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IMMOBILIZATION OF PROTEIN ON ALDEHYDE-CONTAINING GELS—I. ACTIVATION OF HYDRAZIDE GEL WITH GLUTARALDEHYDE

FRITZ PITTNER,[1] TALIA MIRON, GISELA PITTNER, and MEIR WILCHEK

Department of Biophysics, The Weizmann Institute of Science
Rehovot, Israel

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Several carriers containing aldehyde groups are described. The carriers were prepared through spacers containing hydrazide groups, followed by glutaraldehyde. Trypsin was coupled to all the carriers, and the influence of temperature, urea, and organic solvents on activity, as well as kinetic parameters of the bound enzyme, were studied. Since all the carriers were prepared similarly, and the same enzyme was coupled to them, similar behaviors were obtained, e.g., two maxima of activity for temperature and organic solvents. Even though all carriers show similar properties for the immobilized enzyme, their preparation and behavior is not always predictable.

INTRODUCTION

Immobilized proteins have been prepared by covalent coupling of proteins to various insoluble carriers, such as polyamino acids, cellulose, polyacrylamide derivatives, glass beads, maleic anhydride copolymers (EMA), dialdehyde starch, beaded agarose, and cross-linked dextrans and nylons (1,2). Different reactions were used to couple the protein to the carriers, including the azide, carbodiimide, diazonium salt, acylchloride, cyanogen bromide, isothiocyanate, and anhydride (1,2). No attempt was made to compare the efficiency of these polymers as carriers by coupling the proteins to different matrices using uniform coupling methods.

In the following, we describe the conversion of some of the above mentioned carriers to hydrazides and the subsequent coupling of trypsin after activation with glutaraldehyde (3). The gels prepared are summarized in Scheme 1. Some of the physical and chemical properties gels are described and compared with each other.

[1]Permanent address: Institut fur Allgemeine Biochemie der Universitat Wien, Wahringer Strasse 38, A-1090 Wien, Austria.

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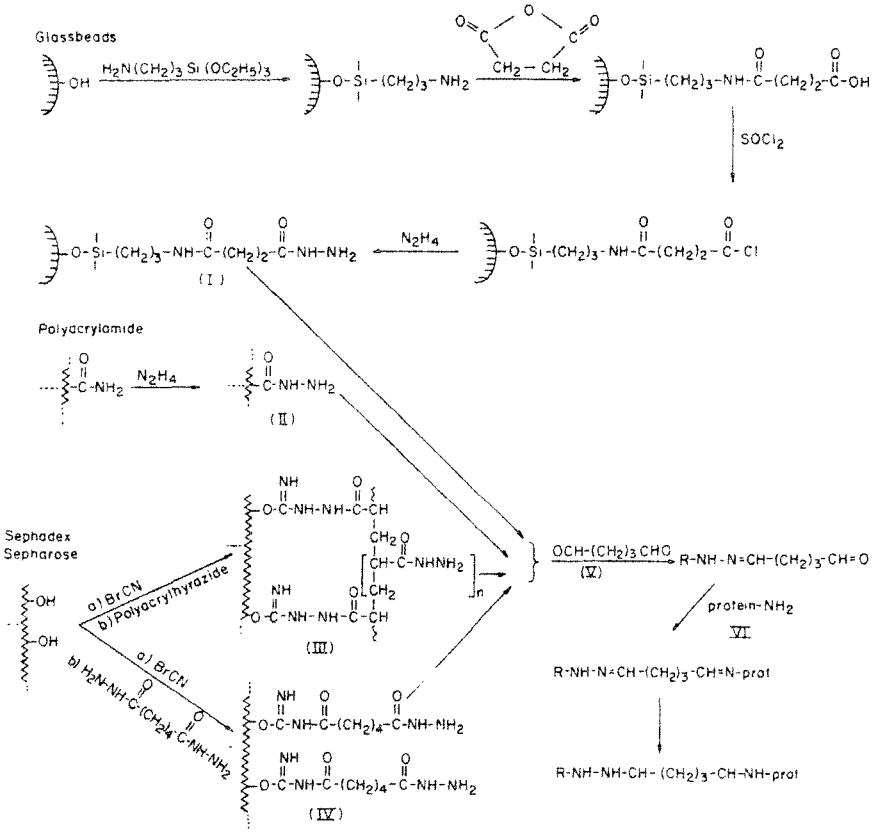

SCHEME 1. Synthesized gels.

MATERIALS

Sepharose 4B, Sepharose CL-4B, Sephadex G25 coarse, and Sephadex G200 were purchased from Pharmacia, Uppsala; Biogel P60 (50–100 mesh) was from Bio Rad, Richmond, Calif.; controlled pore glass PG-1000-200, 2,4,6-trinitrobenzene-sulfonic acid (TNBS), 3-aminopropyltriethoxysilane, and trypsin were from Sigma, St. Louis, Mo.; cyanogen bromide, methylacrylate glutaraldehyde (25%), and thionylchloride were from Fluka, Buchs, Switzerland; hydrazine-hydrate (98%) was from BDH, Poole, England; succinicanhydride and 2,4-dinitrophenylhydrazine were from Merck, Darmstadt; and fluorescamine was from Roche, Basle, Switzerland. *N*-benzoylargininеethylester-HCl (BAEE) was prepared by I. Jacobson at the Weizmann Institute.

