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IMMOBILIZATION OF PROTEIN ON ALDEHYDE-CONTAINING GELS—II. ACTIVATION OF PYRIDINE RINGS WITH CYANOGEN BROMIDE

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Cyanogen bromide was used to convert pyridine rings in polymers to polyaldehyde. By reaction with NH_2-containing substances, the rings are rebuilt, resulting in a pyridinium compound. Thus proteins and other NH_2-containing substances can be covalently bound. This method provides a new means for a immobilization technique. Pyridine-gels based on polysaccharide and polyacrylamide matrices, as well as pyridine glass beads, were synthesized and used to study the conditions necessary for coupling. Trypsin and α-chymotrypsin were used as test substances for immobilization of proteins. Some properties of the bound protein were studied and compared to native enzyme. Some general results on the applicability of these gels for affinity chromatography are also presented.

INTRODUCTION

Of the many methods available for immobilization of biologically active compounds, the most widely used is the cyanogen bromide method for activation of polysaccharides (1). Polymers containing aldehyde groups are also very efficient carriers for immobilization of protein (2, and references cited therein). These carriers were prepared by coupling glutaraldehyde to polymers containing amines or hydrazides. The gels, however, suffer from one disadvantage; the amount of aldehyde groups cannot be controlled, since cross-linking reactions may occur, even when a large excess of glutaraldehyde is used. In this paper, we present a method for the activation of pyridine-containing carriers with cyanogen bromide, which yields aldehyde groups in one step. The amount of aldehyde groups formed can be controlled by the amount of cyanogen bromide added. This method introduces a new application of cyanogen bromide in protein chemistry.

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MATERIALS

Cyanogen bromide, 4-vinyl-pyridine, and pyridine-4-aldehyde were obtained from Fluka, Buchs, Switzerland. Enzacryl (polythiol), Batch No. 54590, was from Koch-Light, Colnbrook, England. All solvents were from Merck, Darmstadt, or Frutarom, Haifa, Israel. Controlled pore glass and trypsin were obtained from Sigma, St. Louis, Mo; Sepharose 4B and CL-4B from Pharmacia, Uppsala, Sweden; 3-aminopropyl-triethoxysilane and 5,5'-dithiobis-(2-nitrobenzoic acid) from Aldrich, Milwaukee, Wis.; chymotrypsin from Worthington, New Jersey; and ϵ-aminocaproyl-D-tryptophan-methylester from Miles Yeda, Rehovot, Israel. α-*N*-benzoylarginine-ethylester (BAEE) and *N*-acetyl-tyrosine-ethylester (ATEE) were synthesized by I. Jacobson at the Weizmann Institute. Polyacrylhydrazide was prepared according to Miron et al. (3) and Cu-lysine according to Winitz and Greenstein (4).

METHODS

Synthesis of the Gels and Coupling of Enzymes

Several gels containing pyridine were synthesized (Fig. 1).

Gel I. Enzacryl (polythiol, Batch No. 54590) was suspended in freshly distilled 4-vinylpyridine (about 20 ml/g gel), incubated in a tightly stoppered flask at 37°C on a shaker for at least 3 h, and kept at room temperature for 2 days. The gel was washed with 95% ethanol, followed by absolute ethanol. The coupling reaction was quantitative, as shown with the Ellman test. The gel (1 g) was activated at room temperature with 1–3 ml cyanogen bromide solution (1 g cyanogen bromide per ml of absolute dioxane) while being stirred. After 5 min of activation, 20 ml water was added and the mixture stirred at room temperature for an additional 20 min. The gel was then filtered as quickly as possible and washed with ice cold water. It was then coupled immediately to 80 mg trypsin dissolved in 20 ml 0.25 *M* phosphate buffer, pH 5, the mixture being stirred for 30 min at room temperature and afterwards overnight at 4°C. The gel was washed with 0.1 *M* NaCl and stored as wet gel at 4°C. It remained stable without loss of activity for several months.

