Biochimica et Biophysica Acta, 358 (1974) 164–177

BBA 67255

PELLICULAR IMMOBILIZED ENZYMES

CSABA HORVATH

Department of Engineering and Applied Science and Physical Sciences Section, School of Medicine Yale University, New Haven, Conn. 06520 (U.S.A.)

(Received December 10th, 1973)

SUMMARY

Methods for the preparation of shell-structured immobilized enzymes supported by a fluid impervious spherical core are outlined. The preparation of such trypsin resins by coreticulation of the enzyme with a copolymer of maleic anhydride and vinyl methylester in situ on the surface of glass beads is described and some properties of the product including its behavior in hydro-organic media are discussed. By using the same reactive polymer pellicular polyanionic conjugates of chymotrypsin, papain, ribonuclease, L-asparaginase and alkaline phosphatase were also obtained. Carbon, nylon, silica and alumina in pellicular form were employed for the preparation of immobilized enzymes by crosslinking with glutaraldehyde. Pellicular carbon was the most suitable carrier for wheat germ acid phosphatase, cathepsin C and chymotrypsin. The activity of the products compares favorably to that obtained with enzymes immobilized on porous glass. The results indicate that a variety of methods can be employed to obtain pellicular immobilized enzymes, which possess high mechanical stability for use in packed beds and offer an efficient utilization of the immobilized enzyme.

INTRODUCTION

Immobilized enzymes have been employed as specific biosorbents in affinity chromatography [1], as heterogeneous catalysts in enzyme reactors [2] and as model systems in studying microenvironmental effects on enzyme action [3]. In many applications, they are utilized in packed beds such as a chromatographic columns or packed reactors.

Recently the properties of pellicular immobilized enzymes, which consist of a solid, fluid impervious core supporting a spherical annulus of an enzymatically active porous medium have been theoretically investigated [4]. Such catalyst particles offer significant advantages for use in packed beds because of their mechanical stability and favorable mass transfer properties.

This approach to enzyme immobilization is an outgrowth of developments in high-performance liquid chromatography [5, 6] where a variety of shell structured

Abbreviations: BAEE, *N*-benzoyl-L-arginine ethyl ester; TEE, L-tyrosine ethyl ester.

sorbents and an inert support, using glass beads as the core material, have been employed.

The name pellicular has been adopted because the active porous medium forms a thin skin on the solid core. In most cases it can be considered as a supported spherical membrane. Thus, pellicular immobilized enzymes can also serve as appropriate models for membrane bound enzymes. Polyanionic trypsin and chymotrypsin conjugates in pellicular form have already been employed to demonstrate the regulatory effect of calcium on bound enzymes by membrane coarctation [7].

Depending on the structure of the porous layer, pellicular immobilized enzymes can be divided into three major classes as illustrated in Fig. 1.

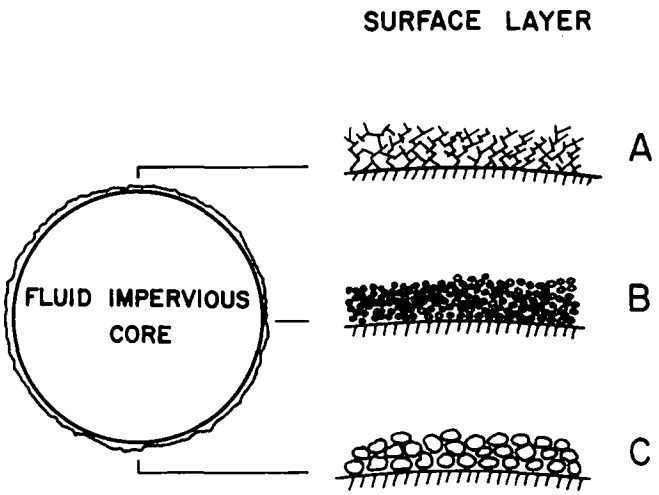

Fig. 1. Schematic illustration of a pellicular immobilized enzyme particle and the various types of surface layers. A. Crosslinked gel containing covalently bound or entrapped enzyme. B. Small-pore rigid polymeric or inorganic layer with the enzyme covalently bound to or crosslinked onto the surface. C. Large-pore layer impregnated with the immobilized enzyme.

(a) The enzyme can be immobilized in a gel matrix that forms a spherical annulus. This configuration makes it possible to make mechanically stable column packing from mucilaginous enzyme resins. The gel layer can be attached first to the glass surface and then used for enzyme immobilization by established coupling methods [8, 9]. Alternatively the enzyme-polymer conjugate can be formed in situ as described below.

(b) The enzyme can be coupled to the surface of a porous matrix layer, which has a sufficiently high surface area, i.e. relatively small pores. The porous layer can be formed from an inorganic sorbent such as silica, alumina or carbon and the attachment of the enzyme can be carried out, for example, via one of the silane coupling methods [10, 11] or by cross-linking onto the surface [12]. Commercially available pellicular sorbents such as Pellosil and Pellumina (H. Reeve Angel and Co.) could also be employed directly in this approach. A polymer can also be used to form a rigid porous layer, which can be activated, in order to covalently link the enzyme to the

matrix. Pellicular porous nylon has been found to be a useful support for a number of enzymes by using coupling methods developed for the attachment of enzymes to nylon tubes [13–15]. The employment of a commercially available pellicular nylon, Pellidon (H. Reeve Angel and Co.), is under investigation.