METHODS

Polyacrylhydrazido-Sepharose (Scheme 1, III) was prepared according to Miron et al. (3). Packed Sepharose 4B and Sepharose CL-4B (10 g) were each washed with water, suspended in 20 ml 2 *M* K_2CO_3 solution, cooled to 0°C, and activated with 1 ml cyanogen bromide solution in DMF (1 g/ml) for 90 s. They were then filtered and washed with 50% aqueous *N,N*-dimethylformamide (DMF), followed by ice water. The activated gel was suspended immediately in 30 ml 0.2 *M* aqueous $NaHCO_3$ solution, containing 0.1–0.6 g water soluble polyacrylhydrazide (3), and the mixture was stirred for 16 h at 4°C. The gel was then filtered off and washed with 0.1 *M* NaCl solution, until samples of the washings showed no color when tested with TNBS (4). Gels containing 30–60 μmol of hydrazide per gram of packed gel were obtained and stored at 4°C as wet filter cakes. The quantity of available hydrazide groups on the matrix was determined by reaction with an excess of TNBS. The quantity of TNBS unreacted was measured and subtracted from its initial concentration.

Adipicdihydrazido-Sepharose (Scheme 1, IV) was prepared according to Lamed et al. (5). Sepharose 4B and Sepharose CL-4B were activated with cyanogen bromide as described above and treated with a 0.2 *M* solution of adipicdihydrazide (13) in 0.5 *M* $NaHCO_3$. Gels containing 4–14 μmol of hydrazide per gram of packed gel were thus obtained and stored at 4°C.

Polyacrylhydrazido-Sephadex (Scheme 1, III) was prepared by allowing either Sephadex G25 coarse or Sephadex G200 to swell in hot water for several hours, before being filtered, suspended in 2 *M* aqueous K_2CO_3 solution, and activated with cyanogen bromide for 90 s as described above. Activated Sephadex (10 g) was coupled immediately to 0.1–0.6 g of polyacrylhydrazide dissolved in 30 ml 0.2 *M* $NaHCO_3$, and the procedure was continued as described for the preparation of polyacrylhydrazido-Sepharose. The gels contained 30–50 μmol of available hydrazide groups per gram packed gel.

Adipicdihydrazido-Sephadex (Scheme 1, IV) was prepared from swollen Sephadex (G25 coarse or G200), which was activated as mentioned above and coupled to adipicdihydrazide in the same manner as with activated Sepharose. The amount of free hydrazide groups on the gel was 60–115 μmol per g packed gel.

Polyacrylamide-hydrazide (Scheme 1, II) was prepared according to Inman and Dintzis (6). Dry Biogel P60 (10 g) was allowed to swell overnight in water at room temperature. It was then filtered, suspended in 75 ml water, heated to 47°C, and then treated with 68 ml 90% $NH_2NH_2 \cdot H_2O$. The mixture was kept at 47°C, and after 1, 6, and 72 h, an amount of gel was removed from the reaction mixture, filtered, and washed with 0.1 *M* NaCl in

order to obtain gels containing different amounts of hydrazide. The amount of hydrazide per gram packed gel was determined with the aid of TNBS as 35–140 μmol.

Hydrazido glass beads (Scheme 1, I). Controlled pore glass was coated with 3-aminopropyltriethoxysilane and reacted with succinicanhydride followed by thionylchloride treatment (7). Hydrazine-hydrate (3 ml) was suspended in 50 ml absolute dioxane, and 3.5 ml glacial acetic acid was added slowly while cooling, followed by the thionylchloride-treated glass beads. The reaction mixture was kept at room temperature for several hours, and then the beads were filtered and washed with chloroform, water, and 0.1 *M* aqueous NaCl until the filtrate gave no reaction with TNBS. The amount of hydrazide groups on the gel assayed by the determination of nitrogen before and after the reaction with hydrazine was 80 μmol per g dry gel.

Glutaraldehyde activation of the hydrazide gels (Scheme 1, V) was performed with an excess of 10% aqueous glutaraldehyde solution for 4 h at room temperature. The gels were then filtered and washed with ice-cold water until the filtrate showed no reaction with 2,4-dinitrophenylhydrazine. The completion of the coupling reaction was indicated by a negative TNBS test.

Coupling of protein (Scheme 1, VI): The gels were shaken overnight at 4°C with a solution of trypsin (5–25 mg per g packed gel) in 0.25 *M* phosphate buffer, pH 7. The filtered gel was washed with buffer followed by 0.1 *M* aqueous NaCl solution until no protein could be found in the filtrate. The gels were stored as wet filter cakes at 4°C. Quantitative determination of immobilized protein was carried out on an amino acid analyzer after acid hydrolysis with 6 *M* HCl in a sealed tube for 22 h at 110°C and by determination with fluorescamine of the soluble protein remaining in the filtrate after coupling (8). Enzyme assay was made on a pH stat (Radiometer Copenhagen model TTT1c) (9,10). For the determination of the 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* NaCl solution, being 20 m*M* with respect to BAEE, and the uptake of 0.1 *M* NaOH was measured at pH 8.5 and 9.5 at 26°C, where the hydrazide gels show a maximum of activity (see Tables 2–4).