Gel II. Controlled pore glass beads (120/200 mesh, mean pore diameter 1038 Å) were treated for 1 h with 3% HNO_3 at 90°C, rinsed with water until the filtrate was neutral, and kept for several days in distilled water (5). The glass beads (10 g) were then mixed with 50 ml 10% aqueous 3-aminopropyltriethoxysilane and the pH adjusted to 3.5 with 6 *N* HCl. The suspension was gently agitated at 75°C for 2 h on a shaker. The coated

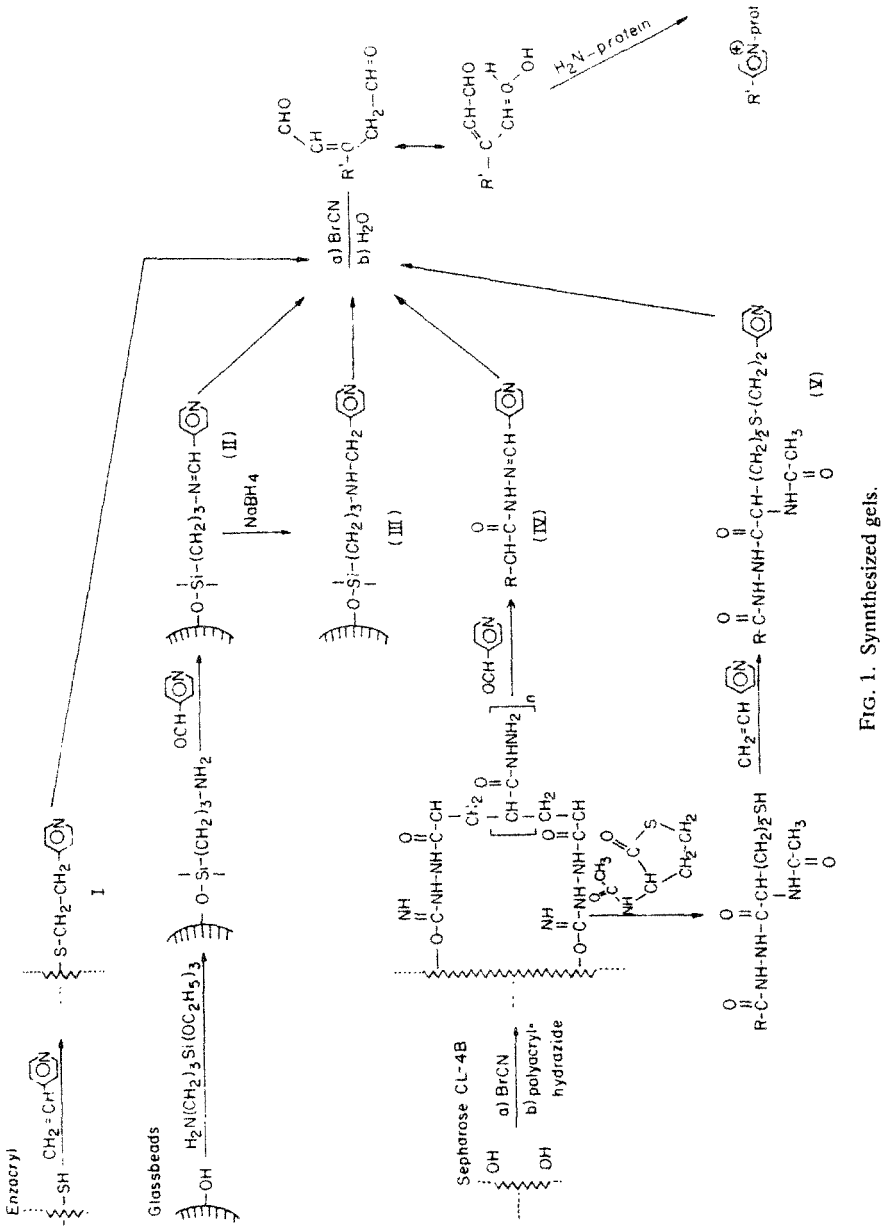

FIG. 1. Synnthesized gels.

glass beads were filtered, rinsed with water, and dried overnight at 115°C. The amount of amino groups was calculated from the nitrogen content determined by elementary analysis, and gave values of 80–180 μmol of amino groups per 1 g dry gel. The coated glass beads (10 g) were treated with 250 ml 2.5% pyridine-4-aldehyde in 0.25 *M* phosphate buffer, pH7, stirred at room temperature for one hour, washed carefully with water and dried in a desiccator over NaOH. The completion of the reaction was indicated by a negative test with trinitrobenzenesulfonic acid (TNBS). The beads (300 mg) were moistened with dry dioxane, and treated with cyanogen bromide (1 g/ml absolute dioxane). After 5 min of activation, 20 ml of 0.2 *M* borate buffer, pH 9, were added and the mixture stirred at room temperature for an additional 20 min, while the pH was kept constant with 2 *N* NaOH by manual titration. The coupling procedure to the protein was the same as mentioned at the beginning of this section.

