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METHACRYLATE GELS WITH EPOXIDE GROUPS AS SUPPORTS FOR IMMOBILIZATION OF ENZYMES IN pH RANGE 3—12

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Summary

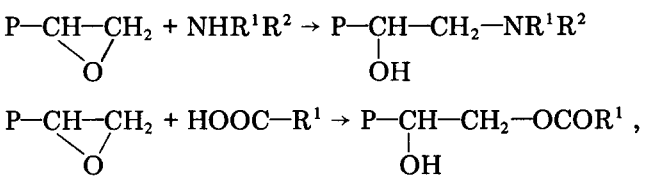
Glycidyl methacrylate gels are carriers suitable for attachment of enzymes and for use in affinity chromatography. Experiments on the coupling of glycyl-L-leucine and acetyl-L-leucine to these gels have shown a high pH-dependence of the bond formation between the support and the α-amino group (pH optimum 9.7); the coupling reaction between the epoxide group and the carboxyl group is practically pH-independent. Serum albumin and trypsin were attached to a greater extent in acidic than in alkaline media. The effects of time and temperature were also studied. The catalytic action of immobilized trypsin, as well as its use for affinity chromatography of trypsin inhibitor, were studied.

Introduction

Widespread application of covalently bonded enzymes and of affinity chromatography depends on the availability of suitable solid supports and of simple coupling reactions. The development in both aspects is far from complete but agarose and glass are two supports of wide applicability. Hydroxyalkyl methacrylate gels (Spherons) and glycidyl methacrylate gels have many features which resemble those of agarose. After these gels have been activated with CNBr they can bind peptides and proteins [1,2]. The mercuri-derivatives of these gels have been employed for isolation of proteins containing thiol groups [3]. By coupling hexamethylendiamine or ϵ-aminocaproic acid to hydroxyalkyl methacrylate gels, two supports (amino-Spheron and carboxy-Spheron) were prepared. These supports have been employed for the immobilization of pepsin by the carbodiimide couping [4], and, after the binding of low molecular weight inhibitors, for the isolation of proteases by affinity chromatography

[5—7]. Proteins can be bonded to these gels using benzoquinone [8] as found with agarose. Trypsin and chymotrypsin were coupled to hydrazide, diazo- and anhydride derivatives of hydroxyalkyl methacrylate gels [1]. Papain was attached to a reactive support, prepared by copolymerization of a mixture containing methacrylic acid *p*-nitrophenyl esters [1,9,10]. These gels (like glass) do not change either their volume or structure with changes in pH, not even in the presence of organic solvents; for this reason they can be used for high-pressure chromatography [11]. Spheron gels are not biodegradable and resist digestion with enzymes. The pore size, specific surface area, and concentration of active groups can be varied over a wide range.

The epoxide (oxirane) group readily reacts with nucleophilic reagents. Under relatively mild conditions, it may react both with amino groups and carboxyl groups as follows:

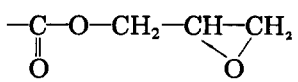


Hence, amino acids, peptides and proteins can be coupled to supports bearing epoxide groups. This type of immobilization has been described earlier in studies on the activation of polysaccharide supports by bifunctional oxiranes [12] and its advantages have been discussed [13]. Favorable properties are also shown by acrylamide gels bearing epoxide groups [14].

Earlier studies from this laboratory were focused on preparation, reactions and application of the macroporous, highly cross-linked copolymers of glycidyl methacrylate with ethylene dimethacrylate [15,16]. The use of these epoxide gels for the immobilization of enzymes and of their inhibitors is investigated in this paper.

