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Azlactone-reactive polymer supports for immobilizing synthetically useful enzymes Part I. Pig liver esterase on dispersion polymer supports

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Abstract

Covalent attachment of pig liver esterase (E.C.3.1.1.1) to cross-linked dispersion polymer supports was effectively accomplished using azlactone [5(4H)-oxazolone] reactive groups. The binding process required about one hour at room temperature, and it was imperative that a relatively high concentration of a salt co-solute be present along with the enzyme. Under these conditions the enzyme rapidly bound onto the polymeric supports via hydrophobic interaction and then covalent attachment proceeded at effective rates. Up to 10 wt.% of the enzyme could be quantitatively bound to supports with retention of high levels of catalytic function, e.g., 68% specific activity at 4 wt.%. The non-reactive content of the polymeric support and especially the hydrophilic–hydrophobic balance were shown to be very important, with the hydrophilic supports providing the more favorable environment for hydrolytic esterase activity.

Keywords: Pig liver esterase; E.C.3.1.1.1; Azlactone; 5(4H)-oxazolone; Immobilized enzyme; Biocatalysis

1. Introduction

Enzyme biocatalysts are predicted to have a significant impact on the chemical industry in the next 10 years [1]. Characteristics of these unique catalysts such as operation at ambient temperature and atmospheric pressure, high degrees of reaction specificities (regio and enantio), phenomenal catalytic turnover numbers, a general reduction in the number of synthetic steps because protection–deprotection operations can often be avoided, and that biocatalytic manufacturing generally leads to "sustainable" operations have long been recognized by industry. More recent developments such as significantly reduced cost of enzymes due to application of recombinant DNA technology and the potential to develop still more specific and powerful catalysts employing mutagenesis and directed evolution techniques will also significantly contribute to increased use of enzymes in general manufacturing of fine and bulk chemicals.

Effective immobilization of synthetic enzymes is extremely important for application in industrial operations. First, an obvious benefit is elimination (or at least a substantial simplification) of separation of the catalyst from the product stream. Second, immobilization often stabilizes the enzyme, thus extending catalyst lifetime and the number of reuses of this still-precious commodity.

The azlactone [5(4H)-oxazolone] heterocycle reacts with appropriate nucleophiles by ring-opening, nucleophilic addition that creates no byproducts [2]. Reaction rates are also generally about where one would like them for enzyme attachment from aqueous media–intermediate between relatively slow-reacting oxirane and highly reactive (and hydrolytically sensitive) isocyanate electrophilic groups.

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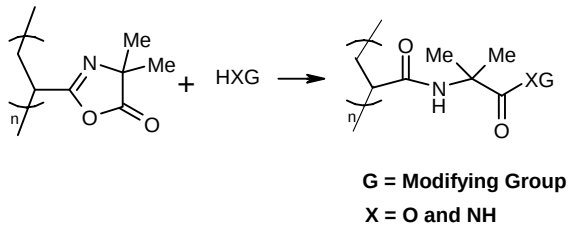

Fig. 1. Overall polymer modification procedure using azlactones.

Fig. 1 depicts this mode of reaction with a pendant azlactone group on a polymer, and when covalently binding biomacromolecules by this method, the HXG moiety is generally a protein containing lysine amino acid residues.

2-Vinyl-4,4-dimethylazlactone (VDMA) has generally been the monomer of choice to prepare azlactone-reactive polymers [3]. It is commercially available and well-behaved in terms of providing high conversion of monomers to copolymer and with an absence of significant chain transfer activity for effective molecular weight control. When cross-linking monomers and appropriate conditions are employed during the copolymerization of VDMA, insoluble supports suitable for covalently attaching proteins can be obtained in various forms including porous beads [4], non-porous filamentous particles [5], or monolithic supports [6].

The porous beads were developed primarily for use in affinity chromatography as 3M's EMPHAZE^{TM} Biosupport Medium and are commercially available through Pierce Chemical Co. (Rockford, IL, USA) as ULTRALINK^{TM} Biosupport Medium. Protein binding capabilities have primarily been restricted to examining proteins useful as affinity ligands, e.g., protein A, and binding capabilities and column performance characteristics have been reported [7].