(c) The porous surface of pellicular support, which has a pore diameter in the range of 100–200 nm can be impregnated with an enzyme–polymer conjugate in situ. The technique resembles the method used for the preparation of certain bonded phases for liquid chromatography [16]. The controlled surface porosity support, Zipax (Du Pont), exemplifies a relative inert pellicular support that can be employed to obtain this type of pellicular immobilized enzyme.

EXPERIMENTAL

Materials

Glass beads (Cataphote Corp.) were screened and washed. For the preparation of polyanionic enzyme conjugates the beads were coated with a thin layer of a strong anion-exchange resin [17]. Pellicular supports have been obtained from Northgate Laboratories. According to the supplier pellicular carbon was prepared by the pyrolysis of polyacrylonitrile coated glass beads; pellicular silica and alumina were prepared by forming the porous layer using a sol-gel proces [18] and pellicular nylon was prepared by precipitating Nylon 6 (DuPont) onto the surface of glass beads and hydrolyzing the porous nylon surface. Gantrez AN, a copolymer of maleic anhydride and vinyl methyl ether, was a gift of the GAF Corp. Polyethylene imine (PEI-60) was a gift of Dow Chemical Co. The enzymes, trypsin (TRL), chymotrypsin (CDI), protease (ATT), ribonuclease (R), papain (PAP), L-asparaginase (ASPC), alkaline phosphatase (BAPC), and wheat germ acid phosphatase (AP) were supplied by Worthington. Cathepsin C was obtained from Sigma. The substrates were purchased from Schwarz-Mann.

Preparation of pellicular polyanionic enzyme resins

The maleic anhydride and vinyl methyl ether (1:1) copolymer, Gantrez AN, was hydrolyzed in boiling water for 6 h, then lyophilized. A mixture of the hydrolyzed and unhydrolyzed polymer was dissolved in acetone containing dimethyl formamide and the glass beads were coated uniformly with the viscous polymer solution. The solvents were subsequently evaporated in vacuo at room temperature. The polymer-coated glass beads were mixed with the solution of the enzyme in 0.2 M phosphate buffer, pH 7.0. With alkaline phosphatase 0.2 M acetate buffer, pH 4.5, was used. In most cases the weight of the beads was equal to that of the enzyme solution. The slurry was shaken at 5 °C overnight and the product was subsequently washed with a series of solutions to remove any enzyme not bound covalently to the polymer [19]. The washing solutions included 1 M NaCl, 2 M urea, 0.01 M EDTA in 0.1 M Tris buffer, pH 8.0, 0.01 M phosphate buffer, pH 7.0, and doubly distilled water. In each washing step the supernatant was decanted. The procedure was continued until the supernatant of the product (about 1 g of beads in 5 ml of buffer) did not show enzymic activity after a contact time of 30 min. Then the product was washed with acetone, filtered and dried at room temperature in vacuo.

Preparation of other pellicular immobilized enzymes

The porous support layer was impregnated with a buffered solution of the enzyme. Subsequently, the enzyme was crosslinked with glutaraldehyde [12, 15]. In some instances the enzyme solution contained dissolved bovine serum albumin, thus, the enzyme was coreticulated [20]. The product was washed according to the same procedure described above.

Measurement of enzyme activity

In most instances the pH-stat method [21] was employed to measure the esterolytic activity of both soluble and immobilized enzymes using an ABU-1 automatic buret, TTT-1 titrator, SBR-2 recorder and a Model 26 pH meter (Radiometer Co.). The reaction vessel was jacketed and the jacket connected to a constant temperature circulator bath. The temperature was kept at 25 °C in all experiments except when the temperature profile of the enzyme activity was determined. The activity of trypsin, protease and papain was measured with *N*-benzoyl-L-arginine ethyl ester (BAEE). The activity of chymotrypsin was measured with L-tyrosine ethyl ester (TEE). Cyclic cytidine-2',3'-phosphate was used to measure the activity of ribonuclease.

The activity of pellicular immobilized acid phosphatase was measured spectrophotometrically by the initial rate of hydrolysis of *p*-nitrophenyl phosphate at 410 nm. The reaction was carried out in acetate buffer at pH 5.0 and the measurement at pH 9.0 after mixing an aliquot of the particle free reaction mixture with borate buffer. Alkaline phosphatase was assayed by measuring the initial rate of hydrolysis of *p*-nitrophenyl phosphate spectrophotometrically at 410 nm in 0.01 M Tris buffer, pH 8.5. In some experiments the rate of production of inorganic phosphate using adenylic acid and *p*-nitrophenyl phosphate as substrates was also measured. The activity of cathepsin C was measured by the rate of hydrolysis of glycylglycine ethyl ester and glycyltyrosine ethyl ester. L-Asparaginase was assayed by Nesslerization.

