Procedure for Covalently Immobilizing Enzymes Which Permits Subsequent Release†

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ABSTRACT: A new procedure for immobilization of proteins through covalent attachment to glass surfaces via thioester-containing coupling chains has been achieved. Porous succinamidopropyl-glass beads were converted into the acyl chloride derivative by using anhydrous thionyl chloride and then treated with either 3-mercaptopropionic acid or mercaptoacetic acid. Thus derivatized, the beads were dried in vacuo; they could then be stored for at least 6 months without detectable loss of reactivity with proteins. Immobilization of protein can be readily achieved simply by suspending the derivatized beads in buffer (pH 5–8) and recycling the protein solution through the beads. In studies with α-chymotrypsin, 10–20 mg/g could be conveniently immobilized by this procedure. Experiments with model compounds revealed that immobilization to such derivatized beads occurred by reaction with amino groups. Following immobilization, protein molecules can be released from the surface by cleavage of the coupling chains at the thioester linkage through treatment with 1.0 M hydroxylamine at pH 7.0. Protein thus released contains a free sulfhydryl group at each site of previous attachment, which can be analyzed quantitatively. Application of the immobilization and release procedures to chymotrypsin permitted direct assessment of the kinetic effects of pore diffusional inhibition on the immobilized enzyme preparations and revealed no inherent alteration of the enzyme's kinetic behavior as a result of the chemical derivatization per se.

Covalent coupling of proteins to solid supports generally relies on common organic reactions. Most of the methods which have been used were reviewed by Mosbach (1976) or by Zaborsky (1973).

Generally, characterization of immobilized enzymes has been limited to kinetic studies or to examination of fluorescent spectra (Gabel et al., 1971; Horton & Swaisgood, 1976; Swaisgood et al., 1978). This restriction severely limits the application of immobilization techniques for studies of reformation of tertiary structure or quaternary structure in proteins. However, the capability of selectively releasing an immobilized protein following various experimental operations would allow the application of many additional biochemical techniques for examination of structural features.

The possibility of releasing protein immobilized through an azo linkage with sodium dithionite or of releasing thioester-linked protein with hydroxylamine or high pH had been suggested by Cuatrecasas (1970a). More recently, Chan & Mosbach (1976) have reported a procedure for reversible immobilization based on a disulfide linkage. An attempt to form a selectively cleavable covalent bond, by incorporating a thioester linkage, was reported by Brown & Horton (1973). Carbodiimide-activated succinylated glass beads (Brown et al., 1972) were treated with 2-mercaptoacetic acid; the derivatized glass was then treated with 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC)[1] and finally exposed to the protein-containing solution. Subsequently, immobilized protein could be removed from the glass surface by cleaving the thioester bonds with hydroxylamine, but the yield of released protein was low (~15% of that immobilized).

One of the problems often encountered in studies of immobilized enzymes is elucidation of the factors involved in altered kinetic patterns. The catalytic rates of enzymes may be affected in several ways by immobilization: (1) by changes in specific rate constants as a result of conformational changes in the enzyme's structure per se, alteration of its microenvironment, or steric hindrance of substrate access; (2) by partitioning of solute molecules (including substrates and products) due to specific interactions with the matrix; and (3) by diffusion inhibition (Engasser & Horvath, 1973; Goldstein, 1976; Kobayashi & Laidler, 1973; Cho & Swaisgood, 1974). It has been very difficult to distinguish among these various kinetic effects while an enzyme remains immobilized. Removal of an immobilized enzyme from its supporting matrix in such a way as to retain a portion of the chain involved in the previous covalent attachment would provide a feasible means for investigation of conformational changes or steric hindrance directly related to the immobilization procedure. Also, the effect of the microenvironment of the matrix surface itself could be thus eliminated, so that the effects of partitioning and diffusion can be clearly separated experimentally from intrinsic changes in specific rate constants.

We describe here a new method for covalent immobilization which allows for subsequent release of the protein by very mild treatment with hydroxylamine. Furthermore, in contrast with immobilization by formation of a mixed disulfide (Chan & Mosbach, 1976), the procedure described herein provides a covalent linkage between the protein and the matrix which is stable in the presence of thiols, a feature of considerable importance when one wishes to examine the aquisition of disulfide bond containing tertiary structures (Janolino et al., 1978).