Scattered and preliminary reports have appeared examining the non-porous dispersion polymer supports as immobilization entities for synthetically useful enzymes in aqueous [8] and non-aqueous media [9]. This report details efforts examining the non-porous azlactone reactive supports as immobilizing media for pig liver esterase (E.C.3.1.1.1).

2. Experimental procedure

2.1. Materials and methods

All dispersion polymer materials were prepared using a literature method [5]. Unless otherwise indicated, the particular support was referred to by its "third" monomer which adjusted hydrophilicity. The VDMA level was constant at 20 wt.%, and the cross-linking monomer, trimethylolpropane trimethacrylate (TMPTMA), made up the balance. The HEMA-30 (hydroxyethyl methacrylate) support, for example, contained TMPTMA—50 parts, VDMA—20 parts, and HEMA—30 parts. Pig liver esterase (PLE) was purchased from Sigma as an ammonium sulfate suspension, nominally possessing 150–200 units/mg of activity (one unit will hydrolyze 1.0 μmole/min of ethyl butyrate to butyric acid and ethanol at pH 8.0 and 25 °C). In order to remove ammonium sulfate, the suspension was dialyzed (6000–8000 molecular weight cut off) three times against 0.9 M Na_2SO_4 and 10 mM KH_2PO_4 (pH 7.4), centrifuged and filtered using a 0.45 μm syringe filter. Enzyme concentrations were determined by measuring the absorbance at 280 nm with one PLE sample, dialyzing that solution against pure water (3×) to remove salts, and drying an aliquot to constant weight at high vacuum to determine percent solids. UV Absorbance was then utilized to compute the concentration of PLE in subsequent commercial samples using a value of 1.48 AU/(mg ml). EPPS buffer [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid] was purchased from Sigma. Propylene glycol methyl ether acetate and Triton X-100 were obtained from Aldrich. Phosphate-buffered saline (PBS) was purchased from Pierce Biotech.

UV spectra were obtained using a Hewlett-Packard 8450A Spectrophotometer equipped with a Diode Array detector. Control of pH in enzyme assays was accomplished using a Kasei GT06 Automatic pH Stat/Titrator. Particle size analyses were conducted using a FRA Microtrac Particle Analyzer. Protein quantitation was also conducted with selected samples (entries 2 and 3 of Table 1) employing a standard amino acid analysis technique [10] in order to obtain corroborating evidence of the quantity of PLE on the support.

2.2. Enzyme assays

2.2.1. p-Nitrophenyl acetate

A constant amount (8 μg) of PLE, immobilized or free, was employed in the assay procedure. With immobilized PLE samples because all supports were dispersed in the same volume of buffer (100 mg of support in 10 ml of EPPS buffer), comparative analyses were conducted employing 20 μl aliquots of shaken slurries using a pipetting tip with the end cut off to better accept and transfer the heterogeneous slurries. It was determined gravimetrically that 200 μg of support were transferred employing this procedure. Simple filtration of a volume of a slurry also indicated a composition of approximately 13 vol.% particles and 87 vol.% supernatant. The PLE sample was added to 2.4 ml of 50 mM EPPS (pH 8.0) buffer contained in a 3 ml UV cell equipped with a magnetic stirring bar and temperature controlled to 25 °C. *p*-Nitrophenyl acetate (80 μl of a 1 mg/ml solution in acetonitrile; providing a substrate concentration in the cuvette of 0.18 mM) was added and the absorbance at 400 nm over a 45 s period was immediately recorded. K_m was determined by a standard double-reciprocal plot method [11] to be 0.25 mM. Although ideally a value above K_m was desirable, the concentration employed was relatively close, obtained values were regarded as comparative and not indicative of actual rate data, and higher substrate concentrations actually

Table 1
p-Nitrophenyl acetate assay results of selected samples[a]