RESULTS

The Matrices

Some known and some new gels containing hydrazide groups were synthesized by the methods described in the experimental section and used

TABLE 1. **Ability to Bind Trypsin to Adipicdihydrazido-Sepharose Activated with Glutaraldehyde**

Storage of activated gels (days)	Trypsin bound per packed gel (mg/g)	Specific activity[a]	
		Trypsin bound (U/mg)	(%)
1	10.7	13.0	87
5	10.7	12.1	80
14	10.0	14.6	97
30	10.2	12.7	85

[a]The specific activity of soluble trypsin used in this preparation was 15 U/mg, as tested on BAEE.

in this work. By reaction of these gels with an excess of glutaraldehyde, free aldehyde groups were introduced into the hydrazide containing matrices, and were utilized for the coupling of proteins and other compounds containing amino groups. The general structures of these compounds are shown in Scheme 1. The gels containing hydrazide and glutaraldehyde can be stored for prolonged periods and used whenever required for coupling of the ligands. The activated derivatives were stored as wet filtered cakes at 4°C. For prolonged storage it is advantageous to keep them in a suspension of 0.1 *M* NaCl to prevent shrinking (1). Table 1 shows the ability of glutaraldehyde-containing derivatives to bind proteins after storage for up to 30 days. Similar results were obtained even after several months of storage. The data shown were obtained with adipicdihydrazido-Sepharose, but other carriers behaved similarly.

The Immobilization of Trypsin

Due to different amounts of reactive groups on the various gels used, different amounts of enzyme could be bound. The data for specific activities, pH optima, apparent Michaelis constants (K_m), V_{max}, and percentage of activity compared with that of the native enzyme are listed in Table 2. The apparent Michaelis constant was measured according to Goldstein et al. (11) in 0.5 *M* NaCl solution, with BAEE as a substrate at pH 9.5, 26°C on the pH-stat. The reason for the high ionic strength of the solution was to prevent the influence of unspecific charge interactions; the pH 9.5 used for measuring the activity was the average pH optimum of the gels. The Michaelis constant of soluble trypsin was determined under the same conditions, but at its pH optimum (pH 8.5), and it showed a value of 1.1×10^{-6} mol. The maximum velocity as determined by statistical analysis of the data is

TABLE 2. **Some Properties of Trypsin Immobilized on Unreduced Hydrazide Gels**[a]

Gels	Hydrazide per packed gel (μmol/g)	Protein bound per packed gel (mg/g)	pH optimum	$K_M \times 10^{-6}$ (mol)	V_{max} (U/g packed gel)	V_{max} (U/mg protein)	Activity at V_{max} compared to native enzyme (%)	Dry weight (mg/g packed gel)
Polyacrylhydrazido-Sephharose 4B	60	12.8	9.5	1.8	23.4	1.8	8.5	78
	42*	10.4	9.5	1.8	29	2.8	12.3	68
	34	8.5	9.5	1.6	32	3.8	17.3	76
Adipicdihydrazido-Sepharose 4B	13	3.9	9.5	1.8	40.6	10.4	47	78
	3.5*	4.5	10	2.2	49.5	11.0	48	82
Polyacrylhydrazido-Sephadex G25 coarse	49	12.5	9.5	3.1	60.5	4.9	22	307
	31*	15.5	10	2.5	84	5.4	25	325
Adipicdihydrazido-Sephadex G25 coarse	115*	10.3	10	4.1	54	5.3	24	302
	71	8.0	10	2.5	44.5	5.6	25	460
Polyacrylamide-hydrazide	141*	14.4	10	1.7	59	4.1	18.1	250
	56	13.0	10.5	3.1	46	3.5	16.2	240
Hydrazido-glass beads	10*	3.2	10	0.6	21.4	6.7	30	420
Soluble trypsin			8–9	1.06		22.1	100	

[a] All the gels mentioned in this table were treated with 20 mg of trypsin after activation with glutaraldehyde.