Material and Methods

Glycidyl methacrylate gels were prepared by radical suspension copolymerization of glycidyl methacrylate gels with ethylene dimethacrylate (7 : 3, v/w, [15,16]). The specific surface area of the gels was 28 m^2/g and the most frequent pore size in dry state was 220 nm (diameter); the pore volume was 1.21 ml/g. The gel was extracted with water and ethanol before use. The support used has an epoxide ring coupled to the polymer backbone through a 3-atom spacer:



Glycyl-L-leucine [17] and acetyl-L-leucine [18] were prepared by conventional methods of peptide synthesis. Trypsin activity (proteolytic activity 3.45 A_{280} units/min per mg at pH 7 determined with benzoyl-L-arginine *p*-nitroanilide [19]) was 1.11 μmol/min per mg at pH 8. Lung trypsin inhibitor, and

hemoglobin were products of Léčiva, Prague. Lyophilized human serum albumin was from Imuna, Šarišské Michalany, Czechoslovakia.

Evaluation of binding capacity

The quantity of compounds bound was determined by amino acid analysis [20]. Before analyses the gel samples were washed with 6 M guanidine hydrochloride, water, and acetone, dried to constant weight at 105°C and hydrolysed for 20 h with 6 M HCl at 110°C.

Coupling of glycyl-L-leucine and acetyl-L-leucine as function of pH

Dry gel (200 mg) was suspended in 1 ml glycyl-L-leucine or acetyl-L-leucine (20 mg/ml) in solution at various pH values and the suspension was stirred for 30 h at room temperature. Citrate buffers were used for coupling at pH 2.2—6.4; coupling at pH values above 6.4 was effected in borate buffers. For the coupling of acetyl-L-leucine, 0.4 ml methanol was added to 0.6 ml buffer (see legend to Fig. 1).

Coupling of glycyl-L-leucine as function of temperature and time

Dry gel (200 mg) was suspended in 1 ml borate buffer (pH 9.7) containing 20 mg of the dipeptide. The suspension was stirred at 4, 25 and 37°C. The quantity of dipeptide attached is plotted vs. time in Fig. 2.

Coupling of serum albumin and trypsin as function of pH

The dry gel (2 g) was suspended in 10 ml 10% serum albumin solutions or in trypsin solutions (3.33 mg/ml) in the proper buffers. 0.1 M acetate buffers were used for coupling at pH 2—5 and 0.1 M borate buffers in the pH range 6—12. All buffers were made to 0.02 M $CaCl_2$ for the coupling of trypsin. The coupling was performed at room temperature (70 h with serum albumin; 24, 48 or 96 h with trypsin, Fig. 3). Immobilized trypsin of higher protein content was prepared by a modified coupling procedure; a 2% trypsin solution was coupled at 4°C and the remaining free epoxide groups were blocked by treatment of the gel with 1 M ethanolamine overnight.

Determination of enzymatic activity of free and immobilized trypsin

The proteolytic activity of free and immobilized trypsin (7.15 mg enzyme/g dry gel) was determined by a modification [2] of Anson's method [21] with solutions of denatured hemoglobin in Britton-Robinson buffers as substrates. The activity of immobilized trypsin was also determined with benzoyl-L-arginine *p*-nitroanilide [19] as follows: the wet gel (7 mg, corresponding to 2.45 mg dry gel) was added to 2.5 ml benzoyl-L-arginine *p*-nitroanilide solution in 0.1 M Tris · HCl buffer (pH 8) or in Britton-Robinson buffer, pH 4—12, placed in a double-jacketed cell maintained at 37°C and provided with a magnetic stirrer. After 10 min 1 ml 30% CH_3COOH in dioxane was added. The suspension was filtered and the absorbance of the filtrate was measured at 410 nm. The activity of trypsin inhibitor was determined to the method of Meloun et al. [22].

Affinity chromatography of trypsin inhibitor on a column of glycidyl methacrylate gel with attached trypsin

Trypsin inhibitor (3 mg) was applied to a 1 × 13.6 cm column of immobilized trypsin equilibrated with 0.1 M Tris · HCl/0.02 M $CaCl_2$ (pH 8). The sample was allowed to soak in and 30 min later the column was eluted by the equilibrating buffer. Trypsin inhibitor was liberated from the complex by elution with 0.1 M acetic acid. Fraction were collected at a rate of 0.2 ml/min and the absorbance at 280 nm measured; inhibitor activity, and pH (after buffer change) were also determined. The chromatography was performed at 23 ± 1°C.