Entry	Sample	Slurry	Supernatant	Particulate	Initial filtrate	Homogenate[f]
1	Free PLE[b]	–	–	–	162	–
2	80:20 ($MeNH_2$) no triton wash	13.9	9.5 (8.3)[c]	5.6	49.1	–
3	80:20 ($MeNH_2$) triton wash	0.9 (0.3)	0.3	0.6	59.3	–
4	80:20 (32.2)[d]	9.7	0.3	9.4	0.2	–
5	LMA[e]-20 (49.6)	6.5	0.7 (0.6)	5.9	11.3	–
6	HEMA-10 (28.7)	13.6	0.2	13.4	0.1	12.3
7	HEMA-20 (25.1)	12.0	0.3	11.7	0.3	15.6
8	HEMA-30 (21.6)	13.9	0.1	13.8	0.2	20.4
9	HEMA-40 (18.0)	9.1	0.3	8.8	10.4	22.1
10	HEMA-50 (14.5)	8.2	0.3	7.9	19.3	27.7
11	VDMA-5[g] (21.6)	–	0.3	–	0.4	14.5
12	VDMA-10 (21.6)	–	0.3	–	0.2	19.5
13	VDMA-15 (21.6)	–	0.3	–	0.2	19.6
14	VDMA-20 (21.6)	–	0.2	–	0.5	19.5

[a] Values are averages of at least three determinations in units of mAU/s.
[b] PLE sample exhibited a specific activity of 16.8 mAU/(s μg).
[c] Values in parentheses are corrected for volume contributions of particulate in the slurry, i.e., corrected supernatant value is 87% of the 20 μl supernatant value. The Particulate value is the slurry minus the corrected supernatant values.
[d] Numbers in parentheses are the lipophilicity indices (LI) for the supports.
[e] LMA means Lauryl Methacrylate.
[f] Homogenate values were obtained by subjecting slurries to a procedure which resulted in physical breakup of the slurry particles.
[g] For entries 11–14 sample designations indicate the wt.% VDMA in the polymer. VDMA-5 = TMPTMA:HEMA:VDMA (55:40:5); VDMA-10 = TMPTMA:HEMA:VDMA (53:37:10); VDMA-15 = TMPTMA:HEMA:VDMA (52:33:15); and VDMA-20 = TMPTMA:HEMA:VDMA (50:30:20) (this support is also the HEMA-30 support).

provided optical densities that were too large for the UV setup employed. The slope of the straight line obtained was the rate of reaction recorded as milliabsorption units per second; the specific acitivity of free PLE was dependent on the particular lot and ranged from 13–17 mAU/(s μg). The non-catalyzed autohydrolysis rate of *p*-nitrophenyl acetate in these determinations at pH 8.0 was <0.3 mAU/s. Each determination was conducted at least three times.

Homogenization of original slurries of supported PLE samples was conducted using a Tissue Tearor Model 985–370 Tissue Homogenizer (Biospec Products, Bartlesville, OK, USA). The procedure was conducted on slurries that were contained in 13 mm diameter glass vials. The tip of the homogenizer was positioned approximately halfway into the suspension, and the slurry was homogenized employing the fastest speed for 5 min.

2.2.2. Ethyl butyrate

Performance of supported and unsupported PLE samples was also assessed using an ethyl butyrate hydrolysis assay. The analysis was conducted using 0.40 mg samples of PLE. The electrode of a pH stat was immersed in 100 ml of 5 mM EPPS buffer (pH 8.0) and stirred magnetically. Ethyl butyrate (700 μl; 0.61 g; 5.25 mmoles) was added, followed by the PLE sample. The volume of 0.10 N NaOH added with time was recorded automatically, and the slope of the straight line obtained was the rate in ml/min. This value could then be utilized to compute the units of activity (in μmol/min), and specific activity (in μmol/(min mg)). Each determination was performed in triplicate.

2.3. Reaction of an azlactone support with methylamine

The TMPTMA:VDMA (80:20) dispersion polymer (2.51 g) was suspended in 100 ml of anhydrous THF. With slow mechanical stirring, gaseous methylamine was bubbled into the suspension at 5–10 °C for 40 min. At this point the suspension was saturated as evidenced by the response of moist pH paper in contact with the effluent vapors. The reaction was stirred at ice bath temperatures for an hour, followed by another hour warming to room temperature. The mixture was filtered, the filtercake washed well with THF, and dried at high vacuum. IR indicated that 92.8% of the azlactone carbonyl at 1823 cm^{-1} had disappeared. This support, now incapable of covalent attachment, was designated 80:20 ($MeNH_2$).