Gel V. Sepharose CL-4B (10 g) was thoroughly washed with water and suspended in 20–30 ml 2 *M* aqueous K_2CO_3. The mixture was cooled in an ice bath; small pieces of ice were then added, cyanogen bromide (1 g/ml dimethylformamide) was added, and the mixture was stirred for 90 s. The gel was washed with a small amount of a 50% aqueous DMF solution followed by ice water. The activated Sepharose was stirred overnight at 4°C with 30 ml of an aqueous solution containing 1.7 g polyacrylhydrazide and 1.3 g $NaHCO_3$. The gel was washed with 0.1 *M* aqueous NaCl followed by water until no hydrazide could be found in the filtrate, as indicated by the TNBS test. The gel contained 17 μmol of free hydrazido groups (also assayed by TNBS). The gel was suspended in 20 ml of ice cold 1 *M* aqueous $NaHCO_3$, stirred overnight with 1 g *N*-acetyl-homocystein-thiolactone (6) and washed again with 0.1 *M* NaCl and water. The gel contained 0.7% sulfur as determined by elementary analysis. The gel was made water free by a stepwise procedure starting by treatment with 50% dioxane and gradually increasing the concentration of dioxane in the washing solution, ending up with absolute dioxane. The water-free gel was treated with 20 ml of 4-vinyl-pyridine for three hours at 37°C on a shaker in a well-stoppered flask, and then kept at room temperature for 24 h. The gel was filtered and washed with 95% ethanol followed by absolute ethanol. The reaction was quantitative, as indicated by the Ellman test. This gel (1 g) was moistened with dry dioxane and treated with 1 ml cyanogen bromide solution (1 g per ml dry dioxane) for 5 min. Then 20 ml water was added and the mixture was stirred for 10–20 min at room temperature. The gel was filtered, washed with cold water, and coupled immediately with 40 mg trypsin dissolved in 20 ml 0.25 *M* phosphate buffer, pH 5. The suspension was stirred overnight at 4°C, and the gel was washed with 0.1 *M* NaCl. A second type of pyridine

containing Sepharose gel (gel IV) could be prepared by coupling pyridine-4-aldehyde directly to the polyacrylhydrazido-Sepharose. In this case, 2 g of polyacrylhydrazido-Sepharose containing 82 μmol hydrazide per g packed gel were dissolved in 50 ml 2.5% pyridine-4-aldehyde and gently stirred overnight at room temperature. The reaction was quantitative, as indicated by a negative TNBS test. The gel was filtered and washed with water until no aldehyde could be found in the filtrate when tested with the 2,4-dinitrophenyl-hydrazide test. The activation and protein coupling procedure was the same as mentioned above.

Reduction of Gels with $NaBH_4$

One g gel was suspended in 10 ml of 0.25 *M* phosphate buffer, pH 7, and treated at 0°C with a solution of 50 mg $NaBH_4$ in 1 ml water for 1 h, filtered, and washed carefully with 1 *M* NaCl. Quantitative determination of immobilized protein was carried out on an amino acid analyzer after acid hydrolysis with 6 *N* HCl in a sealed tube for 22 h at 110°C. Enzyme assay was made on a pH-stat (Radiometer Copenhagen, model TTT1c) according to Levin et al. (7). For the determination of trypsin activity, 100 μl aliquots of soluble enzyme containing 25 μg of protein (or insoluble enzyme in approximately the same amount) were added to 5 ml of a 0.1 *M* aqueous NaCl solution, being 20 m*M* with respect to BAEE, and the uptake of 0.1 *N* NaOH was measured at the pH optimum of the immobilized enzyme at 26°C. For the assay of chymotrypsin, the procedure was used with a 12 m*M* solution of ATEE in 0.1 *M* NaCl as a substrate at pH 8 (the pH optimum of soluble chymotrypsin) and pH 9 (the pH optimum of immobilized enzyme) at 26°C. One enzyme unit (U) is defined as the cleavage of 1 μmol of substrate per minute by the enzyme. The determination of free SH-groups was made according to Ellman (8). Aldehydes were determined by reaction with 2,4-dinitrophenyl-hydrazine (9).

