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Improved Stability of Proteins Immobilized in Microparticles Prepared by a Modified Emulsion Polymerization Technique

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Abstract □ Proteins can be immobilized in spherical microparticles of polyacrylamide gel (having a diameter of about 1 μm) by an emulsion–polymerization technique. Highly cross-linked gels have a structure consisting of relatively large pores. This structure is advantageous when dealing with biologically active proteins acting on molecules dissolved in the surrounding medium. A rapid equilibrium is established between the interior of the particles and the medium, and rate-limiting diffusion is not observed. A suspension of carbonic anhydrase immobilized in microparticles will thus have kinetic properties very much like the free enzyme. In addition to the entrapment of the protein molecules in the three-dimensional network formed by the polyacrylamide threads, protein molecules are also fixed in the cross-linked threads of polyacrylamide. This fixation is probably responsible for the improved stability of the protein molecules against heat denaturation. Not even autoclaving at 110° for 30 min denatured the immobilized enzyme completely (more than 25% of the enzyme activity was left). The higher resistance of molecules in microparticles against proteolytic degradation also is documented.

Keyphrases □ Proteins—immobilized in polyacrylamide microparticles, effect on stability □ Microparticles, polyacrylamide—containing immobilized proteins, effect on stability □ Polyacrylamide microparticles—containing immobilized proteins, effect on stability □ Stability—proteins immobilized in polyacrylamide microparticles

The immobilization of proteins in polyacrylamide microparticles with a high degree of cross-linking (T-C = 8-25[1]) by emulsion polymerization was studied recently (2–4). The macromolecules apparently are immobilized by two mechanisms: fixation in the highly cross-linked threads of polyacrylamide and, if sufficiently large, entrapment in the network formed by the polyacrylamide threads (4). Consequently, the immobilized macromolecules will be localized partly on the microparticle surface, enabling them to react also with substrates or cells that cannot penetrate into the particles (2, 5). The highly cross-linked gels (C = 25%) also produce a network with relatively large pores, which is advantageous with biologically active proteins interacting with molecules dissolved in the surrounding medium because the macromolecules retain most of their activity when bound in the microparticles.

The present work was undertaken to investigate the consequences of the immobilization in highly cross-linked microparticles of polyacrylamide on the activity and stability of proteins, especially carbonic anhydrase. A fraction of the enzyme is remarkably stable, even against heat denaturation, while some enzymatic activity is lost in a normal way. A modified procedure is described for the large-scale microparticle preparation.

EXPERIMENTAL

Materials—Human serum albumin[2], immunoglobulin G[2], bovine β-lactoglobulin[3] (as a mixture of the A and B forms), protease[3] Type VI from *Streptomyces griseus*, and bovine carbonic anhydrase[3] (EC 4.2.1.1) were used without further purification. Acrylamide[3], *N,N'*-methylenebisacrylamide[3], *p*-nitrophenyl acetate[3], *N,N,N',N'*-tetramethylethylenediamine[3], tris(hydroxymethyl)aminomethane[3], and other chemicals were analytical grade.

Determination of Carbonic Anhydrase Activity—The esterase activity of carbonic anhydrase was determined with *p*-nitrophenyl acetate as the substrate (6). The enzyme (50 μg in free solution or in microparticles) was added to 5 ml of 0.05 *M* tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.5. The formation of the hydrolysis products was followed spectrophotometrically at 400 nm. The velocity of the reaction is expressed as an increase in absorbance during 1 min.

Appropriate correction was made for the spontaneous hydrolysis of the substrate in samples not containing any enzyme. Microparticles of polyacrylamide not containing enzyme did not affect the reaction, as found in control experiments. The particles did not cause any light scattering at the wavelength used, and no sedimentation was detectable during the time of the measurement.