Results and Discussion

The main advantage of the immobilization of proteins on gels bearing epoxide groups rests in the simple coupling reaction which involves only the treatment of the dry gel with the solution of the product to be attached. As can be seen in Fig. 1, peptides are bonded predominantly through their α-amino group. This process is strongly pH-dependent, the optimum lying near pH 9.7. It has been shown in experiments with acetyl-L-leucine that peptides are attached also through their carboxyl group. The extent of this reaction is much lower and practically pH-independent. The covalent bond between the α-amino groups of the peptides and the gel is stable and is not split during the acid hydrolysis. We investigated the quantity of glycine and leucine liberated by acid hydrolysis (6 M HCl, 110°C for 20, 88, 120 or 240 h) from the gel to which glycyl-L-leucine had been coupled. In all cases we found 21 μmol/g of leucine and only traces of glycine. On the contrary, the ester bond between the matrix and the carboxyl group is hydrolyzed under these conditions. When the gel, with attached acetylglycyl-L-leucine, was hydrolyzed for 20 h the hydrolysate contained equimolar quantities of glycine and leucine (unpublished data). We

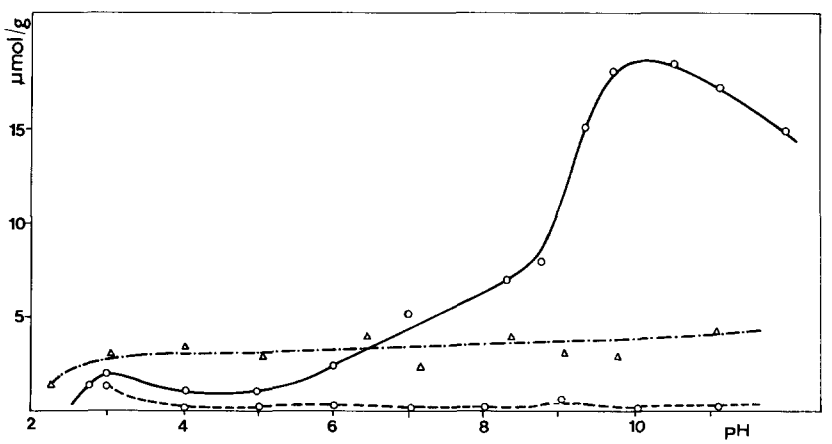


Fig. 1. Quantity of glycyl-L-leucine and acetyl-L-leucine bonded (in μmol of dipeptide or amino acid per g of dry conjugate) as a function of pH of reaction mixture. For pH 2.2—5: citrate buffers; above pH 6.4: borate buffers. The quantity of dipeptide bonded was calculated for glycyl-L-leucine from the analytical values for leucine, — ○ —, bonding through the α-amino group of glycine and for glycine, - - ○ - -, bonding through carboxyl group of leucine. The quantity of acetyl-L-leucine was determined from the analytical value for leucine, - · - △ - · -.

may assume that the small quantity of glycine found in the hydrolysates of the gels with bonded glycyl-L-leucine represents the quantity of dipeptide coupled through its carboxy group. The coupling via carboxyl group is much higher with acetyl-L-leucine than with glycyl-L-leucine. For explanation, we may consider two possibilities: (i) the hydrophobicity of acetyl-L-leucine is much higher than that of glycyl-L-leucine (or of carboxylic acids used as buffer components) and the effective concentration to the acetyl derivative is enhanced near the matrix; (ii) the zwitterionic dipeptide is virtually repulsed from the matrix area and the extent of the coupling reaction is lowered (not only via the carboxylic group but also via α-amino group, unpublished results). As shown in Fig. 2 the binding of peptides onto gels with epoxide groups is relatively slow even at pH optimum (9.7) and is temperature-dependent.