The determination of hydrazido groups was done by reaction with an excess of TNBS; the quantity of unreacted reagent was measured and subtracted from its initial concentration. Thus aliquots of the gels (0.2–0.3 g packed gel) were incubated with 0.5 ml of a 2% aqueous TNBS solution. Saturated sodium tetraborate solution, 1.5 ml, was added and the mixture stirred at room temperature for 1 h. Then the sample was diluted with water to a volume of 25 ml. This solution, 25–200 μl, was incubated with 1.0 ml of 0.02 *M* adipicdihydrazide solution and 0.8 ml of saturated borate solution for 1 h at room temperature. The final volume was adjusted to 2 ml with water and the absorbance measured on a Cary spectrophotometer at 500 nm. A blank was made in the same manner, but without gel. The

amount of hydrazide was estimated according to Lambert–Beer's law, $\varepsilon = 17{,}000$.

The apparent Michaelis constant ($K_{m(app)}$) was measured according to Goldstein et al. (10) in 0.5 *M* NaCl, with BAEE as substrate, at pH 9.5 and 26°C on the pH-stat. For qualitative tests of amino acids and proteins, a ninhydrine reagent was used (11).

RESULTS AND DISCUSSION

Preparation of Pyridine-Containing Gels

Many polymers, copolymers, and membranes containing pyridine have been reported in the literature. These gels were prepared mostly by direct polymerization of vinylpyridine. Due to the hydrophobicity and lack of swelling of these gels in aqueous solutions, they are not suitable to be applied as efficient carriers for enzyme immobilization. The polyvinylpyridine that was prepared by direct polymerization of vinylpyridine and 1,4-divinylbenzene failed to bind enzymes, and the same may be true for other copolymers. Therefore we decided to prepare pyridine-containing polymers on carriers that are known to swell in aqueous solutions, such as polyacrylamides and polysaccharides. Controlled pore glass beads are also suitable. The methods of binding pyridine to such polymers are numerous, since it is possible to link almost any functional group of gels with pyridine derivatives. For our studies, we have chosen only two representative reactions: (1) the coupling of vinylpyridine to polymers containing SH-groups and (2) reacting pyridine-4-aldehyde to polymers containing amino or hydrazido groups. We do not claim that these are the best carriers or that the best reactions were chosen.

Binding of pyridine derivatives to carriers is very simple, and pyridine gels of any kind are easily prepared. The number of pyridine rings on the gels may also be of importance. Too many pyridine rings may be a disadvantage since the aldehyde formed after cleavage of the ring contains a proton on the α-carbon. Therefore aldol condensation may occur on the polymer, thus reducing the number of aldehydes available for reaction. This reaction may also cause cross-linking, which results in shrinkage of the carrier and disturbance of the network. Another reason may be that many pyridine rings disturb each other by steric hindrance. With polyvinylpyridine, even though we could obtain very strong color after reaction with cyanogen bromide and barbituric acid, only a small amount of enzyme was bound (0.1 mg/g packed gel).

Mode of Binding of Compounds Containing Amino Groups to Cyanogen Bromide Activated Pyridine Gels

Reaction of pyridine with cyanogen bromide results in a very polar pyridinium-complex-salt which, when cleaved in the presence of amines (aniline), yields either glutaconic-dianil (12) or phenylpyridinium salt (13). The mechanism of the ring closing reaction was investigated by Marvell et al. (14). Taking these facts into account, two kinds of binding mechanism are possible: a coupling reaction via a Schiff base mechanism or a binding reaction that forms back a pyridine ring, with the nitrogen deriving from the amino groups of the coupled compound, as shown in the lower right side of Fig. 1. Although the reaction of cyanogen bromide treated pyridine with an amine is quantitative and can be used for the quantitative determination of CN^+ (15,16), the cleavage of polymeric pyridines is much more difficult and gives only an 18% yield in the case of poly-(4-vinylpyridine) (17). The same authors obtained a slightly red colored dianil of glutaconic-aldehyde in the polymer when they reacted polyvinylpyridine in ethanol or ether with cyanogen bromide in presence of aniline.