TABLE 3. **Some Properties of Trypsin Immobilized on Reduced Gels**

Reduced gels	Hydrazide per packed gel (μmol/g)	Protein bound per packed gel (mg/g)	pH optimum	$K_M \times 10^{-6}$ (mol)	V_{max} (U/g packed gel)	V_{max} (U/mg protein)	Activity at V_{max} compared to native enzyme (%)	Dry weight (mg/g packed gel)
Polyacrylhydrazido-Sepharose 4B	42	10	10	1.7	34	3.4	15	65
Adipicdihydrazido-Sepharose 4B	3.5	4.5	10	1.4	32.4	7.2	30	67
Polyacrylhydrazido-Sephadex G25 coarse	31	14.4	10	3.5	77.1	5.4	24	356
Adipicdihydrazido-Sephadex G25 coarse	115	4	10	2.6	68.6	17.1	36	350
Polyacrylamide-hydrazide	141	8	10	2.5	32.7	4.1	6	310

TABLE 4. **Influence of the Protein Amount on the Gel Properties**

Gel (μmol hydrazide per g packed gel)	Amount of protein			Percent activity: U/mg enzyme bound / U/mg soluble enzyme		$K_{M(app)}$ (μmol)	pH optimum	Dry weight (mg/g packed gel)
	Used for coupling (mg/g packed gel)	Bound (mg/g packed gel)	Bound (mg/g dry gel)	0.1 *N* NaCl[a]	0.5 *M* NaCl[b]			
Polyacrylhydrazido-Sepharose CL-4B (60 μmol/g)	2	2	26	96	51	3.8		
	5	5	66	62	29	4.4		
	10	8.1	106	56	19	6.0	10	76
	15	12.4	163	41	13	4.7		
	25	18.4	242	30	10	6.7		
Adipicdihydrazido-Sepharose CL-4B (10 μmol/g)	2	2	28	37	20	0.86		
	5	3.7	51	32	23	1.6		
	10	5.4	75	18	18	0.93	9.5	72
	15	8.3	113	13	8	0.66		
	25	15	208	7	6	0.73		
Polyacrylhydrazido-Sephadex G200 (45 μmol/g)	2	2	12.4	77	36	5.6		
	5	4	25	70	31	6.6		
	10	8.5	53	36	21	4.1	9.5	160
	15	10.5	66	27	8	5.5		
	25	19.6	122	25	8	12.9		

	2	2	10	60	51	5.1		
Adipicdihydrazido-	5	3.4	17	41	40	1.6		
Sephadex G200	10	5.1	26	12	7	1.8	9.5	193
(60 μmol/g)	15	7.2	37	7.5	7	1.4		
	25	10	52	7.8	5	1.7		
	2	2	12	37	13	2.9		
Polyacrylamide-	5	3.9	23	21	17	4.0		
hydrazide	10	6.9	40	15	5	4.1	9.5	172
(36 μmol/g)	15	8.9	52	13	4	4.2		
	25	8.8	51	10	4	1.5		
	2	2	5	43	9	3.5		
Polyacrylamide-	5	5	13	30	7	6.1		
hydrazide	10	10	26	27	3	4.5	10	380
(450 μmol/g)	15	15	40	21	3	8.3		
	25	14.2	38	17	4	9.5		
Hydrazido-glass beads	2	2	5	63	58	4.8		
(30 μmol/g)	5	5	14	49	28	3.5		
	10	10	27	36	20	2.6	9.5	370
	15	15	40	28	16	4.1		
Soluble trypsin				100	100	1.06	8.5	—

[a]The test carried out in 0.1 *M* NaCl and with excess substrate.
[b]Values calculated from V_{max} in 0.5 *M* NaCl.

22 U/mg enzyme [one enzyme unit (U) is defined as the cleavage of 1 μmol of substrate (BAEE) per minute by the enzyme (trypsin)]. In addition to the gels listed in Table 2, sodium borohydride treated gels were prepared in order to investigate the possible influence on stability and activity of the Schiff-base double bonds. For this purpose, we chose the gels indicated with an asterisk (*) in Table 2, suspended them in 0.25 *M* phosphate buffer, pH 7, and treated the suspension with a 6- to 10-fold excess of sodium borohydride at 0°C for several hours. Their properties are summarized in Table 3.

Capacity

In order to investigate the capacity of the gels to bind protein, batches of glutaraldehyde-activated gels were reacted with increasing amounts of protein. The data are listed in Table 4. Up to 20 out of 25 mg protein added could be bound per gram of packed gel. In order to determine the amounts of enzyme bound per gram of dry weight, batches of the gel were dried, and the weight in mg/g of packed gel was determined (Table 4, column 7).

Stability

No leakage of protein was detected during a period of more than six months. In the same period, no decrease of enzyme activity could be

TABLE 5. **The Change of Activity with Time at Room Temperature and 35°C**

Gels	Treatment[a]	Initial activity (U/g packed gel; 24°C)	Percent activity after 2 months (24°C)	Initial activity (U/g packed gel; 35°C)	Percent activity after 2 months (35°C)
Polyacrylhydrazido-Sepharose 4B	Unreduced	150	61	450	22
	Reduced	115	51	202	63
Adipicdihydrazido-Sepharose 4B	Unreduced	132	55	228	22
	Reduced	90	62	122	40
Polyacrylhydrazido-Sephadex G25	Unreduced	238	78	437	38
	Reduced	152	73	292	54
Adipicdihydrazido-Sephadex G25	Unreduced	172	73	372	45
	Reduced	165	76	282	55
Polyacrylamide-hydrazide	Unreduced	250	54	515	16
	Reduced	70	63	112	51

[a] Reduction was carried out with $NaBH_4$ as described in the text under Methods.

