ENZYME ENVELOPES ON COLLOIDAL PARTICLES

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SUMMARY

A new method has been developed for the preparation of insoluble proteins. The method involves the adsorption of protein as a monolayer onto colloidal silica particles, followed by intermolecular crosslinking with glutaraldehyde. Insoluble bovine trypsin prepared by this method was obtained in about 99% yield with 80% retention of esterase activity. This activity was only partially inhibited by protein inhibitors of trypsin (M.W. 9,000-28,000). Proteolytic activity was approximately 17% that of native trypsin. These particles are easily sedimented, but are nonetheless readily dispersible to form sols. The method appears to be of general applicability for the preparation of insoluble proteins as stable envelopes on colloidal particles.

It has become apparent in recent years that water-insoluble enzymes and other proteins offer certain advantages over their freely soluble counterparts. Their most important operational advantage is their ease of removal from reaction solutions by centrifugation or filtration. However, existing methods suffer from two shortcomings. First, most of them involve the attachment of the protein to a lattice-type matrix, e.g., cellulose (1,2), Sephadex (3), ethylene-maleic anhydride copolymers (4), or polystyrene (5). These, by their very nature, restrict access of substrate to the bound enzyme molecules. This is reflected in a much lower efficiency of such preparations compared to the native enzyme and is especially evident with large molecular weight substrates (1, 3). Second, the recoveries of active protein in the insoluble form by some of the methods have been relatively low (1,6).

The following method, schematically-illustrated in Figure 1, for the preparation of insoluble proteins appears to overcome some of these shortcomings.

The protein is adsorbed as a monolayer onto colloidal silica particles (duPont Ludox HS), and subsequently, covalent crosslinks were introduced

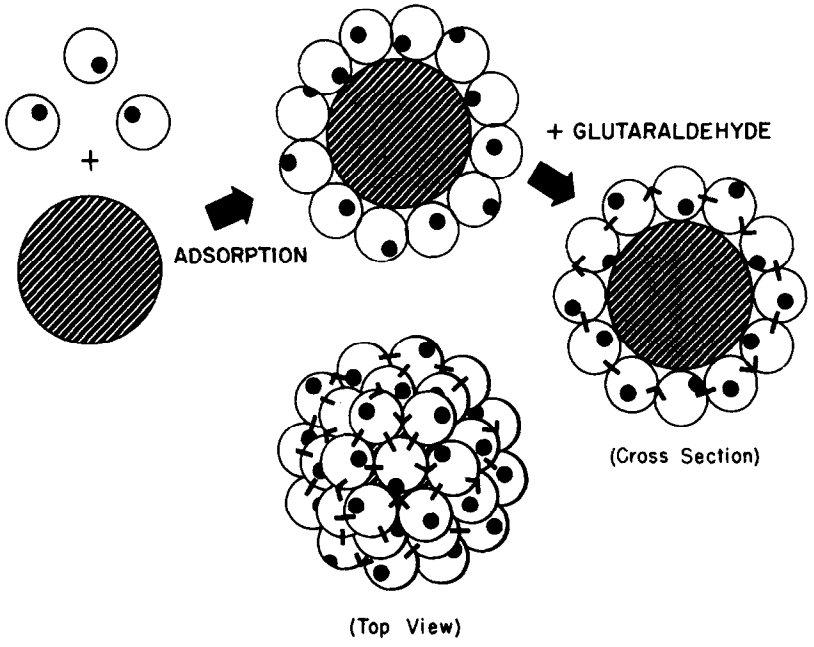

Figure 1. Schematic representation of the method for preparing insoluble proteins as envelopes on colloidal particles. The shaded area represents the silica particle, the dark circles represent the active sites of a protein with biological activity and the bars represent covalent intermolecular crosslinks.

between adjacent protein molecules by treatment with the bifunctional reagent, glutaraldehyde. This paper will describe the preparation and properties of insoluble trypsin using this method.