The quantity of serum albumin and trypsin coupled to epoxide gels is plotted vs. pH in Fig. 3. There is no marked maximum at pH 9.7 which reflect the reaction of the α-amino group, obviously because the α-amino group of the N-terminal amino acid is not the group dominating the coupling. A more important role is played by carboxyl groups and ε-amino groups. The data given in Fig. 1 demonstrate that the coupling through the carboxyl group is practically pH-independent. The extent of coupling through the ε-amino group is by one order lower than the coupling through the α-amino group and is pH-independent in the range pH 7—11 (unpublished data). The largest quantity of both proteins was attached at pH 3. This phenomenon is probably caused by enhanced reactivity of carboxylic groups of proteins in the hydrophobic environment of glycidyl methacrylate gels. Furthermore, trypsin may undergo to some extent conformational changes so that carboxyl groups adjacent to the hydrophobic gel surface are presented to the reactive epoxide groups. In spite of the high content of epoxide groups the amount of protein attached is very

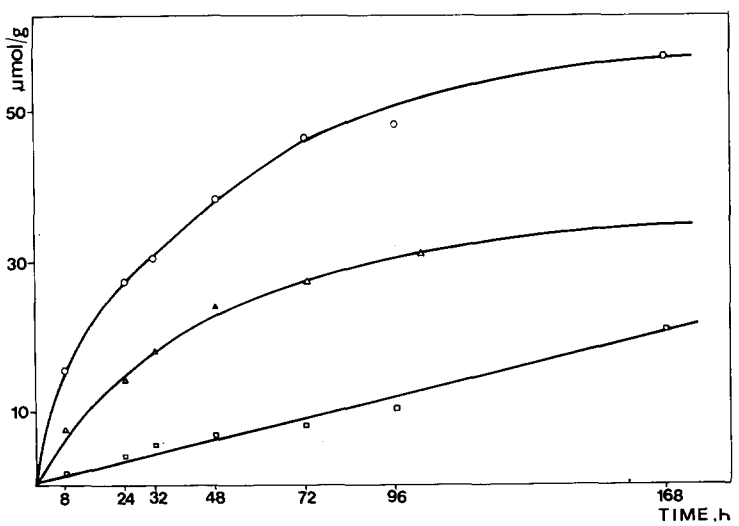


Fig. 2. Quantity of glycyl-L-leucine bonded (in μmol of dipeptide per g of dry conjugate) as function of time and temperature. The quantity of dipeptide bonded was determined from the analytical value for leucine. Temperature: 4°C, — □ —; 25°C, — △ —; and 37°C — ○ —.

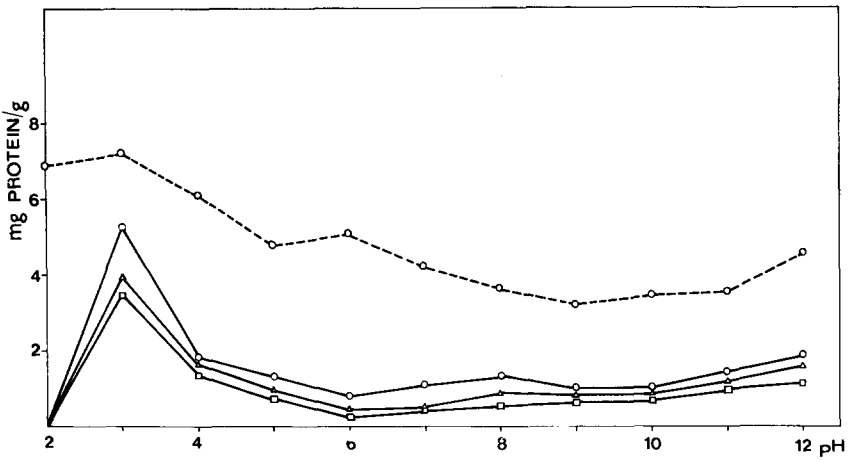


Fig. 3. Quantity of immobilized serum albumin and trypsin (in mg of protein per g of dry conjugate) as function of pH in reaction mixture. For pH 2—5: acetate buffers; pH 6—12: borate buffers. The quantity of serum albumin after coupling for 70 h, - - ○ - -; quantity of trypsin after coupling for 96 h, — ○ —; 48 h — △ —; and 24 h, — □ —, at room temperature.

small and can be only little affected by prolongation of coupling time (see quantities of trypsin attached after 24, 48, and 96 h) or by the enhancement of the concentration of trypsin in the solution.

