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INTERACTION OF TRYPSIN WITH IMMOBILIZED *p*-AMINOBENZAMIDINE DERIVATIVES STUDIED BY MEANS OF AFFINITY ELECTROPHORESIS

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SUMMARY

The strength of the interaction of trypsin with immobilized *p*-aminobenzamidine derivatives was studied by affinity electrophoresis on polyacrylamide gel in Tris–diethylbarbituric acid buffer (pH 8). *p*-Aminobenzamidine was coupled to two kinds of soluble macromolecular carrier: (i) periodate-oxidized Dextran T-500 and (ii) a synthetic copolymer of N-(2-hydroxypropyl)methacrylamide and 4-nitrophenyl esters of 6-(methacroyl)aminohexanoic acid. The enzyme inhibitor was attached to the periodate oxidized dextran either directly or through glycine, 6-aminohexanoic acid or 12-aminododecanoic acid spacer. The strength of the interaction of trypsin with immobilized *p*-aminobenzamidine did not depend on the type of macromolecular carrier that the ligand was bound to but it did depend on the length of the spacer arm. The dissociation constants of the trypsin–immobilized *p*-aminobenzamidine complexes decreased with increasing length of the spacer arm.

INTRODUCTION

Affinity electrophoresis is a type of electrophoretic separation of proteins based on their different affinities to ligands immobilized in the separating gel. This technique is convenient for the quantitative study of interactions of proteins with immobilized and free ligands. So far, affinity electrophoresis in polyacrylamide gels has been used for the study of interactions of phosphorylases with glycogen[1,2], lectins[3–5], enzymes[6] and antibodies[7] with immobilized and free sugars and some enzymes with Blue Dextran[8].

Using this method, the ligand in question can be coupled with dextran or other

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high-molecular-weight water-soluble polymers, and the bound ligand is added to the polymerization mixture used for the preparation of the polyacrylamide gel. Affinity electrophoresis of proteins interacting with Gibacron Blue reported in a previous paper[8] was the first example of the application of this method.

In this work the applicability of affinity electrophoresis for the quantitative study of the interactions of trypsin with its immobilized inhibitor, *p*-aminobenzamidine (PAB), was studied. Two kinds of macromolecular carrier of this ligand were used: periodate-oxidized Dextran T-500 and a synthetic copolymer of N-(2-hydroxypropyl)methacrylamide and 4-nitrophenyl esters of 6-(methacroyl)aminohexanoic acid.

EXPERIMENTAL

Lyophilized trypsin was supplied by Léčiva (Dolní Měcholupy, Czechoslovakia); the activity was determined using benzoyl-L-arginine-*p*-nitroanilide as a substrate according to Erlanger *et al.*[9].

PAB was purchased from Serva (Heidelberg, G.F.R.), Dextran T-500 (molecular weight *ca.* 500,000) from Pharmacia (Uppsala, Sweden) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate from Fluka (Buchs, Switzerland).

Polyacrylamide gel electrophoresis was performed using the apparatus designed by Davis[10] in Tris–diethylbarbituric acid buffer (pH 8.0)[11] or in an acidic buffer system (pH 3.8) as described by Reisfeld *et al.*[12] omitting the large pore gel layers.

Protein samples (50 μg) in 25% glycerol solution (20 μl) were applied per tube (5 × 75 mm). Electrophoresis in the alkaline buffer system[11] was run at 4 mA per tube towards the cathode for 2 h at room temperature. In the acidic buffer system[12] the electrophoresis was carried out at 7 mA per tube for 1.5 h. Migration distances were measured with an accuracy of $\pm$ 0.5 mm after staining with Amido Black.

Dissociation constants (K_i) of the trypsin-immobilized PAB complex were obtained by a modification of our original method[3]. The values of $1/d_0 - d$ were plotted against $1/c_i$ (where d_0 = mobility on a control gel and d = mobility on affinity gel containing a molar concentration c_i of the immobilized ligand). The straight line yields $-1/K_i$ as the intercept with the abscissa and the value of its intercept with the ordinate is $1/d_0 - D_i$ (where D_i = mobility of the enzyme–"immobilized" ligand complex), thus providing information on the degree of immobilization of the ligand[13].