Because enzymes cannot be coupled in water-free solutions, we hydrolyzed the cyanogen bromide activated pyridine rings with water before coupling the protein to the gel. The reaction products obtained were slightly yellow. Pyridine glass beads seemed to be the most convenient medium with which to follow the reaction, because the UV absorption spectra can be measured easily when the gels are suspended in $CHCl_3$ and packed into a cuvette. Measurement was made on a Cary spectrophotometer with microcuvettes of 2 mm thickness. Since the pyridine glass beads (gel II) were prepared from amino containing glass beads and pyridine aldehyde, as described above under Methods, the Schiff base formed was reduced with $NaBH_4$ at pH 7 and 0°C (gel III). The activation with cyanogen bromide in dioxane and the hydrolysis followed by coupling of aniline was carried out in the same manner as described for coupling of trypsin to the same gel. The reduced gel III showed UV absorption in the range of 230 nm when reacted with cyanogen bromide and aniline. Dianil of glutaconic-aldehyde has absorption maxima at 407 and 485 nm (18). In our case, no absorption in this range was observed, indicating the formation of the closed pyridinium structure.

In another experiment, the lysine-Cu-complex (14) was coupled to gel III, and the copper was used to protect the α-amino group during the coupling procedure. The copper was released by rapid treatment with 2 *N* HCl and the beads rinsed with water. A positive ninhydrin test indicated

bound lysine on the beads. The beads were then treated during 24 h at 35°C with 1 *N* HCl, conditions under which a Schiff base is hydrolyzed (19). The gel and the filtrate were tested with ninhydrin reagent. The gel showed strong blue color, while the filtrate showed only a faint color, which had a different absorption spectrum than the product of the reaction between lysine and ninhydrin. In addition, the infrared spectra (KBr tablets) of gel II and gel III coupled to lysine before and after hydrolysis showed no absorption in the range of Schiff base or aldehyde. These results strongly indicate the reconstitution of a pyridine ring.

Binding of Enzymes

Gels based on polysaccharide (Sepharose CL-4B), substituted polyacrylamide (enzacryl polythiol) and controlled pore glass matrices were synthesized as described above under Methods and shown in Fig. 1. As pointed out, the activation with cyanogen bromide has to be carried out under anhydrous conditions. For gels based on polysaccharides and polyacrylamides (Enzacryl polythiol being an exception because it can be stored dry without diminution of its properties), it is recommended to dehydrate the gels immediately before activation to avoid too much shrinking, which results in less capacity for binding of protein. It was observed that with aged gels, stored under absolute dioxane, shrinking continues further and cannot be reversed completely by the short hydrolysis period following the activation. Pyridine-containing gels on rigid matrices (e.g., pyridine glass beads) can be stored for prolonged periods of time under dry conditions and used immediately whenever required for coupling. All the gels prepared were very stable, and the enzyme activity remained constant for at least six months when stored under sterile conditions at 4°C as filtered cakes moistened with 0.1 *M* NaCl solution.

Properties of Bound Enzymes

Dependent on the gel matrix and on the activation, different amounts of trypsin could be bound to the gels (Table 1). Up to 117 mg of enzyme per g dry gel could be bound. A comparison of columns 1, 2, and 3 of Table 1 show that there is no relationship between the amount of enzyme bound and the number of pyridine groups on the gel. The reason may be that there is no quantitative cleavage of substituted pyridine rings to form the reactive aldehyde groups (17). We found that with the reaction conditions used, pyridine rings that have been substituted in the 4-position with $-CH_2-CH_2-$ give much better yields of aldehyde than those which were prepared via pyridine-4-aldehyde (Table 1). Chymotrypsin was bound and