2.4. PLE binding to methylamine reacted support

To 100 mg of the 80:20 ($MeNH_2$) support were added PLE [4.0 mg (0.88 ml of a 4.55 mg/ml stock solution)], 6.0 ml of a high sulfate solution consisting of 0.7 M Na_2SO_4, 0.125 M NaH_2PO_4, and 0.125 M Na_2HPO_4 that had been neutralized to pH 7.4, and 0.13 ml of a PL-31 surfactant solution consisting of 406 mg of PL-31 and 10 ml of deionized water. The mixture was tumbled at room temperature for 2 h and then filtered using a sintered glass funnel (ASTM 10–20 μ). This "initial filtrate" was retained for analysis, while the filtercake was washed with PBS (2 × 25 ml) and water (2 × 25 ml). The filtercake was then quantitatively transferred into 1% Triton X-100 in deionized water (25 ml), tumbled at room

temperature for 16 h, filtered and washed with deionized water (4 × 25 ml). The solid was then quantitatively transferred into 10 ml of EPPS pH 8.0 buffer for storage at 5 °C. All filtrates and washes were retained for enzyme assay investigations. Additionally, a "supernatant" value was recorded by filtering a slurry aliquot using a 0.2 μ syringe filter and subjecting the filtrate to the assay reaction.

2.5. PLE binding to azlactone-reactive supports

A typical binding procedure involved adding 6.0 ml of the high sulfate and 0.13 ml of the surfactant solutions described in the previous section to 100 mg of the azlactone-reactive support. The mixture was shaken briskly to disperse the support, and any remaining clumps were broken up using a spatula. The PLE (4.0 mg) solution was then added, followed by tumbling at room temperature for 2 h, and the remaining washing and re-suspension steps of the procedure outlined in the previous section were applied to obtain the covalently bound PLE-support product.

2.6. Examination of the rate of covalent binding

To 800 mg of the HEMA-30 support were added 48 ml of a 0.7 M citrate/buffer solution (consisting of 0.7 M sodium citrate and 0.05 M NaH_2PO_4 and 0.05 M Na_2HPO_4 neutralized to pH 7.4) and 1.2 ml of a surfactant solution [from a stock solution consisting of Pluronics L-31 (400 mg) dissolved in 10 ml of deionized water]. This mixture was shaken briskly for a few minutes and any remaining clumps of support were broken up with a spatula. The PLE challenge (32.1 mg; 4.4 ml of a stock solution containing 7.3 mg/ml; free PLE activity in the assay was 152 mAU/s) was then added and the resultant mixture was tumbled at room temperature. After 2, 5, 10, 30, 60, and 120 min, 6.7 ml aliquots were removed. The aliquots were filtered quickly through a 10–20 μ filter. The filtrate was additionally filtered through a 0.45 μ syringe filter before analysis in the *p*-nitrophenyl acetate assay as the "initial filtrate"; average values of 5.1, 2.1, 2.1, 1.1, 0.5, and 0.1 mAU/s were observed across the time series. The filtercakes were washed with PBS (1 × 25 ml) and deionized water (3 × 25 ml) before resuspension in 10 ml of 1% aqueous Triton X-100 for 16 h. The mixtures were then filtered, washed well with water (4 × 25 ml), and resuspended in 10 ml of EPPS buffer for "slurry analysis"; average values of 4.8, 10.9, 16.7, 18.9, 21.2, and 21.1 mAU/s were observed with the samples.

2.7. Reuse experiments

The pH stat and an overhead paddle stirring apparatus were utilized with a 200 ml beaker as reaction apparatus and vessel. HEMA-30/PLE(4%) (250 mg of support; 10 mg of immobilized PLE) was added to the beaker providing a total volume of 90 ml with the 0.05 M EPPS pH 8.0 buffer. Ammonium hydroxide (0.271 M) was utilized to neutralize the acetic acid by-product created upon hydrolysis of propylene glycol methyl ether acetate (0.66 g; 0.68 ml; 5.0 mmoles) that was added last ($t = 0$) to the stirred slurry. The rate of the hydrolysis reaction from the period of 2–10 min was the slope of the straight line obtained by plotting the volume of ammonium hydroxide added (also the same number of moles of acetate reactant hydrolyzed) with time for the zero order reaction. The initial value was 0.647 ml/min (175 μmoles/min). The entire contents of the beaker were then quantitatively transferred to a fritted glass funnel (4–8 μ) and filtered. The filtercake was washed with 50 ml portions of 0.005 M EPPS (3×). The moist filtercake was then quantitatively transferred back into the beaker and the volume of 0.05 M EPPS brought up to 90 ml for the next reuse experiment. At the end of the day, the washed filtercake was resuspended in the 0.05 M EPPS buffer and stored at 5 °C. Temperature control over the course of the 30 reuses was not rigorously controlled and may have varied between 21–23 °C.