Affinity gels were prepared by addition of appropriate amounts of the solution of PAB-coupled polymer to the polymerization mixture to give the required concentration (c_i) of immobilized enzyme inhibitor; c_i was chosen within the range of concentrations from $5 \cdot 10^{-5}$ to $3 \cdot 10^{-4}$ *M*. The PAB concentration in the polymer solution was determined spectrophotometrically at 292 nm assuming that the absorptivity of PAB did not change during immobilization.

Preparation of polymers containing coupled PAB

Periodate oxidation of Dextran T-500. To the Dextran T-500 solution (1%), sodium periodate was added to give a final concentration of 0.1 *M*. The oxidation proceeded at 25 °C for 1 h; the reaction mixture was exhaustively dialysed against distilled water.

Coupling of amino acids to oxidized dextran. A solution of an appropriate amino acid in 0.2 *M* borate buffer (pH 9.0) (100 mg in 20 ml of the buffer) was mixed with a 1% solution of periodate-oxidized dextran (20 ml) and stirred at room temperature for 2 h. Then sodium borohydride (10 mg) was added in two portions within 10 min. After stirring for a further 10 min, two drops of acetone were added. The reaction mixture was dialysed exhaustively against distilled water and then lyophilized.

Coupling of PAB to oxidized dextran. A PAB solution in borate buffer (pH 9.0) (10 mg in 10 ml of the buffer) was added to a 1% solution of the oxidized dextran (10 ml). After stirring for 2 h at room temperature, the precipitate formed was discarded and the supernatant was reduced with sodium borohydride (10 mg), which was added in several portions within 30 min. After exhaustive dialysis against distilled water, the reaction mixture was lyophilized. The content of coupled PAB in the dextran derivative was 3.9%.

Coupling of PAB to dextran containing bound amino acid residue. Dextran coupled with the amino acid residue (100 mg) was dissolved in water (10 ml) and then PAB (10 mg) was added; the pH of the reaction mixture was maintained at 4.7 using 0.1 *N*-sodium hydroxide solution. After addition of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate (50 mg), the mixture was stirred for 20 h at room temperature, then dialysed against distilled water and lyophilized. The content of PAB in the dextran derivatives was in the range 1.6–3.4%.

Coupling of PAB to synthetic polymers. Copolymers of the 4-nitrophenyl ester of 6-(methacroyl)aminohexanoic acid (for polymer of molecular weight 39,000) or the N-hydroxyphthalimide ester of 6-(methacroyl)aminohexanoic acid (for polymer of molecular weight 82,000) with N-(2-hydroxypropyl)methacrylamide were prepared as described by Labský and Kálal[14,15]. The molecular weight of the polymers was determined by the light-scattering method.

Coupling of PAB to these polymers was performed by heating the polymer (200 mg) with PAB (100 mg) dissolved in 3 ml of dimethyl sulphoxide in a closed vessel at 50 °C for 5 h. The resulting polymer derivative was dialysed exhaustively against 1% acetic acid in water. The content of coupled PAB in the prepared polymers was about 3 mol.%.

RESULTS

The interaction of trypsin with immobilized PAB was studied in the alkaline Tris–diethylbarbituric acid buffer system. The alkaline buffer system used has been shown not to affect the trypsin activity in the solution. In an acidic buffer system the mobility of trypsin on any type of affinity gel was identical with that on a control gel, indicating an apparent lack of interaction of the enzyme with immobilized PAB at the low pH (3.8). This is in agreement with the pH dependence of trypsin activity.

In the Tris–diethylbarbituric acid buffer system trypsin yielded a single zone, usually accompanied by a faint zone with lower electrophoretic mobility (Fig. 1). The control gels were prepared by addition of an appropriate polymer derivative without coupled PAB, *i.e.*, derivatives substituted by the corresponding ω-amino acid. Addition of these polymer derivatives to the gels had no effect on the trypsin mobility in comparison with gels containing either no macromolecular additions or underivatized dextran or hydroxypropylmethacrylamide polymer. Thus, no "non-specific" inter-