Protein Determination—The concentrations of human serum albumin, immunoglobulin G, and β-lactoglobulin were determined from the optical density at 280 nm. The $E^{1\%}_{1\,cm}$ values were 5.3 (7) for albumin, 13.6 for immunoglobulin, and 7.90 for lactoglobulin (8). The spectra were recorded with an automatic recording spectrophotometer at 400 nm to correct for the light-scattering effect (9). The amount of protein immobilized in the microparticles was determined after hydrolysis in 6 *M* HCl

[1] The nomenclature suggested by Hjertén (1) is used.

[2] KABI AB, Stockholm, Sweden.
[3] Sigma Chemical Co.

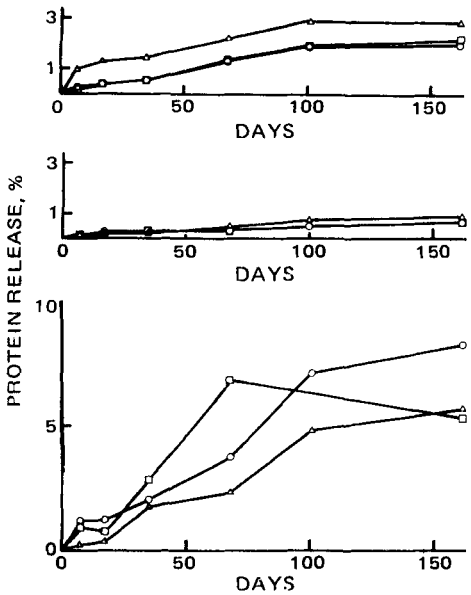

Figure 1—*Release of immobilized proteins during storage at room temperature from microparticles of polyacrylamide with T-C = 8-5 (○), T-C = 15-5 (△), and T-C = 8-25 (□). The immobilized proteins were β-lactoglobulin (top), human serum albumin (middle), and immunoglobulin G (bottom), and the particles were suspended in 0.1 M KCl and 0.005 M phosphate, pH 7.4 with 0.1% sodium azide.*

at 105° for 20 hr and subsequent amino acid analysis with an automatic amino acid analyzer.

Preparation of Microparticles—Microparticles with immobilized proteins were prepared using a modification of the method described by Ekman *et al.* (4). The protein was dissolved with acrylamide (1.5 g) and *N,N'*-methylenebisacrylamide (0.5 g) in 25 ml of 0.005 *M* sodium phosphate buffer, pH 7.4. After addition of the catalyst, ammonium peroxosulfate (100 μl of a solution of 0.5 g/ml), the solution was poured into a mixture consisting of 100 ml of toluene, 25 ml of chloroform, and 0.4 g of a detergent, poloxamer 188[4]. The mixture was homogenized to produce a water-in-oil emulsion[5]. The size of the water droplets was continuously measured in the microscope, and the emulsion was homogenized until a satisfactory size of the water droplets was obtained.

The homogenizer was then removed, and the emulsion was slowly stirred with a magnetic stirrer. The accelerator, *N,N,N',N'*-tetramethylethylenediamine (1 ml), was added to the emulsion, and polymerization started after a few minutes. During the whole procedure, oxygen was excluded from the system by bubbling nitrogen gas through the solution. After 30 min, the nitrogen stream was discontinued, and the phases were allowed to separate. The microparticles were washed with buffer by centrifugation until no more protein could be detected in the supernate.

Determination of Microparticle Density—The density of the microparticles was determined by density gradient centrifugation in a self-generating gradient of silica[6], according to Pertoft *et al.* (10), as previously described (4).

RESULTS

Leakage of Proteins from Microparticles—One early drawback observed in enzymes entrapped in polyacrylamide was loss of activity from the gel because of leakage. However, with highly cross-linked polyacrylamide (*e.g.*, with C = 25%), the immobilization is more stable (Fig. 1). Microparticles were prepared with lactoglobulin, albumin, and immunoglobulin, having Stokes radii of 2.67, 3.34, and 4.92 nm, respectively (4).