The activity of the pellicular immobilized enzymes was compared to that of the corresponding soluble enzymes by measuring the saturation rate of reaction with the enzyme in both the immobilized form and free solution under identical conditions. The activity of the pellicular immobilized enzymes was expressed by the mg equivalents of soluble enzyme per gram of beads. Although this expression can conveniently be used to compare the activity of different products it does not necessarily reflect the actual amount of active immobilized enzyme since the catalytic constant of the enzyme can be influenced by the microenvironment. In some instances the activity is expressed in international units per g of pellicular product.

Some experiments were carried out with pellicular trypsin resins in a continuous flow packed bed reactor, using $5 \cdot 10^{-3}$ M BAEE in 0.2 M phosphate buffer, pH 7.0. The experimental set-up and the measurement of the conversion with the spectrophotometer were similar to that used in a previous study [15] of open tubular heterogeneous enzyme reactors.

PELLICULAR POLYANIONIC ENZYME RESINS

Polymers containing an abundance of carboxylic anhydride groups such as copolymers of maleic anhydride with ethylene [22, 23] and butanediol-divinylether [24] as well as polymetacrylic acid anhydride [25] have been used to immobilize proteolytic

enzymes. The properties of trypsin–maleic anhydride–ethylene conjugates have received the greatest attention [26] and the behavior of enzymes in a polyanionic matrix has been elucidated [27].

The polycarboxylic enzyme conjugates obtained by these methods, however, are mucilaginous materials whose utilization is greatly hampered by their poor handling characteristics. Therefore, their preparation in pellicular form appeared to be particularly attractive. In this study a copolymer of maleic anhydride and vinylmethylether (Gantrez AN) which has been also reported as an enzyme-immobilizing agent [28] was used as the reactive polymer.

In the first experiments the beads were coated with a layer of the pure polyanhydride and contacted with a buffered trypsin solution. Under these conditions the protein reacted at the outer surface of the layer only and a highly crosslinked barrier was formed which was not permeable by the protein molecules. Such products were not only low in protein content but also unstable, because the swelling of the hydrolyzed but not crosslinked polymer in the interior of the layer caused the layer to break off. This difficulty could not be eliminated by using a partially hydrolyzed polymer, which was found to yield better results than the pure polyanhydride in other studies [29].

It was found, however, that when the reactive coating contained an inert water soluble substance such as the hydrolyzed polyanhydride, which could be leached out during the enzyme coupling process, the formation of a crosslinked enzyme conjugate in the entire depth of the polymer layer was facilitated and a stable enzyme resin shell was obtained. In some instances maleic acid has also been successfully used as a substitute for the nonreactive polyacid.

The addition of a crosslinking agent such as hexamethylene diamine or tetraethylene pentamine to the enzyme solution was not necessary to obtain an insoluble protein conjugate. It is noted that the molecular weight of Gantrez AN 169 is over

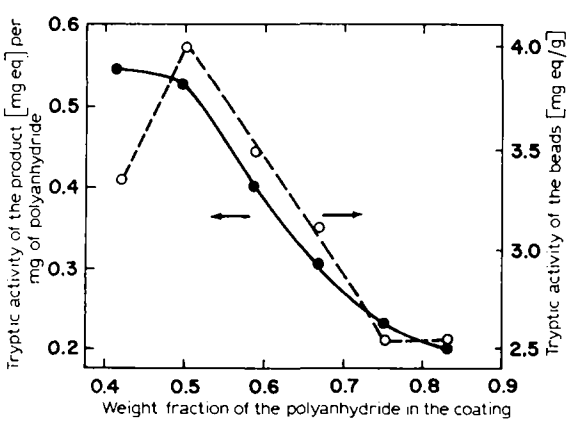

Fig. 2. Graph illustrating the effect of the weight fraction of polyanhydride in the reactive coating on the esterolytic activity of the product. The activity is expressed by mg equivalents (mg eq) of soluble trypsin per mg of polyanhydride employed and by mg equivalents of soluble trypsin per g of beads. 15 mg of polymer mixture were coated onto 1 g of glass beads in each experiment. Reaction mixture: 5 g polymer coated beads and 60 mg trypsin in 5 ml of 0.2 M phosphate buffer, pH 7.0. Activity determination: 10^{-2} M BAEE in 0.2 M phosphate buffer at pH 8.5. The activity of trypsin in free solution was 27 I.U./mg.

one million, thus, it is much higher than that of the ethylene–maleic anhydride copolymers (mol. wt 20 000–100 000) used in previous studies [30].