RESULTS AND DISCUSSION

In preliminary experiments the degree of adsorption of trypsin onto a three-fold weight excess of silica was found to be independent of pH between 6.8 and 8.7, but decreased markedly above 8.7. Further studies of the adsorption and of the crosslinking reaction were carried out at pH 8.5.

The stoichiometry of the adsorption is given in Figure 2. The trypsin concentration, as judged by absorbance at 280 mμ and by activity, were almost identical, indicating that there was no preferential binding of active or inactive trypsin. Initially, the added silica removed proportional quantities of trypsin from the solution, but as more silica was added the curve

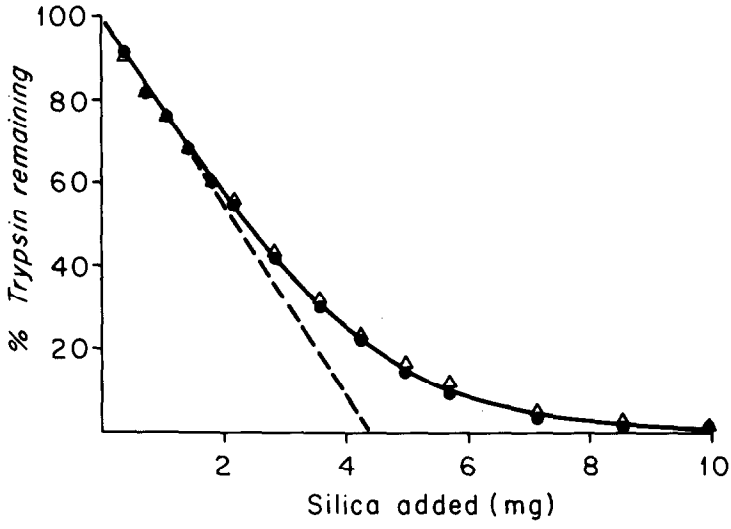

Figure 2. Stoichiometry of adsorption of bovine trypsin on silica. Various quantities of silica were added to 1.9 mg of bovine trypsin in a total volume of 3.0 ml of 0.1 M borate, 0.001 M benzamidine, pH 8.5. After mixing and incubation for 15 min. at room temperature, the solutions were centrifuged at 37,000 g for 20 min. The amount of trypsin remaining in the supernatant was determined both by absorbance at 280 mμ, Δ, and by activity, ●.

deviated from linearity. It is not clear whether this represents a range of binding constants or a perturbed equilibrium. By extrapolating the linear part of the curve to the abscissa, the stoichiometry of the initial adsorption could be estimated. In making this estimation the following figures and assumptions were used: surface area for the silica of 210-230 M^2/g (I. E. duPont, technical bulletin), molecular diameter for trypsin 40 Å and the packing of trypsin molecules as rigid spheres. From the silica:trypsin ratio at the point of stoichiometry, it was calculated that the trypsin could occupy 77-85% of the surface area of the silica. This, together with preliminary electrom micrographs of the final product, support the view that a monolayer of protein is in fact formed.

The effect of silica concentration on the retention of enzymatic activity after crosslinking with glutaraldehyde was then studied. It was found that a five-fold molar excess of glutaraldehyde over protein amino groups was the optimum amount needed to give maximum stability of this product with minimal

inactivation. The results of this experiment are given in Figure 3.

In the absence of silica there was a 55% loss in activity, associated with the formation of large aggregates of polymerized protein. Progressive increases in activity were observed as the silica concentration was increased, until the equivalence point was reached. This equivalence point corresponds to the ratio of silica to trypsin present at the intercept on the abscissa in Figure 2. Above this equivalence point there was essentially no further increase in activity. This is consistent with the conclusion that maximum activity of insoluble enzyme is attained when the protein is in the form of a monolayer.