The microparticles were suspended in 0.1 *M* KCl with 0.005 *M* phosphate buffer, pH 7.4, so that the protein concentration was about 3, 7, and 20 mg/5-ml sample. The leakage was tested with three different gel types, T-C = 8-5, 8-25, and 15-5, respectively. Sodium azide (0.002%) was added as a preservative, and different samples were left at room temperature for the times stated in Fig. 1.

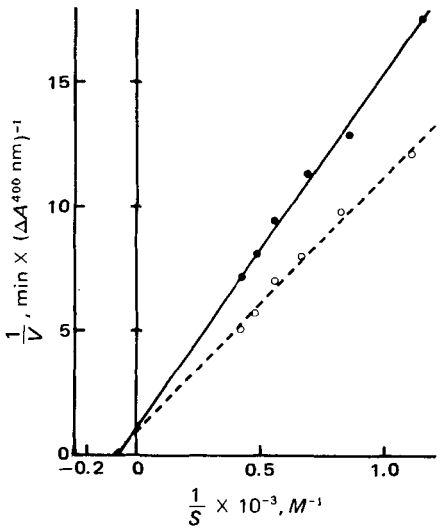

Figure 2—*Lineweaver-Burk plots for the hydrolysis of p-nitrophenyl acetate by bovine carbonic anhydrase. The enzyme was either used free in solution (○) or immobilized in microparticles (●).*

The protein released from the particles was determined (from the optical density) after centrifugation. As seen from Fig. 1, only small amounts of proteins were released. Thus, less than 1% albumin was lost from the particles after 5.5 months, while about 2% of lactoglobulin and 5–7% of immunoglobulin were lost.

Enzymatic Properties of Immobilized Carbonic Anhydrase—The kinetics of carbonic anhydrase in the free state and in microparticles are compared in Fig. 2. The hydrolysis rate of the chromogenic substrate *p*-nitrophenyl acetate was determined with the same total amount of enzyme in the two series. As expected, the velocity was somewhat lower with immobilized enzyme, and the maximal specific activity, as calculated from the *y*-axis intercept, was 12% lower. The Michaelis constant, K_m, was 13.0 m*M* for the immobilized enzyme compared to 10.5 m*M* for the soluble enzyme. The values are close to those found by Pocker and Stone (6).

Thermostability of Immobilized Carbonic Anhydrase—Figure 3 summarizes studies of the stability of carbonic anhydrase when exposed to higher temperatures for 5 and 30 min. After treatment at the temperatures shown, the samples were cooled with an ice bath. After 5 min, the samples were incubated at 25° for 30 min, and the enzymatic activity was then measured. The same amount of enzyme was used in all samples, and the initial velocity was used to calculate the residual enzymatic activity.

All of the free enzyme and a fraction of the immobilized enzyme were denatured at temperatures above 60°, with a "midpoint" at about 63°. However, a substantial fraction of the enzyme in the microparticles resisted the heat treatment; even after 30 min at 90°, more than 30% of the enzyme was still active. Not even autoclaving at 127° for 15 min dena-

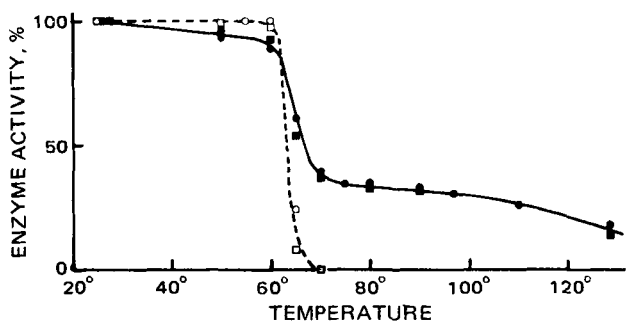

Figure 3—*Heat stability of carbonic anhydrase. The remaining enzymatic activity was measured after heating free enzyme for 5 (○) or 30 (□) min and immobilized enzyme for 5 (●) or 30 (■) min. Enzyme immobilized in microparticles was also kept in an autoclave for 5 and 30 min at 110 and 127°. Prior to the determination of the remaining activity, the samples were kept in an ice bath for 5 min and at room temperature for 15 min.*

[4] Pluronic F 68, Wyandotte Chemical Co., Wyandotte, Mich.
[5] An Ultraturrax TP 18-10 homogenizer was used.
[6] Ludox, E. F. du Pont de Nemours and Co., Wilmington, Del.