The proteolytic activity and the activity determined with benzoyl-L-arginine *p*-nitroanilide are plotted as a function of pH in Fig. 4; The activity of free trypsin at pH optimum was taken to represent 100%. The relative activity of immobilized trypsin determined with benzoyl-L-arginine *p*-nitroanilide was high. There was no shift in pH optimum, which has been observed, for example, by Manecke and Vogt [23] who examined the activity of trypsin coupled to

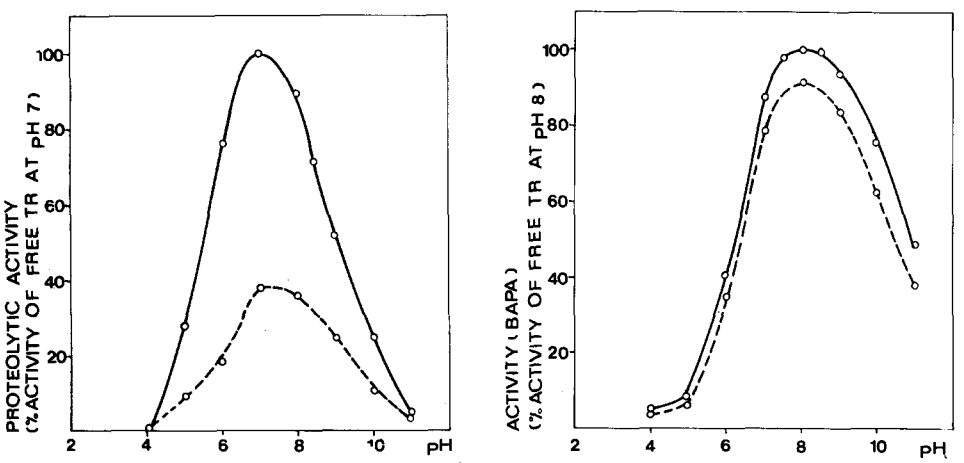


Fig. 4. Catalytical activity (with respect to hemoglobin and benzoyl-L-arginine *p*-nitroanilide (BAPA)) of trypsin (———) and trypsin covalently bonded to glycidyl methacrylate gel (- - - - -) as function of pH. The quantity of immobilized enzyme vs. 7.15 mg per g of dry gel. Activity of free trypsin (TR) 3.45 A_{280} units/min per mg at pH 7, 1.11 μmol/min per mg at pH 8, determined with benzoyl-L-arginine *p*-nitroanilide. Activity ratio of bound to free enzyme 38% (proteolytic activity) and 92% (activity determined with benzoyl-L-arginine *p*-nitroanilide).