TABLE 1. **Some Properties of Pyridine Gels**

Gel	Amount of pyridine per dry gel (μmol/g)	Trypsin bound per wet gel (mg/g)	Trypsin bound per dry gel (mg/g)	pH optimum	$K_m \times 10^{-6}$ (mol)	V_{max} (U/g packed gel)	V_{max} (U/mg trypsin)	Activity: calc. from V_{max} (%)
(I)	800	26.8	117	9.5	0.42	250	9.4	43
(II)	100	2.4	6.2	9.5	0.23	12.4	5.3	23
(IV)	930	3.8	43	9.5	0.32	11.6	3.1	14
(V)	216	12.4	104	10	4.89	109	9	41
Soluble trypsin				8–9	1.06		22.15	100

gave similar results; e.g., 8.5 mg chymotrypsin per g dry gel could be immobilized on (II) with a specific activity of nearly 100% as determined on ATEE as substrate. Chymotrypsin was also coupled to gel V. The best coupling conditions of chymotrypsin to both gels were identical with the best conditions used for trypsin. The pH optimum of immobilized chymotrypsin was shifted to pH 9, while that of trypsin was shifted to pH 9.5–10. In some cases, the apparent Michaelis constant showed values below that of soluble enzymes (Table 1).

It is well known that immobilization of trypsin can enhance its stability against 8 *M* urea, as described for copoly-(maleic acid-ethylene)-trypsin (7) and for hydrazide containing gels activated with glutaraldehyde (2). The pyridine gels were treated with a solution containing 8 *M* urea in 0.1 *M* NaCl at 22°C for several days (Table 2).

To test whether reduction of potential Schiff base bonds alters the stability of the gel against 8 *M* urea, trypsin immobilized on gel IV was treated with the $NaBH_4$ as described above under Methods. Even though in all cases a decrease in activity was observed after storage in 8 *M* urea, some stabilization was also evident, since after three days, the enzyme was still active, while soluble trypsin completely lost its activity within 90 min. The reduction had little effect on the stability since very few, if any, of the lysines of the enzyme are bound through Schiff bases; most of them reformed the pyridine ring. The Schiff base through which gel IV was prepared is stabilized by resonance; therefore its hydrolysis in 8 *M* urea is unlikely and cannot change the behavior of the bound enzyme. A conclusion that can be drawn from these results is that trypsin is bound to the pyridine gels with fewer bonds. Therefore, it behaves more like the native enzyme, whereas in the case of enzymes bound through Schiff bases (2), enhancement of activity was observed by treatment with 8 *M* urea.

TABLE 2. **Stability of Some Gels in 8 *M* Urea**

Gel	Initial activity (U/g gel wet)[a]	Activity remaining after 1 day[b] (%)	Activity remaining after 3 days[b] (%)
(I)	304	17	11
(II)	38	86	70
(IV)	17	24	14
(IV), reduced with $NaBH_4$	13	27	26
(V)	173	25	19

[a]The activity was measured in a solution containing 8 *M* urea and 0.1 *M* NaCl.
[b]Initial activity = 100%.

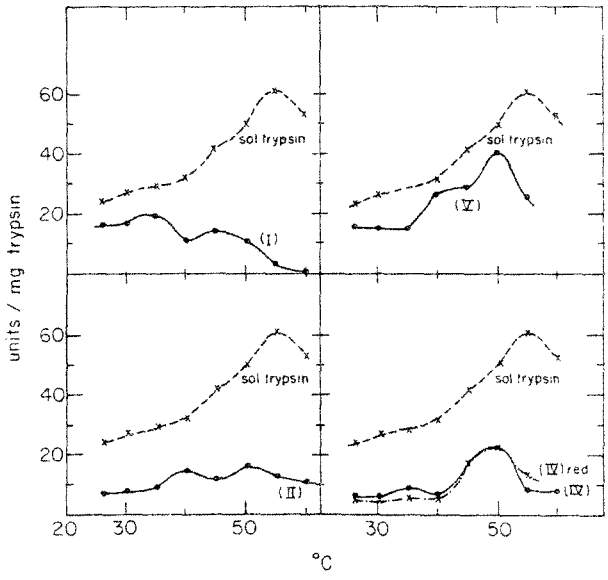

FIG. 2. Influence of temperature on the activity of trypsin immobilized on various preparations.

Influence of Temperature on Enzyme Activity

As described in the preceding paper (2), trypsin immobilized on hydrazide gels always gave two maxima of activity in the 26–60°C range, one being at 35°C and the second one situated between 45 and 50°C. In the trypsin immobilized on the pyridine gels (Fig. 2), two peaks (or at least one shoulder and one peak) are also observed: one is located at 50°C (with only one exception at 45°C), whereas the other peak shifts between 35 and 40°C. In the gels that were treated with $NaBH_4$, the peak at lower temperature was completely absent. The curve of activity resembled very closely that of the soluble enzyme, which has only one peak at 55°C. The possible reason for the double peaks of activity has already been discussed in (2).