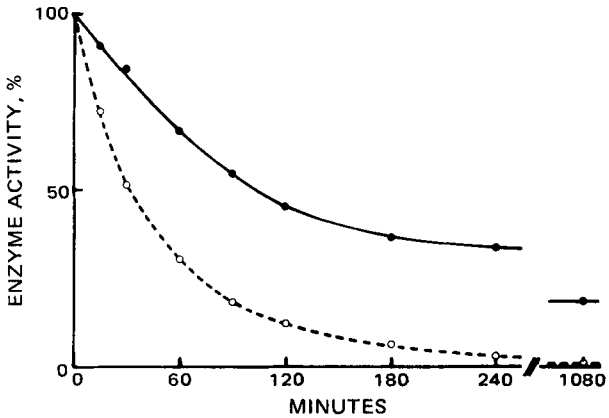

Figure 4—*Inactivation of bovine carbonic anhydrase (0.3 mg/ml) in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.5, by proteolytic digestion with protease VI (2.0 mg/ml). Carbonic anhydrase was either free in solution (○) or immobilized in microparticles (●), T-C = 8-25.*

tured the enzyme completely (about 15% was left), and autoclaving at 110° for 30 min left more than 25% of the enzyme intact.

Proteolytic Degradation of Carbonic Anhydrase—The increased thermal stability was paralleled by an increased stability against proteolytic enzymes (Fig. 4). In free solution, carbonic anhydrase was relatively rapidly inactivated by protease VI, an enzyme with broad specificity. In the microparticles, however, carbonic anhydrase was largely protected; the protease could only attack the protein molecules on the surface or situated close to the largest pores in the interior of the particles penetrable by it. Thus, about 65% of the enzyme activity in microparticles was retained after treatment for 1 hr; about 35% was retained in free solution.

Microparticle Density—In several applications, *e.g.*, in the isolation of specific cells in a cell population, the density of the microparticles is an essential factor (5). The modified procedure yields microparticles of a somewhat higher apparent density than the previously used procedure (Fig. 5). Thus, the density of particles with C = 25% increased from 1.08 to 1.13 g/cm^3. However, the general profile of the density curve was the same as found earlier, with a minimum value at C = 10% (4); particles with very low or very high concentrations of cross-linking agent had the same density as found earlier, about 1.15 g/cm^3, representing the value of the linear polyacrylamide. The reasons for these results are discussed later.

DISCUSSION

Immobilization of proteins in polyacrylamide gels was initially carried out as a block-polymerization procedure, subsequently followed by mechanical degradation of the gel to finer particles. The bead polymerization method constitutes a refinement of the technique, producing spherical particles by polymerizing a water solution of monomers and protein dispersed in an organic phase, as presented by Nilsson *et al.* (11). Enzymes immobilized in this manner show deviations from normal kinetics as exhibited in free solutions (decrease of V_{max} and increase of K_m), which may partly be due to the rate-limiting diffusion processes within the gel granules.

Small-size preparations with immobilized carbonic anhydrase were prepared previously by microencapsulation (12). Also, in this case, the K_m of carbonic anhydrase was increased, probably because of diffusion limitations through the membrane. By preparing particles with a small diameter, an enzyme preparation with kinetic properties very much like those of free enzyme was obtained. However, the small size is not the only explanation to the improved kinetics; the acrylic monomer composition contributes by ensuring a macroporous gel structure. In the microparticles, the K_m for carbonic anhydrase is virtually the same as in solution. However, the V_{max} is somewhat decreased. The results suggest that some enzymatic activity is lost in the microparticles, probably due to steric blockage of the active site by the gel structure or denaturation during preparation.