Methods

α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas was obtained as a lyophilized powder from Miles Laboratories or Sigma Chemical Co. and used without further purification.

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[1] Abbreviations used: EDC, 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide; BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester.

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All chemicals used were reagent grade or better.

Protein was immobilized on the surface of porous glass beads (obtained from Sigma Chemical Co.) by covalent binding via succinamidopropylsilane chains. Beads were cleaned with concentrated nitric acid in a boiling water bath for 1 h and then rinsed until the pH reached 7.0. The beads were silanized and succinylated as described by Cho & Swaisgood (1974). The succinamidopropyl-glass beads were washed with water, dried in a vacuum oven, and then stored dry until needed for protein immobilization. As an indication of completion of the succinylation reaction, a small sample of the beads was treated with 2,4,6-trinitrobenzenesulfonate. Absence of red-orange color was taken as evidence of near completion of the reaction (Cuatrecasas, 1970b). The number of chains immobilized per gram of glass was measured by quantitation of nitrogen by use of the micro-Kjeldahl–microdiffusion technique of Janolino & Swaisgood (1975).

Succinamidopropyl-derivatized glass was converted to the acyl chloride derivative by treatment with anhydrous thionyl chloride. Care was taken to prevent the introduction of water in each of these reaction steps. The reaction mixture was held at 70 °C for 1 h. The solution was then cooled to 25 °C, and excess thionyl chloride was drained from the beads, which were then rinsed thoroughly with dry dichloromethane. The beads were next covered with anhydrous 3-mercaptopropionic acid and again held at 70 °C for 1 h. Following reaction, the excess acid was drained and the derivatized beads were again washed with dichloromethane. After thorough drying at 125 °C in a vacuum oven, the beads were stored in the absence of solvent until needed for protein attachment.

Protein attachment to the thioester beads was achieved by degassing the beads in buffer and then recycling the protein solution through the beads in a fluidized bed configuration for several hours. For determination of the amount of protein immobilized, all protein not covalently bound to the beads was removed before amino acid analysis by rinsing with (per gram of beads) 1.2 L of H_2O (or 0.1 ionic strength phosphate buffer) and then with 400 mL of 3.3 M urea made 0.01 M in imidazole, followed by 1.2 L of buffer. Samples of beads were subjected to hydrolysis in 6 N hydrochloric acid at 110 °C for 24 h. Either a Beckman 116 or a Durrum 500 amino acid analyzer was used for the analysis; both were equipped with peak-integrating computers.

A portion of the immobilized protein was released from the matrix by cleavage of the thioester bond with a solution of 1.0 M hydroxylamine (pH 7.0) at room temperature.

Hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BzTyrOEt) by α-chymotrypsin was assayed according to the method of Hummel (1959). Reaction was initiated by addition of soluble enzyme to the reaction mixture, and reaction progress was monitored spectrophotometrically at 256 nm. In the case of immobilized enzyme, all reactants except the substrate were recycled through the beads and the reaction was initiated by addition of BzTyrOEt. Conversion of less than 2% of the substrate to product per pass through the beads allows the use of differential kinetics (Ford et al., 1972). These conditions were achieved by recycling a volume of 20 mL through less than 0.05 μmol of immobilized enzyme. BzTyrOEt concentrations were varied from 7.0 μM to 1.65 mM, where the limit of measurement by the spectrophotometer using a 1-cm path length cell was reached.[2] Initial slopes were read directly from the recorder chart, and kinetic constants were evaluated by using a computer program to fit data directly to velocity vs. substrate concentration curves.

[2] A 0.2-cm path length cell was used to measure kinetics of soluble enzyme at higher BzTyrOEt concentrations, up to 2.73 mM.

Table I: Covalent Immobilization and Release of α-Chymotrypsin from Thioester-Derivatized Glass Beads

	mg of α-chymotrypsin bound/g of glass[a]		
	before 1 M NH_2OH	after 1 M NH_2OH	% released
"nonactivated"	20.9	6.3	69.9
EDC-"activated"	4.4	4.1	6.8

[a] Solutions containing 1 mg/mL enzyme (50 mg/g of beads) at pH 7.0 (sodium phosphate, $\Gamma/2 = 0.1$) were recycled through both types of beads for 3 h at 8 °C. Half of each sample (0.8 g of beads) was rinsed with 100 mL of 1 M NH_2OH at room temperature. Prior to amino acid analysis, all samples were rinsed thoroughly with 3.3 M urea, 0.01 M imidazole, and water.