observed when the gels were stored as wet filtered cakes under sterile conditions at 4°C. In order to obtain data about the stability of the gels under working conditions, the change of activity with time both at room temperature and at 35°C was investigated. The selected gels (Table 2, *) were suspended and stored in 0.1 *M* NaCl solution, and the pH was adjusted to 9.5. All gels kept at room temperature (24°C) showed a rapid decrease of activity within the first day, which then slowed down to reach values in the range of 50–70% of initial activity, even after storage for two months. Gels that were treated with $NaBH_4$ gave approximately the same values as the unreduced preparations. Storage at 35°C resulted in a rapid initial drop of activity. The values reached after two months storage were in the range of 16–38%. The $NaBH_4$ treated gels, which showed in general lower initial activity than their parent unreduced forms, reached values of 40–63% of initial activity after two months storage (Table 5).

Enzyme Activity

The dependence of enzyme activity on different temperatures was measured by suspending unsolubilized trypsin (4–10 mg packed gel) in 4 ml 0.1 *M* NaCl at various temperatures. The enzymatic activity was followed

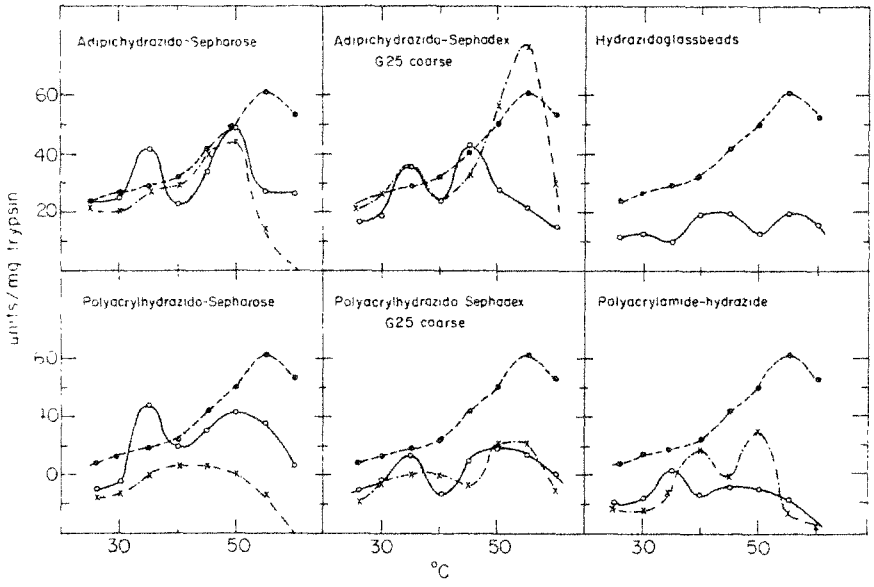

FIG. 1. Influence of temperature on the activity of trypsin immobilized on various preparations: ●--●, soluble trypsin; ○——○, native gel; ×--×, reduced gel.

by adding a solution of 20 mg BAEE in 1 ml of 0.1 *M* NaCl and measuring the uptake of 0.1 *M* NaOH at pH 9.5, at various temperatures (Fig. 1). The range of investigation was between 26 and 60°C, because at higher temperatures, the thermal cleavage of the ester was very fast, and some gel structures appeared to crack. As demonstrated in Fig. 1, the unreduced gels always showed two peaks of activity, one being at 35°C, and the other being at 45–50°C. The activity at the maxima was of comparable intensity. The position of the minimum is situated at 40°C. The hydrazido glass beads also show two maxima. The peak of lower temperature appears at 40–45°C, while the other peak appeared at 55°C. The reduced gels also show two maxima of activity, the second peak was always higher than the first. The first maximum is situated between 35 and 40°C, the second one at 50–55°C, the minimum being at 45°C. Only a shoulder could be observed at lower temperatures for adipicdihydrazido-Sepharose, while the polyacrylhydrazido-Sepharose showed only one peak at 40–45°C. Upon cooling to room temperature, the gels initially contained higher activity, which gradually reached the value of the unheated enzyme after 270 min (Table 6).

It is well known that 8 *M* urea causes a decline of activity, as was shown by Levin et al. (10) in the case of copoly-(maleic acid-ethylene)-trypsin, which resulted in a small residual activity of only a few percent after treatment for 24 h at room temperature. For comparison we decided to study the effect of 6 and 8 *M* urea on enzyme activity of the hydrazido-gels. In 6 *M* urea (0.1 *M* with respect to NaCl), hydrazido-gels showed 40% less activity, compared to a sample that was measured in the absence of urea. During 24 h, no further decrease of enzyme activity could be observed.

TABLE 6. **Behavior of Preheated Immobilized Trypsin Upon Cooling**[a]

Interval (minutes at 26°C)	U/g packed gel after preheating to	
	35°C	50°C
10	101	85
25	92	84
45	81	77
90	78	77
270	71	83

[a]Batches of trypsin immobilized on polyacrylhydrazido-Sephadex G25 coarse (10 mg) were suspended in 4 ml 0.1 *M* NaCl and kept at 35 and 50°C for 10 min. The gels were cooled to 26°C and tested for activity after various periods. Untreated gel at 26°C shows an activity of 69 U/g packed gel.