The effect of the conditions in the preparation of pellicular trypsin resins are discussed below to exemplify the significance of the major factors involved in the coupling process. The data have been obtained with 50-μm beads and the coupling was carried out with trypsin solutions in 0.2 M phosphate buffer at pH 7.0. The composition of the reactive polymer layer, the layer thickness and the amount of enzyme used in the coupling reaction was varied. Fig. 2 shows the tryptic activity per mg of polyanhydride as well as per mg of beads as functions of the layer composition which is expressed by the weight fraction of the polyanhydride. It is seen that optimum results are obtained when equal amounts of polyanhydride and polyacid are used, since the activity of the bulk product is maximum and the tryptic activity calculated per mg of polyanhydride does not increase significantly by further reducing the weight fraction of the polyanhydride in the layer. Fig. 3 shows that the activity of the product and the

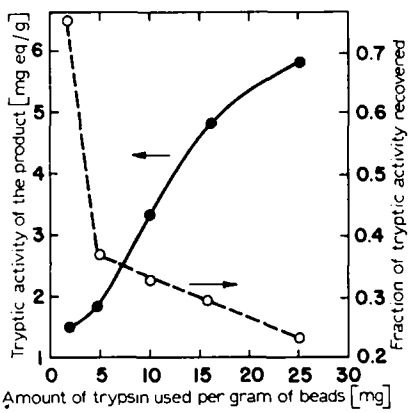

Fig. 3. Graph illustrating the effect of the amount of trypsin used in the coupling reaction on the esterolytic activity of the product as expressed in mg equivalents (mg eq) of soluble trypsin per g of beads and per mg of enzyme employed. Reactive coating: 15 mg of polymeric anhydride and acid mixture (1:1) per g of glass beads. Reaction mixture: 5 g of polymer coated glass beads. 5 ml of 0.2 M phosphate buffer, pH 7.0, containing the appropriate amount of enzyme. Activity determination is given in Fig. 2.

recovery of enzyme activity are affected in the opposite way by the amount of enzyme present in the reaction mixture. A practical compromise is found at about 10 mg of trypsin per g of beads under the reaction conditions stated. This corresponds to the inflection point of the activity curve of the product.

Under the optimum conditions established by these measurements, the activity of the pellicular resins was found proportional to the amount of reactive polyanhydride present in the coating (1:1, polyacid–polyanhydride) when 3–12 mg of polymer was used per g of beads as shown in Fig. 4. This finding suggests that all of the polyanhydride reacts with trypsin in a quasi stoichiometric fashion to form the polycarboxylic conjugate.

Properties of trypsin resin

The chemical properties of the pellicular polyanionic trypsin resin were quite

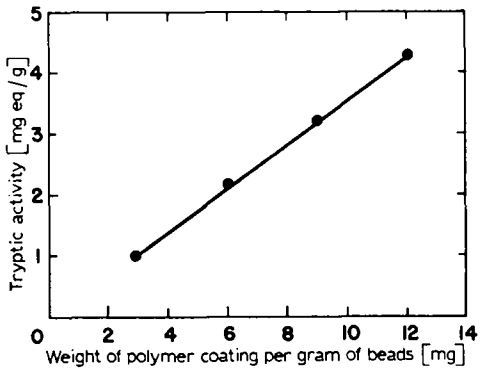

Fig. 4. Plot of the activity of pellicular polycarboxylic trypsin resin against the amount of polymer coating used in the preparation. The weight ratio of the polymer coating to the enzyme was kept constant. The polymer coating was a 1:1 mixture of anhydride and acid. The activity was determined as described in Fig. 2.

similar to those described with polyanionic trypsin gels [23, 26, 29]. Nevertheless, a few observations have been made in the course of our investigation which have not been reported with other polyanionic trypsin conjugates.

Previous studies have shown that the rate of reaction catalyzed by polycarboxylic trypsin and chymotrypsin conjugates increases with increasing ionic strength in the external solution [26]. This phenomenon has been explained by the increase in the internal pH of the enzyme resin. As shown in Fig. 5 this effect is indeed observable at low NaCl concentrations in the surrounding solutions. When the ionic strength is further increased, however, the rate of reaction reaches a maximum at a given NaCl concentration and the activity of the pellicular trypsin resin decreases with further increase in the ionic strength.

This phenomenon can be explained by the gradual shrinking of the gel when the

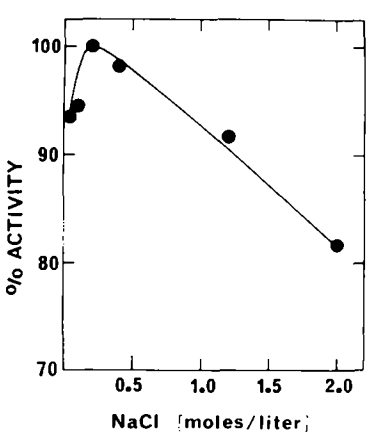

Fig. 5. Effect of the concentration of NaCl in the surrounding solution on the activity of pellicular polycarboxylic trypsin resin. The relative activity with respect to the maximum value measured at 0.2 M NaCl is plotted against the NaCl concentration. Conditions: 10^{-2} M BAEE as substrate in 10^{-2} M phosphate buffer, pH 8.0, having different concentrations of NaCl.

salt concentration increases. Since the substrate diffusivity is reduced in the shrunken gel the overall activity of the resin particles decreases. The effect of shrinking of such trypsin resins by calcium has been discussed elsewhere [7]. It is noted that the attenuation of the overall reaction rate due to shrinking of the gel is less pronounced with shell structured than with conventional gel particles according to a previous theoretical study [4]. From the practical point of view the shrinking of the resin layer can be desirable because it facilitates the filtering and handling of the pellicular material.