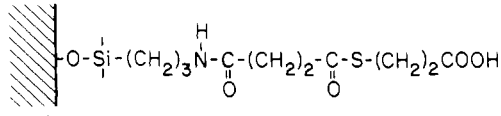

FIGURE 1: Postulated structure of the thioester–glass derivative.

Ellman's procedure was used for determination of total sulfhydryl groups in proteins (Ellman, 1959; Kalab, 1970). Urea was used as a denaturant to expose buried sulfhydryl groups before samples were assayed.

Samples were prepared for mass spectrometry by using a rotary evaporator to remove most of the moisture and then were freeze-dried. High-resolution mass spectra were obtained at Research Triangle Institute, Research Triangle Park, NC. All calculations and predicted structures were verified by computer analysis at Research Triangle Institute.

Results

All of these studies were performed by using 120–200 mesh size porous glass beads (1100-Å mean pore diameter) which were derivatized with succinamidopropyl chains at a level of 88 μmol of N per g. The thioester derivative was prepared, and at first, immobilization of protein was attempted by activating the carboxyl groups of the mercapto acid termini of the covalently attached chains by using a water-soluble carbodiimide (EDC). Surprisingly, however, such activation of carboxyl groups was found to be unnecessary. Indeed, under the conditions used, treating the beads with EDC prior to exposure to protein resulted in only 20% as much protein immobilized as when protein was added directly to the thioester-derivatized glass (Table I). Consequently, studies were initiated to characterize this glass derivative.

Following thioester derivatization, the beads become distinctly yellow to yellow-orange in color. The electronic absorption spectrum, obtained by placing the glass beads in carbon tetrachloride, which has a refractive index close to that of glass, indicated a broad absorption maximum centered around 400 nm. A high-resolution mass spectrum was obtained for the cleavage products resulting from 24-h hydrolysis in 6 N HCl. The largest fragment had a mass of 207.0319. Calculations (based on carbon-12 with all other atoms as their most abundant isotopes) predict a value of 207.0326 for the molecular mass (plus one hydrogen) of *S*-succinylmercaptopropionic acid. These results support the contention that a thioester derivative, as indicated in Figure 1, had been formed on the glass surface.

For investigation of the identity of the amino acid residue(s) which reacts with the thioester-derivatized beads, a number of model compounds, in which all but one of the functional

Table II: Model Compounds Tested for Reactivity with Thioester-Derivatized Glass Beads

compd	functional group available	amount bound (μmol/g)[a]
α-*N*-benzoyl-L-arginine ethyl ester	guanidino	0
α-*N*-(*p*-tosyl)-L-arginine methyl ester	guanidino	0
α-*N*-acetyl-L-lysine methyl ester	ε-amino	0.4
α-*N*-acetylhistamine	imidazole	0
N-acetyl-L-tyrosine ethyl ester	phenolic	ND[b]
glycine ethyl ester	α-amino	0.6

[a] Determined by amino acid analysis following thorough washing with 4 M urea, 0.01 M imidazole, and then water to remove substances not covalently bound. Conditions: 50 mL of 4.0 mM solutions of each compound in sodium phosphate (pH 7.0, Γ/2 = 0.1) were circulated through 0.33-g samples of beads for 3 h at room temperature. [b] Not determinable because the thioester derivative yields, upon amino acid analysis, a peak overlapping the position of tyrosine. (Seemingly none was bound, based on the data given in Figure 2 and the pK_a of tyrosine's hydroxyl group.)

groups were blocked, were exposed to the beads under immobilization conditions. The results, given in Table II, show that compounds having either a free α- or ε-amino group reacted to give a covalently immobilized derivative. The amounts immobilized would correspond to ~15 mg of α-chymotrypsin per g of glass, assuming one site of attachment per protein molecule.