Fig. 1. Affinity electrophoresis of trypsin on polyacrylamide gels containing hydroxypropylmethacrylamide copolymers with coupled PAB. 1, Control gel containing hydroxypropylmethacrylamide copolymer without coupled PAB ($c_l = 0$); 2 and 3, affinity gels containing hydroxypropylmethacrylamide copolymer (mol. wt. 39,000) containing coupled PAB through C_6 spacer ($c_l = 3.0 \cdot 10^{-5}$ and $1.0 \cdot 10^{-4}$ M); 4, affinity gel containing hydroxypropylmethacrylamide copolymer (mol. wt. 82,000) containing coupled PAB through C_6 spacer. Electrophoresis was carried out in Tris–diethylbarbituric acid buffer (pH 8.0).

action of these polymeric derivatives with trypsin was observable (Fig. 1). With dextrans substituted with ω-amino acids the trypsin zone was rather diffuse with a sharp frontal edge; the mobility of the frontal edge was identical with that observed on gels containing hydroxypropylmethacrylamide polymer derivatives or on other controls gels. Similarly, the zones on affinity gels prepared from dextran substituted by PAB bound through an amino acid spacer were diffuse with a sharp frontal edge.

TABLE I

DISSOCIATION CONSTANTS OF TRYPSIN–IMMOBILIZED *p*-AMINOBENZAMIDINE COMPLEXES

*Polymer**	K_l *(M)*
Dextran–PAB	$2.13 \cdot 10^{-3}$
Dextran–glycine–PAB	$4.71 \cdot 10^{-4}$
Dextran–6-aminohexanoic acid–PAB	$2.89 \cdot 10^{-4}$
Dextran–12-aminododecanoic acid–PAB	$1.47 \cdot 10^{-4}$
HPMA–6-aminohexanoic acid–PAB (mol. wt. 39,000)	$2.88 \cdot 10^{-4}$
HPMA–6-aminohexanoic acid–PAB (mol. wt. 82,000)	$2.67 \cdot 10^{-4}$

*PAB = *p*-aminobenzamidine; HPMA = hydroxypropylmethacrylamide copolymer

Incorporation of all of the prepared macromolecular derivatives of PAB into polyacrylamide gel caused a decrease in the electrophoretic mobility of trypsin in the alkaline Tris–diethylbarbituric acid buffer system. The decrease in electrophoretic mobility was dependent on the concentration of immobilized PAB in the gel. The dissociation constants of the trypsin-immobilized PAB complexes obtained on various types of affinity gels are given in Table I.

Clearly, the strength of the interaction of trypsin with immobilized PAB does not depend on the type of macromolecular carrier used: very similar values of K_i were obtained using the hydroxypropylmethacrylamide copolymers and periodate-oxidized dextran substituted with PAB bound through a hexamethylene spacer. On the other hand, K_i decreases with increasing spacer length.

Surprisingly, effective immobilization of PAB was achieved even with relatively low-molecular-weight hydroxypropylmethacrylamide copolymer derivatives; a negligible mobility of D_i of the enzyme complex with immobilized ligand is obtained from the $1/d_0$—d *versus* $1/c_i$ plot (Fig. 2) for both types of polymer used. Moreover, no leakage of the polymers from the gel into the electrode buffer compartments during the electrophoresis was observable by spectrophotometric examination.