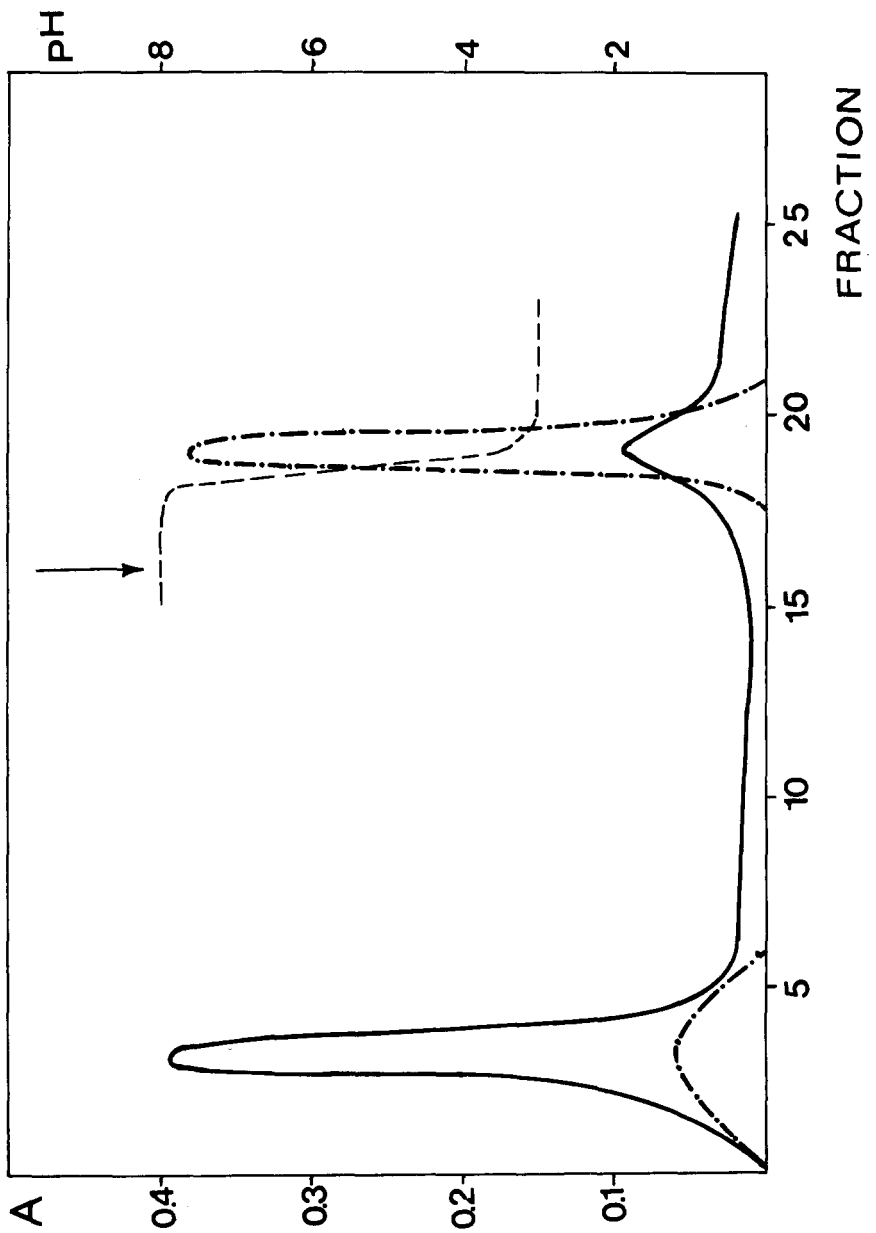


Fig. 5. Affinity chromatography of trypsin inhibitor on glycidyl methacylate gel with immobilized trypsin. Trypsin inhibitor (3 mg) was applied to a 1 × 13.6 cm column of immobilized trypsin equilibrated with 0.1 M Tris · HCl buffer, pH 8 (0.02 M in $CaCl_2$) and eluted by the equilibrating buffer. Fractions were collected at a rate of 3 ml/15 min. ———, absorbance at 280 nm. - - - - - -, inhibitory activity. . . . . , pH. The arrow denotes a change in eluting buffer (0.1 M acetic acid).

polyvinyl supports with the same substrate. The reason is obviously the relatively low trypsin content of the immobilized preparation; there is no cumulation of products as a result of its catalytic action. The preparations of immobilized trypsin are stable when stored in suspension in the presence of calcium ions at 4°C and lose about 30% of their activity after lyophilization.

The use of trypsin coupled to glycidyl methacrylate gels for affinity chromatography of trypsin inhibitor is shown in Fig. 5. The course of the chromatography was analogous to the course of chromatography of trypsin inhibitor on hydroxylalkyl methacrylate gel to which chymotrypsin has been coupled after CNBr activation [2]. Amino acid analysis of the isolated trypsin inhibitor was in good agreement with that of the authentic specimen [24]. A nonspecific adsorption of other proteins is therefore not probable.

We can conclude that the hydrophobic character of the glycidyl methacrylate gels is enhanced and its influence on the coupling reactions could be more pronounced, when compared with agarose. However, the enhanced hydrophobicity does not disturb its application in affinity chromatography.

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