Influence of Ethanol on Enzyme Activity

Soluble trypsin shows an increase of activity in presence of ethanol, having a maximum in a solution containing 30% alcohol, followed by a rapid decline of activity at higher concentrations (Fig. 3). Trypsin immobilized on gel V also shows an increase of activity, one maximum at a concentration of 20% ethanol and a smaller peak at a concentration of 60% ethanol,

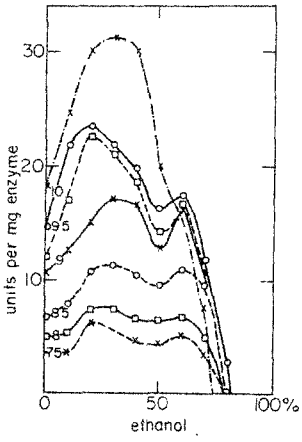

FIG. 3. Influence of ethanol on the activity of trypsin immobilized on gel V at pH 7.5–10.

followed by a rapid decrease of activity in solutions of higher concentration. Even at the minimum between these two peaks, the enzyme still had a higher activity than in the absence of ethanol. When immobilized trypsin is stored overnight in 96% ethanol at room temperature, the enzyme activity could be fully restored by removing the alcohol and washing the gel with 0.1 *M* NaCl. This is an indication that even though the enzyme was inactive in 96% ethanol (Fig. 3), it was not denatured. The native enzyme treated in the same manner was precipitated, but no efforts were made to redissolve the enzyme.

Applicability of Pyridine Gels to Affinity Chromatography

As already demonstrated by Tsuji et al. (20), polyvinylpyridine directly quaternized with chloroethylnucleotide bases can be utilized for affinity chromatography. It was therefore of interest to see if the Koenig reaction (12) could be used to modify the pyridine-containing polymers for use in affinity chromatography. ε-aminocaproyl-D-tryptophan-methylester-containing gels were used to purify chymotrypsin (21). $NaBH_4$-treated gel IV (5 g) was made water free, activated with cyanogen bromide, and reacted with 100 mg ε-aminocaproyl-D-tryptophan-methylester in 5 ml DMF and 7 ml 0.25 *M* phosphate buffer, pH 5. Small columns containing 200 mg of packed gel were equilibrated with 0.05 *M* buffer solutions of pH 5–9. Through each column, 5 mg of chymotrypsin in 2 ml of 0.05 *M* buffer was passed and the columns washed with buffer. Only at pH 7.5 was the amount of chymotrypsin adsorbed to the gel significant (64%), while at other pH values, the gels did not adsorb chymotrypsin more than the parent gel (10–20%). It seems that between pH 7 and 8 there is some binding due to

affinity, although in no case can hydrophobic and ion exchange interactions be fully excluded. On the other hand, the pyridine gel itself may behave as an affinity gel for chymotrypsin due to its aromaticity.

CONCLUDING REMARKS

In the preceding paper (2), we described some aldehyde-containing gels that were prepared by reaction of hydrazides with glutaraldehyde, and here we have described gels containing aldehyde that were prepared directly from compounds on the polymer. In both cases, there are advantages and disadvantages. In the case of glutaraldehyde, large amounts of protein can be coupled, but its preparation causes some difficulties due to cross-linking. In the case of pyridine-containing gels, even though the amount of protein coupled is not very high, the amount of aldehyde formed can be controlled, and several enzymes can be coupled consecutively, since we have shown that the enzymes are not active, but stable, in organic solvents. Another great advantage of cyanogen bromide activated pyridine derivatives for coupling of protein (and other NH_2-containing substances) is the fact that this procedure can be carried out not only under neutral conditions, but also at pH 5. In most cases, this results in the coupling of even larger amounts of protein to the gel. These conditions are very important for proteases like trypsin or chymotrypsin, which undergo self-digestion under neutral conditions. At pH 5, almost no self-digestion occurs; therefore it is possible to bind more intact protein to the carriers. Further studies on the improvement of these methods are in progress.

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