Covalent immobilization of compounds which leave the thioester bond intact should yield derivatives which could be released in 1 M hydroxylamine. Furthermore, the released derivative should contain one sulfhydryl group for each site of attachment. α-Chymotrypsin, which had been immobilized by this procedure, was released by washing the beads with 1 M hydroxylamine and isolated by concentrating the solution containing the enzyme by ultrafiltration through an Amicon PM 10 membrane. Measurement of the free sulfhydryl groups on the released protein gave an average of 3.3 mol/mol of protein.

For establishment of the optimum conditions for immobilization with respect to both the amount of protein immobilized and the amount which could be subsequently released in hydroxylamine, experiments were performed in which the pH and temperature of the immobilization reaction were varied. Thus, α-chymotrypsin was immobilized by using a 3-h reaction period at pH values of 5, 7, and 9 in phosphate buffers of 0.1 ionic strength and at temperatures of 10, 25, and 40 °C. Each immobilized enzyme sample was divided into two portions, one of which was washed with 1 M hydroxylamine (pH 7.0). All samples were washed with 3.3 M urea, 0.01 M imidazole, and water prior to amino acid analysis. Statistical least-squares analysis of the results produced the surfaces shown in Figure 2. Relationships shown are significant with a probability level of $P < 0.0001$ with $R^2 = 0.73$. Increasing the pH between 5 and 8 did not greatly affect the amount of protein immobilized; however, above pH 8 the amount was dramatically reduced. Although increasing the temperature also increased the amount immobilized, it appeared to decrease the percentage which could be subsequently released by treatment with 1 M hydroxylamine.

Both the storage stability of the thioester–glass derivative and that of the covalent protein attachment were investigated. Derivatized beads were held dry at room temperature for 6 months between preparation and protein immobilization without affecting their capacity to both bind and release protein. After immobilization, samples of α-chymotrypsin–beads were rinsed and held in one of the following solvents (in the presence of 0.02% sodium azide) at room temperature for 1 month: sodium phosphate solutions of pH 5.0, 7.0, and 9.0, respectively; 6 M guanidinium chloride; and distilled water. Following storage, the samples were divided into aliquots, half of which were treated with 1 M urea and water in preparation for amino acid analysis. Results, listed in Table III, indicate no significant loss of protein during storage, except for that in guanidinium chloride. However, the percentage releasable appeared to decrease as a result of storage for 1 month.

Table III: Effects of Various Storage Conditions on Thioester-Immobilized Protein[a]

solvent	mg of protein/g of beads: before 1 M NH_2OH	mg of protein/g of beads: after 1 M NH_2OH	% released
distilled water	12.9	9.9	23
6 M guanidinium chloride	9.6	9.4	2
pH 5 sodium phosphate	14.2	11.7	18
pH 7 sodium phosphate	14.0	12.2	13
pH 9 sodium phosphate	12.7	11.2	12
not stored	14.0	8.1	42

[a] All stored samples were allowed to stand at room temperature for 1 month. Conditions of hydroxylamine treatment were as in Table I.

Table IV: Effects of Binding and Removal of Protein Caused by Variation of Chain Length in the Thioester Procedure

parameter	$HS(CH_2)_2$-COOH	$HSCH_2$-COOH
protein bound (mg/g of beads)	14.0	16.4
protein removed (mg/g of beads)[a]	5.9	11.6
percent removed	42.3	70.5

[a] Protein was removed by washing with 100 mL of 1 M hydroxylamine at pH 7 and room temperature. All bead samples were washed with 3.3 M urea, 0.01 M imidazole, and distilled water prior to amino acid analysis.

Table V: Kinetic Parameters of α-Chymotrypsin

enzyme prepn	apparent K_m (mM)	apparent k_{cat} (s^{-1})	apparent k_{cat}/K_m (mM^{-1} s^{-1})
soluble enzyme before immobilization	2.74 ± 0.72	54.5 ± 9.0	19.9
immobilized (20.2 mg/g)	4.43 ± 2.46	3.9 ± 1.7	0.9
immobilized (9.5 mg/g)[a]	6.69 ± 2.02	6.9 ± 1.7	1.0
soluble enzyme released by hydroxylamine	3.35 ± 0.68	64.3 ± 8.8	19.2

[a] The same immobilized enzyme preparation (20.2 mg/g) after brief treatment with 1 M hydroxylamine.