As shown in Table I the activity of the pellicular trypsin conjugate was adverse-

TABLE I

EFFECT OF POLYAMINES ON THE ACTIVITY OF PELLICULAR POLYANIONIC TRYPSIN CONJUGATE

All experiments were carried out with $5 \cdot 10^{-2}$ M BAEE at pH 8.0 in the different solutions given below. The activity of trypsin in free solution was the same under these conditions.

External solution	Tryptic activity (units/g)
Phosphate buffer, $5 \cdot 10^{-2}$ M	95
Polyethylene imine hydrochloride, 1% (w/v) of the free amine	28
Tetraethylene pentamine hydrochloride, 1% (w/v) of the free amine	93

ly affected by the presence of polyethylene imine, a polyamine having a molecular weight of approximately 60 000, in the substrate solution. On the other hand tetraethylene pentamine which has a relatively low molecular weight had no significant effect. It is assumed that the accessibility of the trypsin molecules in the interior of the layer is reduced by the interaction between the soluble positively charged polyamine and the negatively charged polymer matrix at the surface of the particles. No effect of polyethylene imine or tetraethylene pentamine has been found on the activity of trypsin in free solution.

The use of immobilized enzymes in hydro-organic media could be of technological significance when the substrate or product is sparingly soluble in water but soluble in organic solvents. Conceivably, the catalytic action of the immobilized enzyme could also be favorably modified under such conditions for some technical applications. Therefore, experiments have been carried out to measure the effect of a few organic solvents on the activity and stability of the polyanionic trypsin resin.

Goldstein [30] found that the catalytic constant of a polyanionic chymotrypsin resin was not significantly perturbed in a 9% (v/v) dimethyl formamide solution. As shown in Table II, the polyanionic trypsin conjugate retained a significant fraction of its activity even in a 50% (v/v) aqueous dimethylformamide solution. In comparison, soluble trypsin lost 80% of its activity in a 50% (v/v) dimethylformamide solution. Aqueous dimethylformamide appears to be a particularly useful solvent for the substrate when such trypsin resin is employed. First, as seen in Table II, 77.5% of the actual enzymic activity can be obtained in a 50% (v/v) dimethylformamide solution. Second, the resin lost only 6% of its initial activity after incubation in a 50% (v/v,

TABLE II

ACTIVITY OF PELLICULAR POLYANIONIC TRYPSIN CONJUGATE IN WATER–DIMETHYLFORMAMIDE MIXTURES

Substrate: $5 \cdot 10^{-2}$ M BAEE, 10^{-2} M phosphate buffer, pH 8.0. Soluble trypsin retained only 20% of its original activity in 50% (v/v) dimethylformamide solution.

Dimethylformamide (%, v/v)	Activity (units/g)
0	95.4
50	73.7
60	27.5
70	8.1

dimethylformamide solution over four days at room temperature. In comparison, 76% of the initial activity was lost when the trypsin resin was incubated in a 50% (v/v) solution of dimethylsulfoxide.

The exposure of dry pellicular polyanionic trypsin resin to pure organic solvents resulted in a significant decrease in the enzymic activity as shown in Table III. It is

TABLE III

RESIDUAL ACTIVITY OF PELLICULAR POLYANIONIC TRYPSIN CONJUGATE AFTER INCUBATION IN ORGANIC SOLVENTS AT 25 °C

Substrate: $5 \cdot 10^{-2}$ M BAEE; 10^{-2} M phosphate buffer, pH 8.0. The original activity of the dry beads was 95 units/g. The enzymic activity of the incubated beads was determined after removing the organic solvent.

Solvent	Incubation time (h)	Residual activity (%)
Dimethyl formamide	24	53
	120	12
Dioxane	24	65
	120	22
Hexamethyl phosphoric triamide	24	18
	144	12

noted that in agreement with earlier observations with soluble trypsin [31] aqueous dioxane solutions had the smallest effect on the activity of the polyanionic trypsin conjugate. These and similar results with other types of pellicular immobilized enzymes (unpublished results) indicate that the properties of enzymes in non-aqueous solvents can be studied more conveniently with immobilized than with soluble enzymes [32] and the immobilized enzymes can have much greater resistance to denaturation by organic solvents than soluble enzymes. Thus, the reaction mixture in heterogeneous enzyme catalysis could contain a substantial fraction of organic solvents without adverse effects.

The investigation of the storage stability of the trypsin resin showed a much higher stability in lyophilized form than in aqueous media. The dry product retained

its original activity at room temperature for at least four months. Upon storage in 0.1 M NaCl solution for 21 days at 10 °C, the assay showed that only 35% of the original activity remained. These results are in agreement with data obtained with other polyanionic enzyme gels [33]. In the presence of calcium the wet stability was significantly higher.