3. Results and discussion

PLE is an enzyme that is relatively pure, inexpensive, and has fairly high activity per unit weight, e.g., 150–200 U/mg. It is stable when refrigerated for prolonged periods, does not require any cofactors or metal ions, and can be conveniently assayed spectrophotometrically by examining hydrolysis reactions of *p*-nitrophenyl esters and carbonates [12]. Pertinent technical specifications for PLE include: pI = 5.0; monomeric molecular weight = 62,016 with 566 amino acid residues and 35 lysine residues available for reaction with the azlactone groups (Expert Protein Analysis System; http://www.us.expasy.org/), and the monomer oligomerizes in concentrated solution to form active quaternary structures. Additionally, the enzyme has been utilized extensively in asymmetric synthesis [13], an active-site model has been elaborated that allows for the interpretation and prediction of the stereochemical result [14], and a recombinant PLE has been expressed in a microbial stain [15].

The filamentous, coral-like azlactone supports obtained by dispersion polymerization [5] were examined as binding supports for PLE. The insoluble polymers were attractive candidates as immobilizing supports for synthetically useful enzymes for several reasons. First of all from physical perspectives, they had relatively high surface areas and were non-swelling, even in organic solvents. As cost control measures, reactor efficiencies were quite high in terms of quantities of material obtained per unit reactor volume and the dispersion polymers required no sizing operation. The hydrophilic–hydrophobic balance of the support backbone could also be manipulated with relative ease by inclusion of a hydrophilic or hydrophobic comonomer with the 2-vinyl-4,4-dimethylazlactone (VDMA) and cross-linking monomers, and the very important pre-covalent association of the enzyme with the reactive support, either by

hydrophobic interaction [16] or by ion exchange [17], was readily addressable.

An issue with many of these heterogeneous materials, however, and especially the more hydrophobic ones, was that they were not "wetted" by aqueous media. A surfactant was required that would permit wetting but would not possess chromophores that would absorb at 280 nm and interfere with UV measurements associated with determination of protein concentration in filtrates and supernatants obtained during binding steps. The Pluronics^{TM} line of surfactants [poly(oxyethylene-b-oxypropylene) copolymers] was identified that met the UV criterion, and the L-31 material was selected because it was liquid at room temperature and easily dispensed. The concentration of the surfactant was found to be fairly critical for effective enzyme binding, and a quantity between 0.10–0.20 wt.% allowed for wetting the dispersion polymers and provided high levels of enzyme binding and retention of catalytic function.

3.1. Binding protocols

It was imperative that procedures within a binding sequence provide for the elimination of non-covalently or non-specifically bound PLE, i.e., by hydrophobic or ion exchange interactions, that could eventually leach out into the reaction solution, contaminating the product and reducing catalytic potency on reuse. To formulate an appropriate procedure, a representative support was pre-reacted with excess methylamine to eliminate azlactone groups. This reaction was conducted in an anhydrous organic solvent, and methylamine was selected as the amine nucleophile because resultant *N*-methyl amide product groups should have minimal hydrophobic impact. The anhydrous reaction condition was selected to eliminate hydrolysis side reactions, since resultant carboxylate groups could engage PLE in ion exchange binding interactions. This latter concern, however, was shown to not be very important; after 2 h at room temperature at pH 7.4, a HEMA-20 support showed no evidence of any hydrolysis by IR.

It was desirable to select a somewhat "neutral" support that was not too hydrophobic or hydrophilic, and the TMPTMA : VDMA (80:20) support was chosen for reaction with methylamine in anhydrous THF. The 20 wt.% VDMA level was probably not optimum and on the high side, but a number of these supports had already been prepared [5] and "erring" on the high side could have advantages for multipoint attachment and stabilization of the PLE, especially given its quaternary subunit structure [18]. Also, residual azlactone groups could be modified after immobilization affording a more or less hydrophilic support medium and possibly creating a more active immobilized enzyme [19].