An experiment was performed to investigate the effect of chain length in the thiol acid attached to the glass surface. 2-Mercaptoacetic acid, like 3-mercaptopropionic acid, produced a reactive surface derivative. Thus, shortening the chain by one methylene group did not significantly affect the amount of protein which could be immobilized; interestingly, however, there appeared to be an increase in the amount subsequently released with hydroxylamine (Table IV).

The effects of immobilization on the kinetic parameters of α-chymotrypsin were investigated by using the techniques described above for immobilization and release of the enzyme. As shown by the data given in Table V, the measured parameters for the native soluble enzyme and the released enzyme samples did not differ significantly. Hence, the

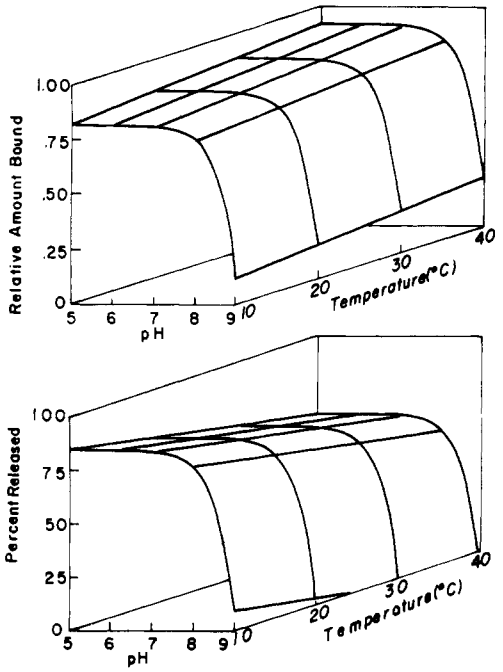

FIGURE 2: Prediction surfaces for binding and release of protein on thioester-derivatized glass as a function of pH and temperature. Conditions: α-chymotrypsin (1.0 mg/mL) was recycled for 3 h through reactors containing 0.25 g of derivatized beads in sodium phosphate ($\Gamma/2 = 0.1$) buffered at pH 5, 7, and 9 and at constant temperatures of 10, 25, and 40 °C. Each reactor was then washed with 250 mL of the buffer. A portion of each preparation of the enzyme-attached beads ($\sim$0.17 g) was then treated with 100 mL of 1.0 M NH_2OH (pH 7.0) at room temperature to assess releasability.

covalent linkage(s) of the enzyme to the coupling chain(s) per se did not appear to critically modify the conformation or sterically hinder access to the catalytic site, so as to alter the kinetic constants of those enzyme molecules which were subsequently released. These kinetic studies were performed under conditions which should have minimized external diffusion effects; therefore, the observed dramatic changes in the kinetics of the immobilized enzyme preparation are most likely the result of internal diffusion limitations. Effects of internal diffusion in a spherical particle have been quantitatively described (Engasser & Horvath, 1973; Goldstein, 1976). The Thiele modulus ϕ is given by

$$\phi = \frac{r}{3}\sqrt{\frac{V_{max}'}{K_m D_{eff}}} \quad (1)$$

where r is the particle radius, V_{max}' is the saturation velocity per unit volume of catalyst, K_m is the Michaelis constant, and D_{eff} is the effective diffusion coefficient for substrate within the matrix. Departure of the observed rate from the kinetic rate can be expressed in terms of an effectiveness factor η

$$V' = \eta V_{kin}' \quad (2)$$

where V' is the observed rate and V_{kin}' would be the true kinetic rate. Graphical relationships between the effectiveness factor, η, the Thiele modulus, ϕ, and the dimensionless effective substrate concentration, σ ($[S]/K_m$), have been obtained (Engasser & Horvath, 1973; Goldstein, 1976).