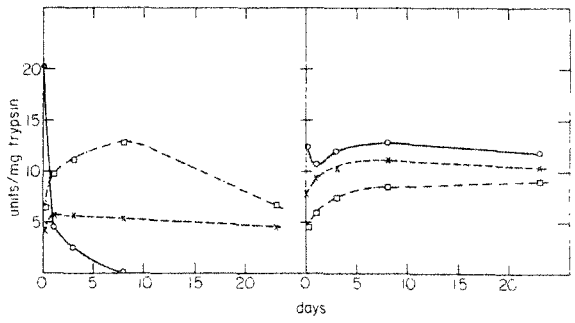

FIG. 2. Effect of 8 *M* urea on the activity of immobilized trypsin. Left: O——O, hydrazido glass beads; × – – ×, polyacrylamidehydrazide; □ – – □, polyacrylhydrazido-Sepharose. Right: O——O, adipichydrazido-Sepharose; × – – ×, adipichydrazido-Sephadex; □ – – □, polyacrylhydrazido-Sephadex.

Treatment of the hydrazide gels at room temperature with 8 *M* urea (being 0.1 *M* with respect to NaCl) also resulted in an initial loss of activity of 60% compared to a sample measured in the absence of urea. This amount of activity remained constant for several hours and showed a slight increase by the end of 24 h. The increase of activity continued, in most cases reaching its maximum after 3–8 days, depending on the gel (Fig. 2). The only exception to this was with the hydrazido-glass beads, which showed a rapid and continuous decrease in activity. The native enzyme, treated in the same manner, was completely inactive after only 90 min. To investigate the activity after removal of urea, two experiments were carried out. In the first, the polyacrylhydrazido-Sephadex gel was incubated with 8 *M* urea for 3 days, and then assayed in 8 *M* urea. Then the gel was filtered, washed with 0.1 *M* NaCl, and the activity measured in 0.1 *M* NaCl. In the second

TABLE 7. **Reactivation of Immobilized Trypsin in 8 *M* Urea**

Incubation	U/g packed gel
0.1 *M* NaCl at $t = 0$	128
8 *M* urea at $t = 0$	110
8 *M* urea at $t = 3$ days	147
0.1 *M* NaCl after quick removal of urea at $t =$ days	115
0.1 *M* NaCl after slow removal of urea at $t = 3$ days	115

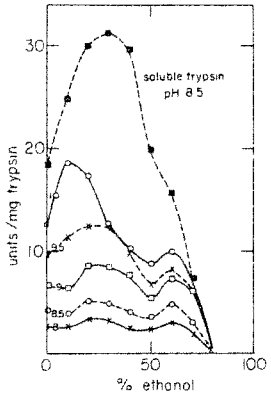

FIG. 3. Influence of ethanol on the activity of trypsin immobilized on polyacrylhydrazido-Sepharose at pH 8–10.

attempt, after incubation for 3 days in 8 *M* urea, the concentration of urea was reduced gradually down to 0.05 *M*. Then the gel was filtered off, washed with 0.1 *M* NaCl, and the activity assayed in 0.1 *M* NaCl. In both cases the original activity was regained (Table 7).

To study the influence of other organic solvents on the immobilized enzyme, the activity of trypsin bound to polyacrylhydrazido-Sepharose was measured in the presence of increasing amounts of ethanol and dioxane (dielectric constants of 24.3 and 2.2, respectively). Two distinct maxima, depending on ethanol concentration and slightly shifting with pH, were observed (Fig. 3). A similar behavior was found with dioxane, the bound enzyme having two maxima of activity, one at 10% and the other at 30% dioxane (Fig. 4). With increasing concentration of organic solvents, the native trypsin also showed increased activity, the maximum being at 30% in the case of ethanol and at 20–30% with dioxane (Figs. 3 and 4). No change of the apparent pH optimum by the organic solvents could be detected. Treatment of the gel with 96% ethanol and absolute dioxane overnight at

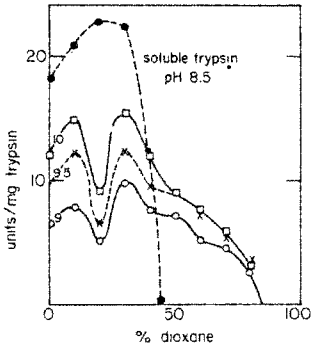

FIG. 4. Influence of dioxane on the activity of trypsin immobilized on polyacrylhydrazido-Sepharose at pH 9–10.

room temperature, removal of the organic solvents, and careful washing with 0.1 *M* NaCl did not result in any loss of activity.