The PLE challenge was somewhat arbitrary at this point, and 4 wt.% was selected. PLE is a relatively active enzyme possessing >150 U/mg of hydrolysis activity, and, given adequate retention of enzyme function after immobilization, a 4% loading would provide a synthetically useful level of catalytic function. As a binding solvent, 0.70 M sodium sulfate (or citrate worked equally well) and 0.25 M phosphate at pH 7.4 were determined to not adversely affect PLE activity and provided the "theta" [20] or relatively poor solvent condition necessary to hydrophobically drive the PLE solute onto the supports as an important pre-step to covalent binding. Binding time should be relatively fast given the reactivity of the azlactone groups, and an initial condition of 2 h at room temperature was employed.

In order to examine the effectiveness of the various washing steps to eliminate non-covalently bound PLE, the unreactive 80:20 ($MeNH_2$) was utilized to examine the effectiveness of the support workup procedures, and filtrates and slurries were examined at various stages. Support workup involved filtration, followed by washing steps with PBS and water. The support was then tumbled for at least 16 h in 1% Triton X-100 in deionized water to remove any remaining hydrophobically bound PLE, followed by washing with water and quantitative transfer into 10 ml of EPPS buffer (pH 8.0) for refrigerated storage. In Table 1, as indicated by the significant particulate activity value of entry 2, it can be concluded that some nonspecific binding did occur even with the *N*-methyl amidated support, but the activity was reduced to acceptably low levels (entry 3) when treated with Triton X-100. These results were confirmed by another set of experiments using sodium dodecyl sulfate (SDS; 5% aqueous for 2 h) as washing solution rather than Triton. SDS is known to be a very effective detergent but can be deleterious to enzyme function due to disruption of tertiary and quaternary structure. Protein contents (determined by hydrolysis, amino acid identification, and quantification) for the supports of entries 2 and 3 were 1.01 and 0.08%, respectively, with 4.00% as the protein challenge and upper theoretical limit. Therefore, the procedure of utilizing Tritron X-100 as a surfactant to remove nonspecifically bound PLE without having adverse effects on enzyme function was adopted as part of the protocol for all subsequent work involving the dispersion polymer supports.

To summarize the determined binding protocol briefly, the following procedure allowed for removal of greater than 95% of all nonspecifically bound PLE. Furthermore, adjustments to the protocol for hydrophilicity variations contributed by the third monomer were unnecessary, at least within the range of concentrations examined. Highest covalently bound activity was observed when the following elements were present in the binding protocol:

- [PLE] challenge = 4.0 wt.% (based on dispersion support weight)
- Two hour binding reaction at room temperature in 0.7 M sodium sulfate or citrate/phosphate buffer (pH = 7.4) solution also containing 0.1 wt.% Pluronic L-31 surfactant
- Filtration and washing with PBS and deionized water
- Resuspension in 1% Triton X-100 and tumbling at room temperature for 16 h

- Filtration, washing with water, and resuspension for refrigerated storage and ultimate dispensation in 0.50 M EPPS buffer (pH = 8.0).

3.2. Immobilized PLE performance

3.2.1. Support composition

Significantly, when the azlactone-reactive 80:20 support was subjected to the same binding protocol as the pre-reacted supports (entry 4; Table 1), a high level of activity was associated appropriately with the particles, and essentially background levels were detected in the supernatant and very low levels (ca. 1%) of activity remained unattached in the initial filtrate (unbound PLE from the challenge solution). Examination of the supernatant of the slurry initially and as the sample aged also provided an assessment of any leaching of the PLE that occurred and a further indication of whether non-specific binding persisted throughout the washing procedures or whether PLE was being released hydrolytically. The 80:20 slurry was examined after refrigeration at 5 °C for 7 weeks, and the particulate value in the aged sample remained at 94% of its initial value and with no increase in supernatant activity, indicating an absence of leaching of PLE from the particulate.