In our case, due to experimental limitations, substrate concentrations were limited to values of $\sigma \leq 0.55$; hence, all of the measurements were made under conditions approaching first order, as exemplified by the near linearity of the observed rates as a function of substrate concentration (Figure 3). Consequently, the actual saturation rate, V_{max}', could not be experimentally obtained. However, as expected for the case

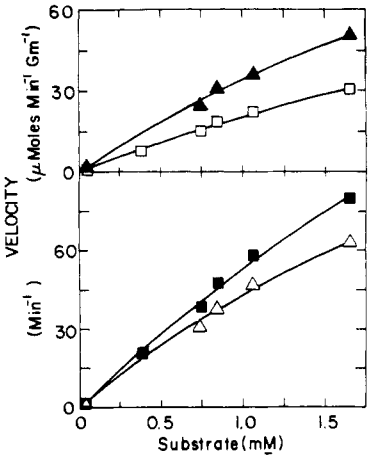

FIGURE 3: Effect of releasing a portion of the α-chymotrypsin from thioester–glass beads on the total and specific activity. Top: activity per gram of glass. Bottom: activity per micromole of enzyme. (▲, △) 20.2 mg of enzyme per gram of glass; (■, □) 9.5 mg of enzyme per gram of glass. Solid lines are least-squares fits of the points to a hyperbola. Each point is the mean of two measurements.

of diffusional limitations, removal of roughly one-half of the enzyme (by treatment with hydroxylamine) resulted in an increase in the specific activity for the remaining enzyme immobilized as shown in Figure 3 (lower curves). Comparison of the extrapolated values for the k_{cat}/K_m ratio for soluble and immobilized enzymes yields a first-order effectiveness factor of roughly 0.05 for the preparation containing 20 mg of enzyme per g.

Discussion

Amino acid analysis of thioester-derivatized beads which had been incubated with protein or model amino acid derivatives and subsequently washed with denaturants under conditions shown to remove all adsorbed material clearly demonstrated that such beads were capable of covalently immobilizing protein without the addition of carbodiimide. Because of the mild conditions and fairly wide range in pH and salt concentration which are compatible with the immobilization reaction, this method appears very attractive for attachment of biologically active proteins to solid surfaces. Furthermore, thioester-derivatized beads remain stable for long periods of time and the immobilization procedure simply requires incubation of a solution containing the protein with the thioester–glass derivative. It should be noted that there is no exposure of the protein to immobilization reagents such as CNBr or EDC in solution, thereby minimizing undesirable side reactions, protein cross-linking, and secondary chemical modifications.

The amount of protein which can be immobilized by using this procedure, viz., 10–20 mg/g, is comparable to that attainable by other commonly used methods (Mosbach, 1976) and to that obtained in our laboratory with carbodiimide activation of succinamidopropyl-glass (Cho & Swaisgood, 1974; Horton & Swaisgood, 1976). By acceptance of the value for total surface area supplied by the manufacturer, 50 m^2/g, an area of 94 $Å^2$ for each site can be estimated from our data. By the assumption of an even distribution of protein molecules on the surface and a diameter for α-chymotrypsin of 40 Å, protein molecules would be separated by roughly two diameters at a level of loading of 20 mg/g; lower levels of loading would result in proportionately greater intermolecular distances.

An additional versatility of this method is provided by the potential for subsequent release of immobilized protein, also under very mild conditions, due to the susceptibility of thioester

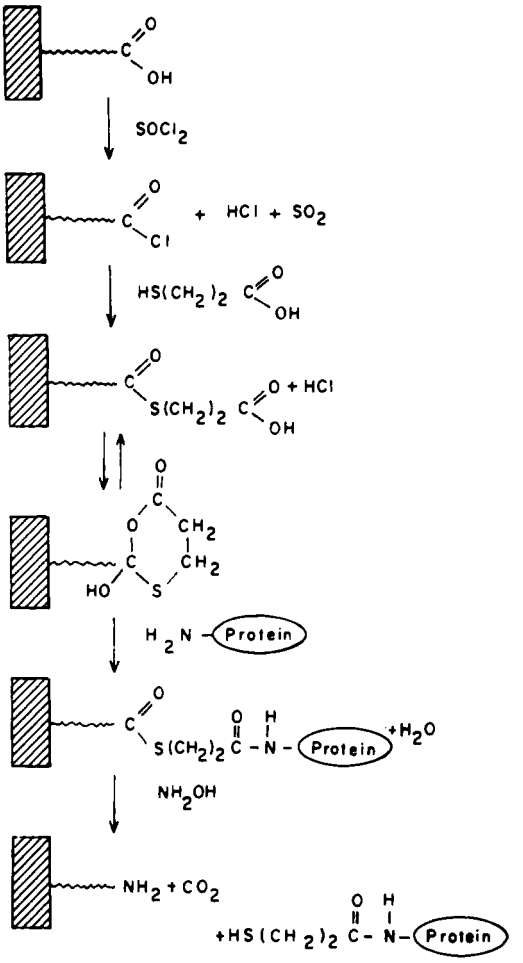

FIGURE 4: Proposed reactions for the formation of the thioester–glass derivative, its reaction with protein, and release of the immobilized protein by hydroxylaminolysis.