DISCUSSION

The purpose of this study was to compare different carriers containing the same functional group to which trypsin was bound by the same bond in order to determine which carrier could be considered the carrier of choice when one immobilizes an enzyme. Unfortunately, it was impossible to draw such a conclusion from our studies, since each gel behaved differently, and sometimes the same gel gave different results when reacted under exactly the same conditions, which might result from the fact that the reaction of glutaraldehyde is not always reproducible. Though used in large excess, cross-linking reactions may occur, and the amount of hydrazide groups on the matrix is no criterion for the aldehyde groups, which can react with the enzyme. The hydrazide- and glutaraldehyde-containing gels are very convenient, since they can be stored for prolonged periods and used for immobilization of proteins under mild conditions.

Properties of Various Matrices

Polyacrylamide hydrazides are easy to prepare, and the amount of hydrazide on the matrix can be regulated by changing the time of hydrazinolysis. On the other hand, this type of gel contains large amounts of water (especially when hydrazinolyzed during longer periods), which results in a shrinking process during storage. These aged gels showed less ability to bind trypsin, and also the specific activity of the immobilized enzyme derived from aged gels was lower than that of freshly prepared hydrazides. One explanation for this effect might be cross-linking reactions between aldehyde groups and the amide groups of the polymer. This well-known reaction was used by Weston and Avrameas (12) for activation of polyacrylamides with glutaraldehyde.

Hydrazido glass beads are more difficult to prepare, because under alkaline conditions (pH > 10), the coating with triethoxysilanes is not stable, and mechanical stirring destroys the beads. However, they bind comparatively large amounts of protein (15 mg trypsin per g wet beads out of 15 mg; Table 4). Concerning preparation of aldehyde gels based on polysaccharides (Sepharose or Sephadex), one can conclude that Sepharose CL-4B is more convenient than the noncross-linked form of Sepharose 4B, since more protein can be bound.

Polyacrylhydrazido-Sephadex matrices (G200 and G25) generally bind more trypsin than the adipicdihydrazido-Sephadex. Unexpectedly, several attempts to bind active trypsin to Sephadex G25 fine failed completely, even though one would expect higher binding due to the larger surface available compared to Sephadex G25 coarse. When reacted with small amounts of enzyme (2–5 mg), all the protein was bound, but the behavior of the gels containing 2 mg of enzyme was different. The specific activity varied from as high as 96% to as low as 37%, while the $K_{m(app)}$ varied from a value which was slightly better than that of the soluble enzyme to one which was sixfold worse. When the specific activity was calculated from V_{max}, the changes were even more drastic: from 51% to as low as 9%. There was no dependence or correlation of variation of specific activity on the change with the K_m. The results show that with an increase of trypsin bound, the specific activity gradually decreases. Gels with large pores that contain only low amounts of hydrazide show apparent Michaelis constants in the range of the soluble enzyme or even slightly better. When higher amounts of enzyme were added, not all the protein added was bound, and there was no strong dependence on the amount of reactive groups (hydrazide) present, since gels containing up to 450 μmol of hydrazide per g, e.g., polyacrylamide hydrazide, bound less protein than a gel that contained only 10 μmol hydrazide per g. The only property that was common to all gels was the shift in the pH optimum from 8.5 in solution to about 10 for the immobilized product. The reason for these irregularities may vary: not all of the hydrazide groups may be available to interact with glutaraldehyde, cross-linkage of the gels could occur, and the different shapes of the gels as well as smaller or bigger pore size may affect the results. In general, each polymer must contain a different network and therefore exhibit different behavior. Reduction of the gels with sodium borohydride did not significantly influence the pH optimum, the $K_{m(app)}$ nor the specific activity.

Table 2 also shows that in most cases, more hydrazide on the gel causes less specific activity of bound trypsin. A significant exception is adipicdihydrazido-Sephadex G25. This gel has very small pores, which do not allow proteins to penetrate, so that most of the aldehyde groups attached to the small adipicdihydrazido residues are hidden and are not available for reaction with trypsin; only the groups at the surface can react. The large polyacrylhydrazide, however, may have enlarged the surface of Sephadex G25.

Effect of Heat

Studies on the change of activity with time (Table 5) at pH 9 showed an initial drop of activity during the first day both at room temperature and at

35°C. The reason might be that not all enzyme molecules are tightly bound and some are released from the gel under these conditions. The reduced gels are more stable to prolonged storage at 35°C than their unreduced parent forms.

Investigations on the change of enzymatic activity with temperature showed two maxima of activity in all cases. Soluble trypsin has only one distinct maximum at 55°C. The reason for this might be a change of conformation of the bound trypsin at only discrete temperature values because of its immobilization, an effect that cannot be detected in the native enzyme, which is not hindered to change its conformation gradually with increasing temperature. Another possibility is that during immobilization of the protein, some of the enzyme is bound in such a manner that it results in destabilization at higher temperatures. It is active only up to 35°C, while the second peak results from an enzyme that behaves similar to the native one.

The first peak might represent activity of both the less stable and the more stable form. With increasing temperature, the less stable form loses it's activity, and only the activity of the more stable one remains. It starts to increase with temperature until it comes close to the temperature where the native enzyme has its maximum activity. Also, after this peak the activity drops fast. Reduction may stabilize the enzyme, since the specific activity is sometimes higher, as in the unreduced form (e.g., adipicdihydrazido-Sephadex, polyacrylamide hydrazide), while in some other cases it has the opposite effect.