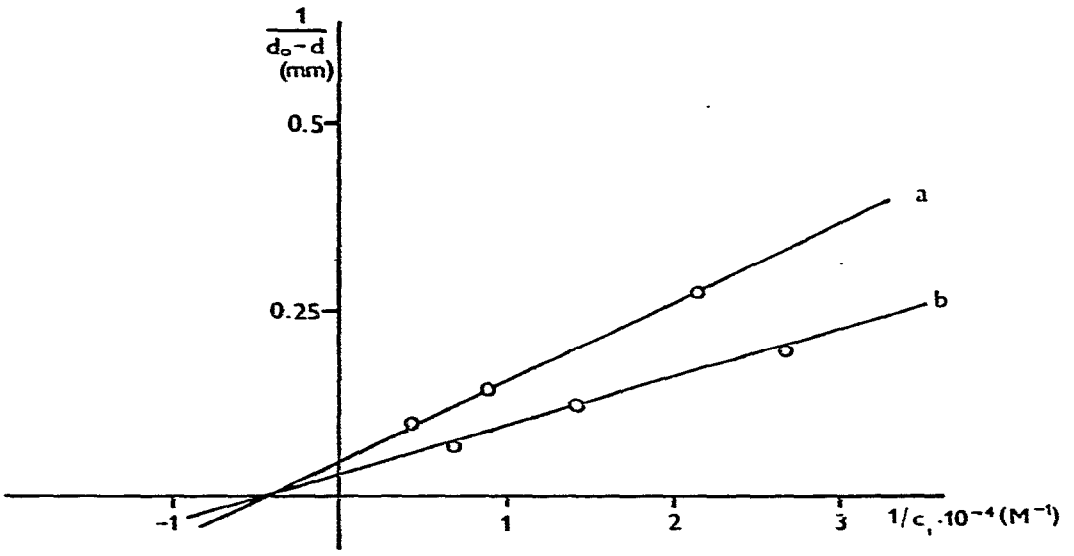

Fig. 2. Determination of dissociation constant of the trypsin–immobilized PAB complex. d_0 = electrophoretic mobility of trypsin on control gel ($c_i = 0$); d = electrophoretic mobility of trypsin on affinity gels containing immobilized PAB; c_i = concentration of immobilized PAB in affinity gels. (a) PAB coupled to periodate-oxidized dextran through 6-aminohexanoic acid residue (Dex–C_6–PAB); (b) PAB coupled to hydroxypropylmethacrylamide copolymer through 6-aminohexanoic acid residue (HPMA–C_6–PAB)

DISCUSSION

The results demonstrate that affinity electrophoresis can be used for the quantitative study of the interaction of trypsin with immobilized inhibitors. This technique seems to be a convenient tool for rapid and simple comparisons of the

effects of carrier macromolecules and spacer arms on the strength of an interaction of an enzyme with an immobilized ligand.

The increase in strength of the interaction of an enzyme with an immobilized ligand with increasing spacer length is a general phenomenon observed in affinity chromatographic experiments, and can be explained either by an improved steric accessibility of the ligand bound through a longer spacer and/or by an interaction of the enzyme with the spacer itself[16]. In our study no non-specific interaction of trypsin with the spacer molecules coupled to the polymer was observed.

It is interesting that relatively low-molecular-weight synthetic hydroxypropylmethacrylamide copolymers (molecular weight 39,000) served well for the effective immobilization of PAB. The immobility of these copolymers cannot be explained by simple entrapment in the gel network, but some kind of interaction of these copolymers with polyacrylamide gel must be assumed.

The interactions of trypsin with immobilized PAB under the conditions of affinity electrophoresis are, even in optimal cases (long spacers), approximately one order of magnitude weaker than those with free PAB (measured by affinity chromatography or from kinetic data[17]. Unfortunately, we were not able to measure the interaction of trypsin with free PAB by affinity electrophoresis because of the high electrophoretic mobility of free PAB in the buffer system used. The decrease in trypsin affinity towards immobilized PAB in comparison with free PAB (2–5-fold) has been observed by other workers[18].

In contrast to these findings, the reverse relationship between the affinity of trypsin towards immobilized and free PAB was found using quantitative affinity chromatography[17].

The weaker interaction of trypsin with immobilized PAB in comparison with free inhibitor might be explained by the possible inaccessibility of a fraction of the immobilized PAB residues to trypsin molecules. Also, the possible effect of the presence of by-products being formed in the reaction of inhibitor binding to polymer could be considered. In general, attachment of PAB to activated esters or carboxyl groups on polymers is assumed to occur simply through an amino group with amide formation[19].

Differences in the relationship of the interaction of trypsin with immobilized and free inhibitor determined by quantitative affinity chromatography[17] and by affinity electrophoresis are given by the different values of the concentration of immobilized ligand (c_l) used in the two methods. The dissociation constant of the enzyme–immobilized ligand complex (K_l) is greatly dependent on the value of c_l used for the K_l calculations. In quantitative affinity chromatography[17] the effective concentration calculated on the basis of the working capacity[20] was used, whereas in affinity electrophoresis the total concentration of immobilized ligand was employed. At present there is apparently no direct method that allows the determination of the effective concentration of immobilized ligands in affinity gels. The concentration of immobilized ligand calculated on the basis of the working capacity[20] is much lower than the total concentration of enzyme inhibitor determined analytically.

Although the values of dissociation constants of protein–immobilized ligand complexes obtained by affinity electrophoresis, as well as by affinity chromatography, are less exactly defined, their usefulness for comparative purposes is nevertheless obvious.

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