (62 μg/ml), PEG2000PAB (192 μg/ml) and PEG9000PAB (660 μg/ml) in aqueous solution.

PEG are almost identical. An elution diagram of PEG 9000 and PEG 9000 PAB on Sephadex G-75 column is shown in fig. 2.

Finally, the rate of hydrolysis of BAPNA by trypsin decreases sharply when PEGPAB is present in the medium indicating strong inhibition.

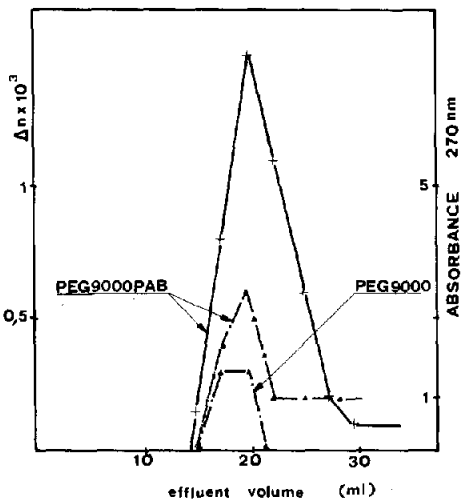

Fig. 2. Elution diagram for the PEG9000 and the PEG9000PAB on a 1 × 50 cm column of Sephadex G-75; 0.05 M phosphate buffer pH 7.0. +—+—+—+ absorbance at 270 nm; ▲—▲—▲—▲ refractive index.

3.2. *Kinetic properties of inhibitors*

For the evaluation of the K_i values of PEGPAB with DL-BAPNA as a substrate, a kinetic treatment was developed which takes into account the presence of two inhibitory sites.

The system can be described by:

$$E + S \rightleftarrows ES \rightarrow E + P$$
$$E + I \rightleftarrows EI$$
$$EI + E \rightleftarrows E_2I,$$

where the dissociation constants are:

$$K_s = \frac{(E)(S)}{(ES)} \quad (1)$$

$$K_i = \frac{(E)(I)}{(EI)} \quad (2)$$

$$K'_i = \frac{(EI)(E)}{(E_2I)} \quad (3)$$

The concentration of ES is given by the following equation:

$$p^2 \frac{K^2_s}{s^2} \frac{i}{K_i \cdot K'_i} + p\left(1 + \frac{K_s}{s} + \frac{K_s}{s}\frac{i}{K_i}\right) - e = 0 \quad (4)$$

where p is the concentration of ES, i that of I, s that of S and e the total concentration of E. Assuming that

$$4\frac{K_s^2}{s^2} \cdot e << \frac{K_i K'_i}{i}\left[1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)\right]^2 \quad (5)$$

the equation (4) can be solved easily, we get

$$p = \frac{e}{1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)} \quad (6)$$

and

$$v = \frac{V}{1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)} \quad (7)$$

Eqs. (6) and (7) are those obtained in the case of an inhibition by a mono-inhibitor. Therefore, Dixon's graphical method can be used to obtain the K_i values.

The experimental data are shown in fig. 3. The K_i values of the three PEGPAB are not very far from that of PAB itself. This may be explained by the fact

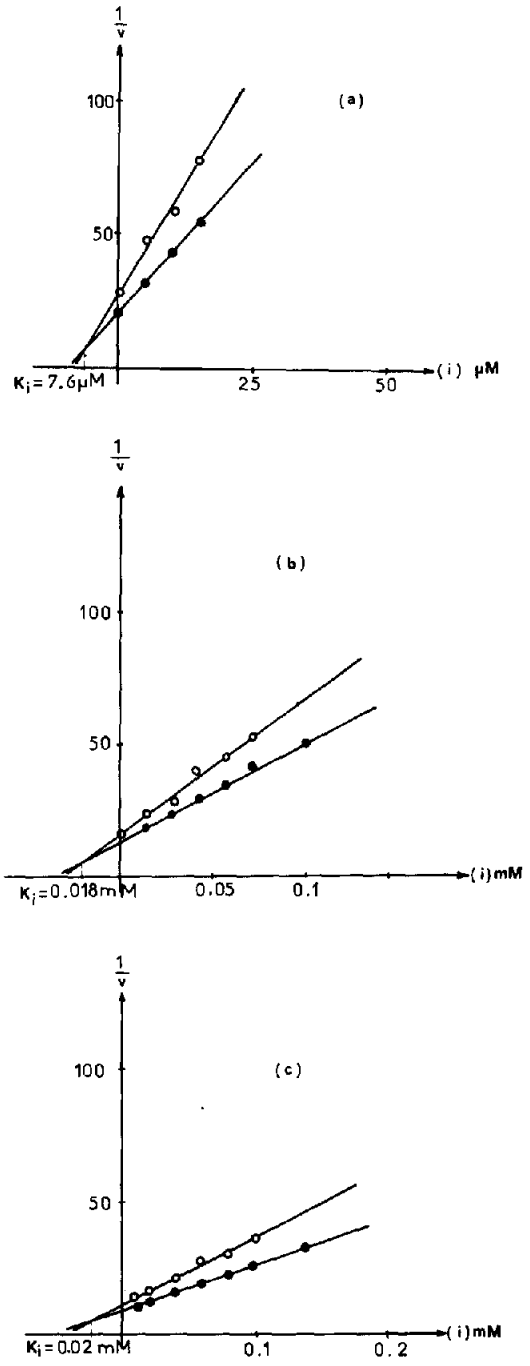

Fig. 3. The competitive inhibition of trypsin by PEGPAB: (a) PEG200PAB, (b) PEG2000PAB, (c) PEG9000PAB. The concentration of DL-benzoylarginine p-nitroanilide was 2.3×10^{-4} M in each of the upper curves and 3.3×10^{-4} M in each of the lower curves. Trypsin concentration, 7×10^{-7} M; 0.05 M Tris buffer; pH 8.15.

that the positive charge of the PAB still exists in the three new inhibitors. It is known that the effectiveness of cationic compounds as trypsin inhibitors is based to a large degree on the attraction of the positive charge to the anionic site of the enzyme (6).

Under our experimental conditions ($K_s = 10^{-3}$ M [5] and $K_i \simeq 10^{-5}$ M) in eq. (5) becomes

$$K_i' \gg 10^{-7} \text{ M} \quad (8)$$

As $K_i \simeq 10^{-5}$ M and as the same inhibitor is bound at each end of the PEG chain, it is obvious that eq. (8) is always satisfied.

Thus, a symmetrical arrangement of the inhibitory group at each end of the PEG leads to a simple inhibitor. In other words, nearly no enzyme bound EI to yield E_2I. Geratz and Whitmore [8] reported a similar conclusion with diamidino-α,ω-diphenoxy-alkanes. These compounds function as reversible inhibitors of thrombin, pancreatic kallikrein and trypsin, and each molecule appears to bind only a single enzyme molecule.

PEGPAB have been used to investigate proteins partition in two-phase systems obtained by mixing dextran and PEG solutions. The results will be published in a following paper.

Acknowledgement

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This work has been registered: French patents no. 72.45695 on December 21, 1972 and no. 73.42320 on November 28, 1973.

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