A third explanation for the two peaks is a transformation from the first peak to the second one during the heating. In the range of the first peak, the enzyme may be bound through many Schiff bases, which are gradually hydrolyzed during the heating, thus binding the enzyme through less bonds and making it more similar to the native form. This may also explain the higher activity in some cases of the borohydride reduced gels, since the treatment with borohydride, which is usually done in alkaline conditions, may also be accompanied by hydrolysis of Schiff bases.

During the immobilization of protein, some of the enzyme might be bound to the gel in a nonnative conformation, while heating gives the enzymes a chance to rearrange themselves to the native state.

Effect of Organic Solvents

When the enzymatic reaction was carried out in solutions containing different amounts of ethanol or dioxane, two maxima of activity of the immobilized enzyme appeared. Only one maximum was observed with soluble trypsin. The two peaks in Figs. 3 and 4 could be explained in a similar manner as for Fig. 1. The first peak of activity represents a less stable

immobilized form, since already at low concentrations of organic solvent, activity is lost compared to native enzyme. The second peak of activity represents the most stable form, which is even more stable than the normal enzyme. This is shown by the slow tailing of activity, up to 80% in the case of dioxane. From here, one can see clearly that enzymes are sometimes stabilized by immobilization. The first peak of activity may also represent a form in which the organic solvent is more accessible to the binding site, maybe because some of the hydrophobic residues of the protein have been exposed. The reason for the tailing in Fig. 4 could also be attributed to the fact that the gels by themselves contain water, which helps the catalytic reaction, while the environment of the native form, after a certain concentration of organic solvent, becomes disordered. Another explanation for the increased stability of the immobilized enzyme in organic solvents might be the fact that after a certain concentration of organic solvent, a partitioning of the water between the organic solvent and the carrier can occur. Therefore, there is always some amount of water available for the catalytic reaction. In the case of ethanol, the pH also has a profound effect on its stability. It seems that at pH 10, most of the enzyme is present in exposed form, since the highest activity is already obtained at concentrations of ethanol that should not effect the stability of the native enzyme. A comparison of Figs. 3 and 4 indicates that the penetration of ethanol into the carrier, and thus the removal of its water, is much more efficient and is comparable to the penetration into the native enzyme. Comparing Figs. 3 and 4, it is very likely that the carrier can continue to keep water in the presence of dioxane, while it cannot do so efficiently in the presence of ethanol. At a concentration of about 80% of either solvent, all the activity is lost. The influence of temperature, ethanol, and dioxane on immobilized trypsin is reversible, showing that even though the activity was lost, the enzyme was not denatured. This again indicated that some water remaining on the gel was available for the bound enzyme.

Effect of Urea

Studies on the influence of 8 *M* urea showed an increased enzyme activity after several days storage in this medium (hydrazido glass beads being the only exception). This increase of activity may be due to the first peak that we have shown in Figs. 1, 3, and 4, and with 8 *M* urea a rearrangement to the more stable form occurs. The initial decrease of activity in the case of adipicdihydrazido-Sepharose might be due to a small amount of protein that is bound less tightly and is removed from the gel by the action of urea. This solubilized enzyme is lost by the action of urea, while the tightly

bound enzyme remaining on the gel is activated by the urea, which yields an increase of activity after one day's storage.

The influence of urea was also reversible. Since it is not logical that urea will enhance enzyme activity over the activity of the native form, we suggest that removal of urea causes the enzyme to go back to a disturbed conformation. This may indicate that there is a strong effect on the enzyme by the carrier, because it is unreasonable that without influence of the carrier the enzyme will go back to a less favorable situation. Only hydrazido glass beads show a continuous decrease in activity. There might be less multisite attachment to the glass beads than to the other carriers, and thus the enzyme behaves more like the native one, with some stabilization, since the activity is not decreasing immediately as in the case of the soluble trypsin. Another explanation is that due to the rigidity of this carrier, the enzyme is not affected by it, as is observed with the other more flexible gels. An additional reason for the decline of enzyme activity of trypsin bound to hydrazido glass beads might be the fact that coating of the glass beads with 3-aminopropyltriethoxysilane might not result only in a covalent coating. Some small clusters of silicium-containing polymer may also be entrapped in holes on the gel surface. These are capable of binding protein, but are only attached to the gel matrix by noncovalent bonds. Because of the action of urea, such bonds can be weakened, and the protein thus released from the gel gradually undergoes a loss of active structure. It cannot lose its active configuration as quickly as the native enzyme, because it is still immobilized on smaller, more flexible particles with a slight protective effect. Therefore, it takes a few days until it loses its activity.

To summarize, we have introduced stable and efficient new gels containing aldehyde groups. The immobilized trypsin, even though it behaved differently from the enzyme in solution in regard to its activity in the presence of organic solvents and 8 M urea, was more stable and therefore can be more efficiently used. The use of these gels in immobilization of other proteins and ligands is still in progress.

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