bonds to hydroxylaminolysis. Over half the protein immobilized could be released simply by washing with 1 M hydroxylamine at room temperature and pH 7.0. Such treatment did not affect the activity of native α-chymotrypsin. Another important feature of this release process is that sulfhydryl groups remain associated with the protein, one for each site which has been bound to the matrix via a thioester linkage. This provides an easy means of measuring the number of binding sites per molecule of protein, especially in proteins with no naturally occurring free sulfhydryl groups. This "tagging" procedure could also be useful for chemically determining the specific site(s) of reaction within protein chains.

Figure 4 presents a postulated reaction sequence for immobilization and release based on the following observations: (1) mass spectra indicated that *S*-succinylmercaptopropionic acid chains were attached to the aminopropyl derivative as expected from well-known organic reactions; (2) only those model compounds having free amino groups were immobilized to the derivatized beads; (3) catalytically active protein was released by hydroxylamine, which has a demonstrated specificity for thioesters (Bruice, 1961); and (4) the released α-chymotrypsin contained free sulfhydryl groups whereas none are present in the native enzyme. For an explanation of the unanticipated reactivity of the thioester–glass derivative, the formation of a ring is suggested, as shown in the figure. Construction of space-filling models indicates that such a ring (five- or six-membered) is sterically possible for each of the two mercapto acids used, although, as yet, there has been no direct demonstration of such a cyclic structure on the surface of the reactive beads. It is postulated that formation of such a ring, to create an anhydride-like derivative, would increase the susceptibility of the terminal "carboxyl group" to nucleophilic attack. With a lower pK_a and, hence, a greater nucleophilicity of the α-amino group under the conditions employed, somewhat greater immobilization of glycine ethyl ester occurred than of the lysyl derivative (via its ε-amino group). Above pH 8, the glass-bound derivative loses its reactivity, possibly due to hydrolysis of the ring. Incomplete release of protein in hydroxylamine could result either from a limited extent of hydroxylaminolysis of the thioester bonds or from initial partitioning of nucleophilic attack by the protein on the carbon in the thioester bond as well as on the terminal carbon during the immobilization procedure.

Hydroxylaminolysis of the thioester bond in the coupling chain of the immobilized protein yields the thioacylated protein and the corresponding hydroxamic acid derivative. The latter may undergo a Lossen rearrangement (Fieser & Fieser, 1961), giving the isocyanate which, upon hydrolysis, would yield the amine and CO_2. Such reaction could be the basis for an observed evolution of gas during the release reaction.

The decrease in the amount of protein immobilized upon treatment of the thioester derivative with carbodiimide may also be due to formation of the postulated ring structure. If some of the mercapto acid was displaced by carbodiimide, forming the *O*-acylisourea derivative at that point in the chain, subsequent reaction with protein would yield immobilized protein which would not be susceptible to release by hydroxylamine; such decreased release was observed (Table I). The fact that less protein was immobilized in these experiments may be due to the time which elapsed between treatment with carbodiimide and exposure to the protein, during which partial hydrolysis of the *O*-acylisourea probably occurred (Cho & Swaisgood, 1974; Swaisgood & Natake, 1973).