For further characterization, 100 mg lots of trypsin were rendered insoluble by treatment with a stoichiometric amount of silica (silica:trypsin ratio 2.3:1 w/w, 15 min., room temperature) and 125λ of 25% glutaraldehyde for 1 hr. at room temperature. Both reactions were carried out in 50 ml of 0.1 M borate, 0.001 M benzamidine, pH 8.5. The product, in this case, was

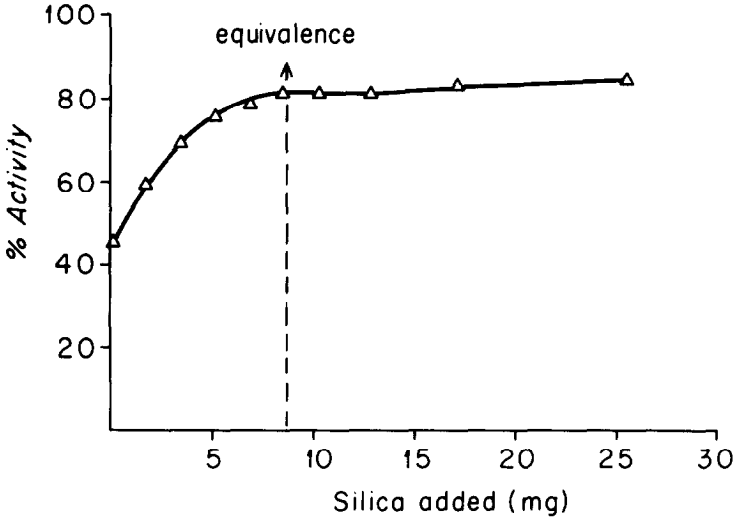

Figure 3. Effect of silica concentration on activity after crosslinking with glutaraldehyde. Various quantities of silica were added to 3.8 mg of trypsin in a total volume of 3.0 ml of 0.1 M borate, 0.001 M benzamidine, pH 8.5. After mixing and incubation for 15 min. at room temperature, 25λ of 2.5% of glutaraldehyde was added to each sample. After 60 min. at room temperature, the remaining estrolytic activity was measured.

then further treated with 0.05 M $NaBH_4$ for 20 minutes at 0° C. to reduce the remaining aldehydic groups and thus prevent covalent crosslinking to the protein inhibitors used in subsequent experiments. The recovery of insoluble trypsin after the glutaraldehyde reaction was greater than 99%, as judged by activity towards BAEE. However, losses in subsequent transfers reduced the overall yield to 80%. Some enzymatic properties of a typical preparation are given in Table I.

TABLE I

	MOLES ACTIVE SITE[a] / MOLE PROTEIN	BAEE ACTIVITY[b] MOLECULES/ ACTIVE SITE/ MINUTE	RELATIVE CASEINOLYTIC ACTIVITY[c] PER ACTIVE SITE
SOLUBLE TRYPSIN	0.74	1700	100
INSOLUBLE TRYPSIN	0.60	1500	17.5

a. Active site titrations by the method of Chase and Shaw (7) using p-nitrophenylguanidobenzoate (NPGB). The insoluble enzyme was removed by centrifugation just prior to reading the absorbance.

b. 0.01 M benzoyl arginine ethyl ester (BAEE) in 0.01 M Tris, 0.05 M $CaCl_2$, 0.1 M KCl, pH 7.8, 26° C in a pH stat.

c. Method of Laskowski, Sr. (8) using 0.1 M borate, 0.002 M $CaCl_2$ in place of phosphate.

The number of moles of active site per mole of protein was reduced from 0.74 in the lot of native trypsin used to 0.6 in the insoluble derivative. The turnover of the small substrate, BAEE, was also lower. The pH-activity profile with BAEE was almost identical to that of the native enzyme. As expected, the activity towards the large substrate, casein, was greatly reduced, presumably due to both a lower availability of active sites and to a lower efficiency at each site.

To further explore the question of steric availability, we examined the degree to which the insoluble trypsin could be inhibited by various protein inhibitors. The results are given in Figure 4.