Other polyanionic enzyme resins

A variety of pellicular polyanionic enzyme resins have been prepared by the same technique used for trypsin. Table IV shows the activity of the products as expressed by the mg equivalents of soluble enzyme per g of beads measured at the saturation rate under otherwise identical conditions. The coupling reaction was carried out without a cross linking agent other than the enzyme itself.

All enzymes were coupled to the polyanhydride in 0.2 M phosphate buffer at pH 7.0 with the exception of alkaline phosphatase which was coupled in 0.2 M acetate buffer at pH 5.0. In agreement with earlier observations [28] alkaline phosphatase did not give active products at pH 7.0, presumably due to the unavailability of the ϵ-amino groups of lysine.

As seen in Table IV, the recovered enzymic activity varies from enzyme to

TABLE IV

ACTIVITY OF PELLICULAR POLYANIONIC ENZYME CONJUGATES

Particle diameter: 50 μm. Reactive coating: 6 mg of Gantrez AN 169 and 6 mg of hydrolyzed Gantrez AN per g of beads. 12 mg of soluble enzyme per g of beads.

	mg equivalents of soluble enzyme per g	Substrate*	pH of assay
Trypsin	5.5	BAEE	8.0
Protease	6.2	BAEE	8.0
Chymotrypsin	1.8	TEE	7.0
Papain	2.3	BAEE	7.0
Ribonuclease A	2.5	CCP	7.0
Ribonuclease T	1.9	CCP	7.0
L-Asparaginase	2.1	Asn	8.5
Alkaline phosphatase	2.8	PNPP	8.5

* CPP, barium salt of cytidine-2',3'-cyclic phosphate; Asn, L-asparagine; PNPP, sodium salt of *p*-nitrophenylphosphate.

enzyme. It is expected, however, that by optimizing the coupling conditions for each enzyme individually, a higher recovery can be obtained. On the other hand, by optimizing the assay conditions for the immobilized enzyme with respect to pH, ionic strength and some additives such as EDTA, higher activities could have been measured. Nevertheless, the activity values shown in Table IV illustrate the fact that such products are sufficiently active for most practical applications.

The addition of hexamethylene diamine to the coupling mixture resulted in products of lower activity, as shown in Table V, than the coupling process without added crosslinking agent. Presumably due to the high molecular weight of Gantrez AN

TABLE V

EFFECT OF ADDITIONAL CROSSLINKING ON THE ACTIVITY OF PELLICULAR POLYANIONIC ENZYME CONJUGATES

The coupling conditions were the same as stated in Table IV except that 0.625 mg of hexamethylene diamine per g of beads were added to the enzyme solution. The activity is expressed by the percent of activity given in Table IV as measured by using the same assay procedure.

Enzyme	Activity (%)
Trypsin	67.0
Protease	71.0
Chymotrypsin	57.0
Ribonuclease	100.0

169, however, an insoluble product was easily obtained by using the enzyme as the sole cross-linking agent. In addition, the pellicular structure afforded sufficient mechanical stability and convenient handling characteristics to the products.

IMMOBILIZED ENZYMES ON PELLICULAR SUPPORTS

Table VI shows a number of pellicular supports employed in the preparation of immobilized wheat germ acid phosphatase. The enzyme has been crosslinked by glutaraldehyde in the porous surface layer. As seen the pH of the reaction had a great influence on the activity of the product and consistently higher enzymic activity had been recovered when the crosslinking was carried out at pH 4.65 rather than at pH 8.0. Pellicular carbon has been found to be the best support. This has been confirmed by using this support for the immobilization of trypsin, chymotrypsin and papain. The activities of the products were comparable to those obtained with the pellicular polyanionic conjugates.

TABLE VI

ACTIVITY OF ACID PHOSPHATASE IMMOBILIZED ON VARIOUS PELLICULAR CARRIERS

Particle size: 60 μm. Approximate layer thickness: 3 μm. The enzyme (12 mg/g of beads) was dissolved in water, then adsorbed onto the support. Subsequently it was crosslinked with 3% glutaraldehyde in 0.2 M phosphate (pH 8.0) or acetate (pH 4.65) buffer.

Support	pH of coupling	Activity (mg equivalents of soluble enzyme per g)
Pellicular carbon	4.65	5.7
Pellicular carbon	8.0	2.2
Pellicular alumina	4.65	4.1
Pellicular alumina	8.0	1.6
Pellicular silica	4.65	3.0
Pellicular silica	8.00	1.4
Pellicular nylon	4.65	3.1
Pellicular nylon	8.00	1.7

Pellicular carbon has also been used to prepare immobilized cathepsin C. The esterase activity of the product was 0.16 and 0.22 units/g beads using glycyltyrosine ethyl ester and glycylglycine ethyl ester as the substrate, respectively. These values represent 8.3 and 8.6% recovery of the enzyme activity, respectively. Nevertheless, the recovery was about four times higher than that recorded for the immobilization of cathepsin C on enzacryl polythiolactone [34]. With the exposure of the product to substrate solution at pH 6.5 and above, a rapid polymerization of the dipeptides was observed as expected from the behavior of the soluble enzyme [35]. As a result the beads agglomerated and showed little or no activity at pH 5.0 after removal from the solution and thorough washing. It is assumed that the polymer precipitated in the pores and made the enzymic sites inaccessible to substrate molecules.