Several studies have been reported in which attempts were made to examine experimentally the effect of the chemical attachment of the coupling chain on enzyme activity. In all of the cases, soluble enzymes were treated with soluble reagents under conditions as similar as possible to those used for immobilization. Thus, Swaisgood & Natake (1973) found a linear relationship between the activity remaining and the number of glycine methyl ester residues incorporated onto carboxyl side chains of glutamate dehydrogenase by using carbodiimide activation of the protein's carboxyl groups. These authors also found subtle changes in the kinetic behavior of enzyme preparations which had only three to four modified residues per subunit. Cho & Swaisgood (1974) compared the kinetics of native lactate dehydrogenase with those of soluble enzyme containing an average of five modified carboxyl groups and enzyme which had been immobilized to aminopropyl-glass by using similar reaction conditions. In that case, the kinetic parameters of the native and the soluble, modified enzyme were essentially the same. Later, Ollis & Datta (1976) compared derivatized soluble forms and similarly immobilized forms of lysozyme, lipase, and α-chymotrypsin. They found α-chymotrypsin to be less sensitive to modification; but in all three cases high degrees of modification were logarithmically related to loss of activity.

The method reported here has the distinct advantage that there is no question about the chemical identity of the derivatized functional groups of modified, soluble and immobilized enzyme preparations. Kinetic parameters for the hydroxylamine-released α-chymotrypsin derivative, which had an average of about three chains incorporated per molecule under the conditions reported, were essentially the same as

those for native enzyme. Hence, the observed kinetic differences between the immobilized enzyme and soluble enzyme must be attributed to effects of diffusion and, possibly, partitioning of substrates, products, etc.

The ability to remove a portion of the immobilized enzyme by hydroxylamine treatment also allows comparison of the kinetics of the same immobilized preparation at two levels of enzyme loading. Hence, the intrinsic K_m values, particle radii, and D_{eff} should be identical for a sample of immobilized enzyme before and after partial release of enzyme. Consequently, the method for assessing the effect of diffusion, suggested by Engasser & Horvath (1973), should be directly applicable to the present derivatives, and some of the problems inherent in comparisons of different preparations (i.e., preparations using more and less heavily derivatized bead samples) should be avoided. The only quantity required by this method which is not available by direct measurement from our data is V_{max}', since sufficiently high substrate concentrations to allow this extrapolation were not accessible. Nevertheless, a good approximation can be obtained from the measured enzyme concentrations in the matrix and the apparent k_{cat} values determined for native enzyme or released enzyme samples. The use of such values would appear reasonable since (a) theoretically the intrinsic value for k_{cat} is approached at high substrate concentrations and (b) the intrinsic value for the immobilized enzyme appears to be similar to that for the native form, in that the value for soluble, released enzyme (with immobilization chains still attached) is similar to that of native enzyme. By identification of the preparation with 20.2 mg of α-chymotrypsin per gram with subscript 2 and that with 9.5 mg/g with subscript 1, the value for

$$\phi_2/\phi_1 = \frac{r_2}{r_1}\left(\frac{V_{max,2}'}{V_{max,1}'}\right)^{1/2} = (V_{max,2}'/V_{max,1}')^{1/2}$$

was calculated to be 1.46. Also, values for V_{max}'/κ, where $\kappa \equiv K_m/\epsilon$, were obtained from linear computer fits to both double-reciprocal plots and Hofstee–Eadie plots for both preparations; in this definition, ϵ is the first-order effectiveness factor, defined as

$$\epsilon = \frac{1}{\phi}\left(\frac{1}{\tanh 3\phi} - \frac{1}{3\phi}\right)$$

From the values of κ obtained, the ratio $\epsilon_2/\epsilon_1 = \kappa_1/\kappa_2$ was calculated to be 0.85. From these various ratios a value of $\phi_1 \simeq 5$ was estimated from the relationship given in Figure 7 by Engasser & Horvath (1973), and from it a value for ϕ_2 of 7.5 was estimated. By use of the expression for the substrate modulus (eq 1), values for K_m and D_{eff} can then be calculated for each preparation. The resulting values for K_m are 3.3 and 2.5 mM for the preparations with 20.2 and 9.5 mg/g, respectively, which agree within experimental error. The corresponding values for D_{eff} are 1.4×10^{-5} and 1.7×10^{-5} cm^2/min, respectively. Hence, the values obtained for K_m are in rather good agreement with that for the native enzyme. Also, the values for D_{eff} appear to be reasonable in comparison to values reported for other substrates in gel matrices (Regan et al., 1974). From these results it can be concluded that the reduction in chymotryptic activity following immobilization to porous glass particles can be entirely accounted for by pore diffusional inhibition.

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