The increased stability of the immobilized compounds against heat denaturation and proteolytic enzyme degradation is dramatic. A large fraction of the immobilized carbonic anhydrase resisted treatment at 110° for 30 min. Possibly the imbedding of the enzyme within the polyacrylamide threads (4) stabilizes the conformation of the enzyme and protects the enzyme against proteolysis. Naturally, the fraction of the protein immobilized on the surface of the microparticles or close to the macropores in the gel structure has the same properties as soluble enzyme and will be lost during heat treatment and by proteolysis in a similar manner.

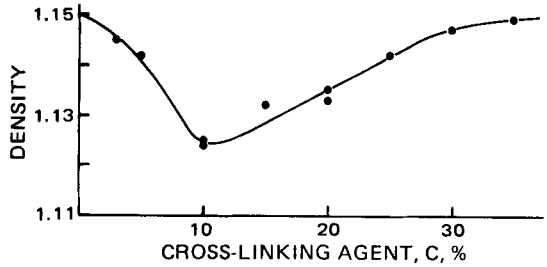

Figure 5—*Density of microparticles of polyacrylamide prepared from monomer solution containing different amounts of cross-linking agent (C). The total amount of monomer (T) was 8% in all cases.*

The modification of the method for the preparation of microparticles presented here eliminates some major difficulties of the previous method (4). In the earlier procedure, the catalyst–accelerator system is added immediately before homogenization of the water phase with the monomers in an organic phase. Since the polymerization proceeds very rapidly when once initiated, not even cooling of the mixture slows down polymerization markedly. Therefore, it is practically impossible to obtain a reproducible and narrow degree of dispersion, even with powerful homogenizers, before polymerization is completed. Moreover, the homogenizer easily ruptures the polymer spheres formed, resulting in a polymer preparation partly consisting of broken spheres of irregular size.

In the modified procedure, which can also be applied in large-scale preparation of microparticles, the accelerator is added to the mixture when a stable emulsion of desired dispersion degree is obtained. The size of the water droplets of the emulsion can be checked in the microscope to ensure that the particles formed have the wanted size within a narrow size distribution. In addition, the slow stirring during polymerization causes the particles to assume a spherical rather than an ellipsoidal form.

That the particles prepared by the modified procedure are more spherical and uniform is also apparent from the results of the density determinations. With C = 25%, the density found in the self-generating silica gradient is somewhat higher with the modified procedure. The higher density, *i.e.*, the smaller buoyancy effect during the centrifugation, is due to the "excluded volume effect" (10), which is smaller with a sphere than with an irregular particle of the same mass in the silica gel.

Earlier work in the field of immobilized enzymes has almost entirely been concerned with the use of enzymes as enzyme reactors in column chromatography. However, immobilized enzymes can also be conveniently used in batch procedures. The use of lactate dehydrogenase for the analysis in the picomole range of the pyruvate–lactate system has already been reported (3). The microparticles have a diameter of less than 1 μm and thus settle very slowly. They are easily pipettable in suspension. The immobilization also stabilizes the enzymes against heat denaturation and proteolytic degradation. Lactate dehydrogenase particles have been stored as a suspension at 4° for many months.

One drawback is the relatively small amount of protein immobilized in the microparticles during the preparation (about 3–10%). However, the protein is more effectively utilized in the small beads, where steric hindrance is minimized, and protein not immobilized can be regained from the washings of the microparticles after the preparation. The protein immobilized is very accessible to smaller molecules, *e.g.*, substrates. Moreover, the stability of the immobilized protein against proteolytic breakdown permits the use of microparticles in highly contaminated systems. Finally, the possibility to sterilize microparticles containing biologically active proteins by autoclaving may open up wide perspectives for the future use of immobilized proteins *in vivo*.