Chymotrypsin immobilized on pellicular carbon (50 μm particles) yielded a product which had the activity of 2 mg of soluble enzyme as measured with $3.3 \cdot 10^{-2}$ M L-tyrosine ethyl ester at pH 6.5 using the pH-stat. The K_m value for the soluble and immobilized enzyme was found to be approximately $9.5 \cdot 10^{-3}$ M and $7.5 \cdot 10^{-3}$ M, respectively. Thus, the K_m value did not change significantly by the immobilization. Fig. 6 shows the pH profile of chymotrypsin in free solution and on pellicular carbon

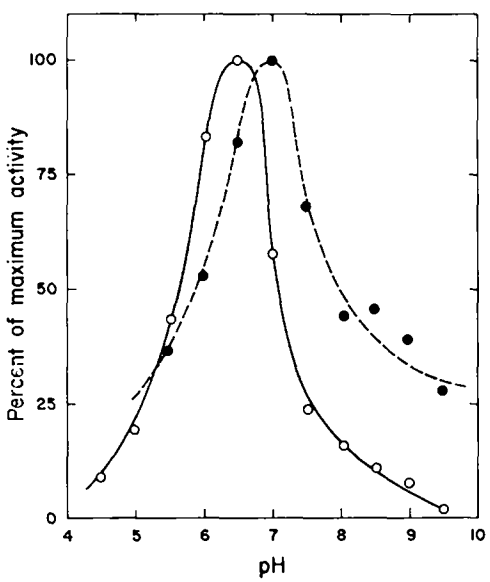

Fig. 6. pH profile of chymotrypsin immobilized on pellicular carbon (broken line) and in free solution (solid line) as measured with $3 \cdot 10^{-3}$ M TEE in 10^{-2} M phosphate solution.

beads as measured with 3×10^{-3} M L-tyrosine ethyl ester. The shift in the maximum activity from pH 6.5 to 7.0 suggests that the microenvironment of the immobilized enzyme is negatively charged. The shape of the curve obtained with the immobilized enzyme at pH values higher than 7 suggests that the removal of the product (H^+) from the heterogeneous enzyme catalyst is diffusion limited. This effect is discussed in a forthcoming paper [36].

PELLICULAR IMMOBILIZED ENZYMES IN PACKED BEDS

As it has already been pointed out, the major use of pellicular immobilized enzymes is expected in packed beds. In such applications larger particles are preferable in order to obtain sufficiently high bed permeability. Therefore pellicular polyanionic trypsin and chymotrypsin resins have been prepared by using 150 μm diameter glass beads. The activity of the products per unit weight was about half of that obtained with the 50-μm beads as determined by the pH-stat assay. Trypsin has also been immobilized on 250 μm diameter pellicular silica. The esterolytic activity of the product per g was equivalent to that of 1.2 mg of soluble enzyme.

Columns were packed with these immobilized enzymes and perfused with phosphate buffers at pH 7. The buffer concentration was changed from 0.01 to 0.3 M and then reversed. No shrinking or swelling of the bed was observed even after several cycles in the buffer concentration and no bed compression was noticed by operating the column at 10 atm inlet pressure over a day.

The steady state conversion measured intermittently did not change in reactors containing polyanionic or siliceous pellicular immobilized trypsin after continuous perfusion with 0.1 M phosphate buffer, pH 7.0, at room temperature over a period of 12 days. It is concluded, therefore, that pellicular materials yield indeed, a stable column packing for enzyme reactors.

CONCLUSIONS

In many respects the pellicular configuration represents a combination of the advantages of porous glass as a mechanically stable carrier of favorable handling characteristics with those inherent in the various immobilized enzyme structures based on organic or inorganic supports. It has been shown that mucilaginous enzyme conjugates can be prepared in pellicular form, which makes possible their use in packed columns. In addition, support materials such as silica, porous carbon and nylon in pellicular form can yield uniform spherical immobilized enzyme particles for use in enzyme reactors. Their activity, both on a weight or volume basis, compares favorably with that of enzymes attached to porous glass. It is noted, however, that the pellicular immobilized enzymes consist of about 95–98% (w/w) solid glass and the comparison of their activity on a weight basis with that of other immobilized enzymes can be misleading.

Since the catalytic behavior of a particular enzyme in immobilized form is largely dependent on the microenvironment under a given set of conditions, the pellicular configuration per se does not impart chemical properties to the product which would be significantly different from the conventional immobilized enzyme made by using the same chemistry. On the other hand, pellicular immobilized enzymes possess significant advantages with respect to the utilization of the enzyme as has been shown in a previous theoretical paper.