Entries 5–10 explore the effects of incorporation of a third monomer into the dispersion polymer. The Particulate results clearly showed a proclivity for PLE and the more hydrophilic supports, indicated by increased activity with lower Lipophilicity Index (LI) [5], i.e., more hydrophilic or polar, supports. A point of diminishing returns, however, was observed in terms of efficiently binding all of the PLE challenge on supports of increasing hydrophilicity (decreasing LI). This was evident with entries 9 and 10 in which the initial filtrate values increased, indicating significant quantities of unbound PLE in the initial filtrate or remaining challenge solution.

Comparison of the *p*-nitrophenyl acetate assays conducted to this point over a series of supports was somewhat problematic, primarily due to variations in support surface areas and densities with changes in composition. With the HEMA series (entries 6–10), for example, the HEMA-10 support had a surface area of 152 m^2/g and a particle bed volume of 16.6 ml/g whereas the HEMA-50 support had corresponding values of 36 m^2/g and 8.8 ml/g [5]. As a means of examining and minimizing effects of these differences, the HEMA series was subjected to physical degradation using a hand homogenizer and the resulting slurry product was referred to as the "Homogenate". The procedure worked quite well; the HEMA-30 sample, for example, exhibited a monomodal particle size distribution with a median particle size of 76 μm and a 90% particle size range of 28–124 μm *before* and 38 μm and 14–62 μm *after* homongenation. The resultant activities of the Homogenates (entries 6–10) were increased compared to non-homogenized starting Slurries and some quite substantially (yet with background activity levels being observed in homogenate supernatant samples). The HEMA-50 sample, for example, increased more than threefold from 8.2 to 27.7 mAU/s. A separate experiment determined that there were no significant differences in the masses of materials transferred volumetrically. It would appear, then, that these results suggest some sort of mass transfer limitation that is particularly operative in the non-homogenized, denser particles. A similar result was observed and diffusional limitations were implicated when significantly increased activity was observed with an immobilized penicillin acylase biocatalyst upon crushing the support into smaller particles [21].

The results depicted in Fig. 2 indicate the superior performance of the most hydrophilic supports. The Homogenate

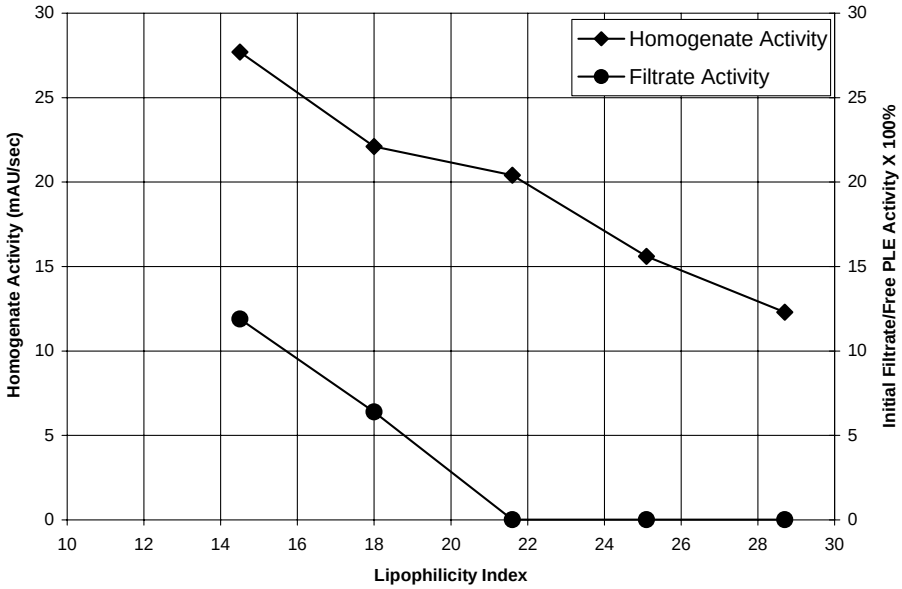

Fig. 2. Effects of support polarity on immobilized activity and binding efficiency.

activity levels essentially increase linearly (and inversely) across the LI range despite actually lower amounts of PLE being present in the HEMA-40 and HEMA-50 supports which bound only 93 and 87%, respectively, of the PLE challenge. These results suggest that even higher specific activities might be achieved on binding PLE to even more hydrophilic supports. With the dispersion polymer supports of the present study, however, the HEMA-50 composition represented a point of diminishing returns because increasing the HEMA concentration beyond 50 wt.% was observed to be accompanied by problems such as increased swelling and diminished surface area [5].