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Permeability of Interstitial Space of Muscle (Rat Diaphragm) to Solutes of Different Molecular Weights

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Abstract □ The transport characteristics of muscle interstitial space were determined using an isolated rat diaphragm preparation. Permeability of the interstitial space for extracellular solutes is one-thirtieth to one-fiftieth that of an equivalent thickness of water. However, most of this low permeability can be accounted for by correcting for the tortuosity and relative volume of interstitial space. The estimated diffusivity of solutes (mol. wt. 100–70,000) in the interstitial space of muscle is only about one-half to one-fifth less than in water alone.

Keyphrases □ Permeability—interstitial space of rat diaphragm muscle to solutes of different molecular weights □ Transport characteristics—interstitial space of rat diaphragm muscle, permeability to solutes of different molecular weights

The prediction of the transport rates at which solutes and drugs cross interstitial space—either from blood to tissue or from tissue to circulation—is dependent on a knowledge of the relative resistances of these regions to transport.

Although several studies focused on the capillary endothelium (1–3), less work has been done on defining the role of the interstitial space and how it affects the net transport rate between blood and tissues. In nonsteady-state experiments using tracers, a temporary interruption in blood flow affected solute transport in a manner attributed to diffusion resistance in the interstitial space (4). By treating muscle with hyaluronidase, the absorption rate of intramuscularly injected dextrans was accelerated (5). Other experiments (6, 7) involved measurement of permeation rates through polyglycon gels prepared to simulate the composition of interstitial polysaccharides; it was suggested that diffusion through muscle tissue might be highly restricted, especially with macromolecules.

The objective of this study was to find the relationship between solute size and solute mobility in the interstitial space of muscle. Tracer solutes were used which, except for tritiated water, are retained in the extracellular space of muscle tissue. And by imposing a dextran gradient across a muscle preparation, the influence of osmotic forces in interstitial transport was evaluated.

Because the diaphragm is constituted of striated muscle and because the flat, sheet-like configuration lends itself to measurements of transient and steady-state transport rates by conventional membrane-testing techniques, the diaphragm of the rat was used in these experiments. This muscle has been used extensively as an experimental preparation in transport studies (8), cellular uptake studies (9), and work on hormonal influences on transport and uptake (10).

EXPERIMENTAL

Rat Diaphragm Preparation—Sprague–Dawley male rats, mostly retired breeders[1], 250–500 g, were injected with 35 mg ip of pentobarbital.

After the abdominal cavity was opened with a midline incision, the vena cava was clamped about 1 cm caudal to the vena caval hiatus. A 1.0-mm longitudinal incision was made in the vessel, and a catheter[2] was introduced and secured with 3-0 silk suture. The thorax was opened just above the sternum, and the thoracic vena cava was tied. The catheter was connected to a syringe pump equipped with a glass 30- or 50-ml syringe, and retrograde perfusion of the diaphragm vascular bed with Krebs–Ringer bicarbonate buffer was begun (11).

The diaphragm was removed by cutting the abdominal wall with scissors just caudal to the costal border, girdling the animal. A similar girdling cut was made just cephalad to the costal rim, and the trunk of the rat was disconnected above and below the diaphragm with bone shears and scissors.

The excised diaphragm, still undergoing retrograde perfusion, was mounted on a rigid, flat, 1.6-mm polycarbonate ring, about 7 cm o.d. and 5 cm i.d., using straight pins at the costal margin which fit into predrilled 1-mm holes in the ring. Once mounted, the diaphragm was either suspended vertically above a specimen dish for the permeability experiments or submersed in a cocktail of buffer, radioactive tracer, and dye for the equilibration experiments.

[1] Spartan Research Animals, Inc., Haslett, Mich.
[2] Intramedic polyethylene tubing PE 160.