It can be seen from this figure that the effectiveness of the inhibitor is inversely related to its size. In each case the percent inhibition reached a plateau indicating that only a limited number of sites were available to each inhibitor. In contrast, the much smaller inhibitor NPGB inhibited all active sites. The catalytic efficiency of sites not inhibited by soybean trypsin inhibitor, presumably those not directly on the surface of the particles, was then determined by active site titrations in parallel with activity determinations in the presence of the inhibitor. Within experimental error there was no difference in the turnover of BAEE by these "inner sites" when compared to the total available sites. However, the apparent Km values have not been examined in these preparations.

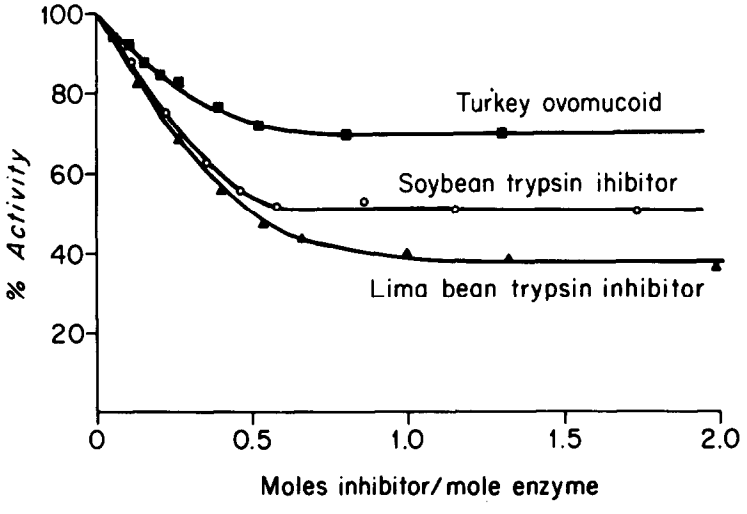

Figure 4. Inhibition of insoluble trypsin by protein inhibitors. Samples of insoluble trypsin containing 1 mg of active enzyme were incubated with increasing quantities of each inhibitor in a total volume of 1.0 ml of 0.1 M Tris, 0.05 M $CaCl_2$ pH 7.8 at room temperature. After 15 min. all samples were assayed for remaining tryptic activity with BAEE as substrate. ■, turkey ovomucoid, M.W. 28,000 (9); 0, soybean trypsin inhibitor, M.W. 21,000 (10); ▲, lima bean trypsin inhibitor, M.W. 9,000 (11).

Glutaraldehyde has been used previously for the preparation of insoluble trypsin (6), subtilisin (12), papain (13), and several other proteins (14,15). In addition, glutaraldehyde has been used to prevent the resolubilization of protein crystals (16). These preparations, however, were in the form of aggregates of polymerized protein and thus offered only a small surface area for the contact of enzyme molecules with the solvent containing the substrate molecules (13). The present method minimizes this shortcoming. Since the enzyme is in the form of an envelope around the particles, each protein molecule is in direct contact with the surrounding solvent. This then increases the probability that the active center is freely exposed and readily accessible to substrate molecules. Such preparations retain high biological activity and are obtained in high yield. An unique characteristic of these preparations is their ease of dispersion. On the one hand, the particles can be sedimented in 10 min. at 5,000 g; on the other hand, 30 seconds in a small volume of water on a Vortex mixer completely disperses the centrifuged pellet.

The concept of forming crosslinked protein envelopes on colloidal particles appears to show promise of general applicability. The method, with slight modifications, has been applied to the preparation of several other proteins, e.g., lysozyme, bovine serum albumin, soybean trypsin inhibitor and α-chymotrypsin. All of these insoluble derivatives were obtained in high yield (greater than 90%) and were highly efficient as immunoadsorbants, as a trypsin inhibitor, and as an enzyme, respectively. The preparation and properties of various proteins by adaptations of this technique are being investigated.

ACKNOWLEDGMENTS

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