Nonetheless, the technological application of pellicular immobilized enzymes will apparently be determined by practical considerations when packed bed enzyme reactors claim a major role in the laboratory and industry.

ACKNOWLEDGEMENT

The author is indebted to Mojmir Sovak for his invaluable assistance and gratefully acknowledges the cooperation of Jean-Marc Engasser, Richard T. Light, Hannah Wan, and Kevin Ward. This work was supported in part by a Grant No. RR 00356 from the National Institutes of Health.

REFERENCES

1 Cuatrecasas, P. and Anfinsen, C. B. (1971) in Methods of Enzymology (Jacoby, W. B., ed. Vol. 22, pp. 345–378, Academic Press, New York
2 Lilly, M. D. and Dunhill, P. (1972) Biotech. Bioeng. Symp. No. 3, 221–227
3 Katchaski, E., Silman, I. and Goldman, R. (1971) in Advances in Enzymology (Nord, F. F., ed.) Vol. 34, pp. 445–536
4 Horvath, C. and Engasser, J.-M. (1973) I & EC, Fundamentals 12, 229–235
5 Horvath, C. (1973) in Methods of Biochemical Analysis (Glick, D. ed.), Vol. 21, pp. 79–154, Wiley, New York
6 Horvath, C., Preiss, B. A. and Lipsky, S. R., (1967) Anal. Chem. 39, 1422–1428
7 Horvath, C. and Sovak, M. (1973) Biochim. Biophys. Acta 298, 850–860
8 Axen, R., Porath, J., and Ernback, S. (1967) Nature 214, 1302–1304
9 Cuatrecasas, P. (1971) in Biochemical Aspects of Reactions on Solid Supports (Stark, G. R., ed.) pp. 79–110, Academic Press, New York
10 Weetall, H. H. (1969) Science 166, 615–617
11 Weetall, H. H. and Mason, R. D. (1973) Biotech. Bioeng. 15, 455–466
12 Haynes, R. and Walsh, K. A. (1969) Biochem. Biophys. Res. Commun. 36, 235–242
13 Hornby, W. E. and Fillipusson, H. (1970) Biochim. Biophys. Acta 220, 343–345
14 Inman, D. J. and Hornby, W. E. (1972) Biochem. J. 129, 255–262
15 Horvath, C. and Solomon, B. A. (1972) Biotech. Bioeng. 14, 885–914
16 Kirkland, J. J. and DeStefano, J. J. (1970) J. Chromatogr. Sci. 8, 309–315
17 Horvath, C. (1973) in Ion Exchange and Solvent Extraction (Marinsky, J. A. and Marcus, Y. eds) Vol. 5, 207–260, Dekker, New York
18 Hermans, M. E. A. (1973) Powder Met. Int. 5, 137–140
19 Kay, G. and Lilly, M. D. (1970) Biochim. Biophys. Acta 198, 276–285
20 Thomas, D., Broun, G. and Selegny, E. (1972) Biochimie 54, 229–244
21 Jacobsen, C. F., Leonis, J., Linderstrom-Lang, K. and Ottensen, M. (1957) in Methods of Biochemical Analysis, (Glick, D., ed.) Vol. 4, pp. 171–210, Wiley-Interscience, New York
22 Levin, Y., Pecht, M., Goldstein, L. and Katchalski, E. (1964) Biochemistry 3, 1903–1913
23 Goldstein, L. (1970) in Methods in Enzymology (Perlmann, G. E. and Lorand, L., eds) Vol. 19, 935–962, Academic Press, New York
24 Brümmer, W., Hennrich, N., Klockow, M., Lang, H. and Orth, H. D. (1972) Eur. J. Biochem. 25, 129–135
25 Conte, A. and Lehmann, K. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 533–541
26 Goldstein, L., Levin, Y. and Katchalski, E. (1964) Biochemistry 3, 1913–1919
27 Katchalski, E. (1970) in Structure-Function Relationship of Proteolytic Enzymes (Desnuelle, P., Neurath, H. and Ottensen, M., eds) pp. 198–221, Academic Press, New York
28 Zingaro, R. A. and Uziel, M. (1970) Biochim. Biophys. Acta 213, 371–379
29 Fritz, H., Gebhardt, M., Fink, E., Schramm, W. and Werle, E. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 129–138
30 Goldstein, L. (1972) Biochemistry 11, 4072–4084
31 Inagami, T. and Sturtevant, J. M. (1960) Biochim. Biophys. Acta 38, 64–79
32 Singer, S. J. (1962) in Advances in Protein Chemistry (Anfinsen, C. B., Anson, M. L., Bailey, K. and Edsall, J. T., eds) Vol. 17, pp. 1–68, Academic Press, New York
33 Weetall, H. W. (1970) Biochim. Biophys. Acta 212, 107
34 Calam, D. H. and Thomas, H. J. (1972) Biochim. Biophys. Acta 276, 328–332
35 Heinrich, C. P. and Fruton, J. S. (1968) Biochemistry 7, 3556–3565
36 Engasser, J.-M. and Horvath, C. (1974) Biochim. Biophys. Acta 358, 178–192