To assess a more optimum level of VDMA in the dispersion supports, a series of copolymers was prepared with varying levels of azlactone functionality but with equivalent LI values. Homogenate values of the corresponding polymers that were treated with 4.0 wt.% PLE under standard conditions are given in Table 1 (entries 11–14). The data suggest that a lower level of azlactone in the support, even half the amount, is sufficient to attach equivalent levels of PLE as the 20 wt.% materials, thus far examined. Having additional azlactone groups present in the support, however, was not shown to be deleterious, and stability issues related to multi-point attachment as well as potential performance issues related to post-modification with hydrophilic nucleophiles provided the motivation to remain with the 20 wt.% level of VDMA in the supports for more detailed studies.

3.2.2. PLE support loading

Having determined the HEMA-30 support to be essentially optimum for both activity and quantitative binding at the 4 wt.% challenge level, it was of interest to examine just how much PLE could effectively be bound. The corresponding activities of slurries and initial filtrates from experiments in which the PLE challenge ranged from 0–17 wt.% are shown in Fig. 3. The results indicate that a PLE challenge of up to about 10 wt.% produces slurry activities that are still increasing with increasing PLE concentration and yet with relatively low levels of free, unbound PLE remaining in the challenge solutions.

3.2.3. Rate of covalent binding

In order to examine the relative rates of hydrophobic and covalent binding interactions with the dispersion supports, the following experiment was conducted, with the HEMA-30 support and a 4% PLE challenge. Aliquots were removed at various times and were examined in the *p*-nitrophenyl acetate assay at the “initial filtrate” (to indirectly examine hydrophobic binding by observing PLE activity remaining in the challenge) and Triton-washed “slurry” activity (to examine covalently immobilized PLE performance) points in the sequence. The data presented in Fig. 4 indicated that hydrophobic binding occurred essentially within the time of mixing of the reactive support and PLE dissolved in the high salt buffer—more than 96% of the free PLE activity had been removed from the challenge solution within 2 min. of mixing. Covalent binding, on the other hand, required about 1 h at room temperature, as the Triton-washed slurry activity reached maximum levels within that time. This relatively rapid rate of covalent attachment was expected with the azlactone supports and may be important in binding certain enzymes. While the rate is not especially critical with PLE because the free enzyme is quite stable in solution, proteases such as trypsin, chymotrypsin, pepsin, and papain are not stable in solution and will require a relatively fast immobilization procedure to impart high levels of stability and performance to an immobilized protease.

3.2.4. Specific activity

Ethyl butyrate is the defining substrate utilized to assess activity of PLE. This plus the relative lack of reproducibility

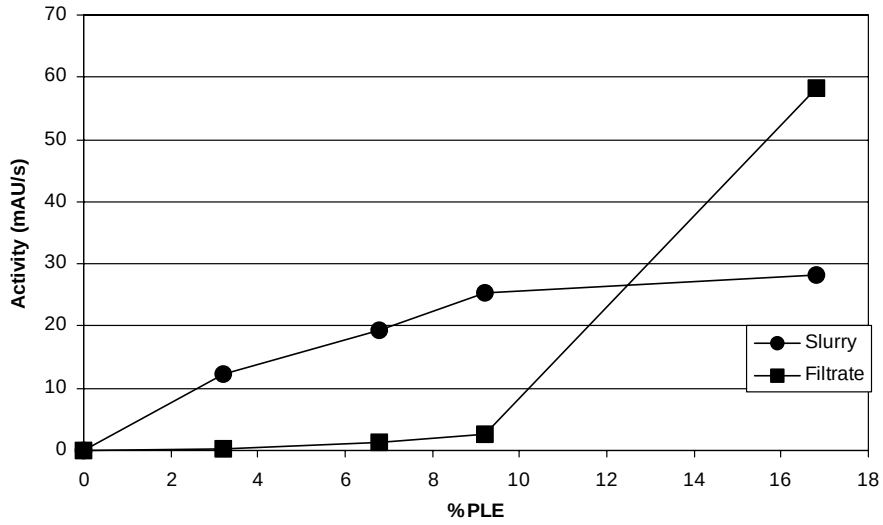

Fig. 3. PLE loading and corresponding immobilized performance.

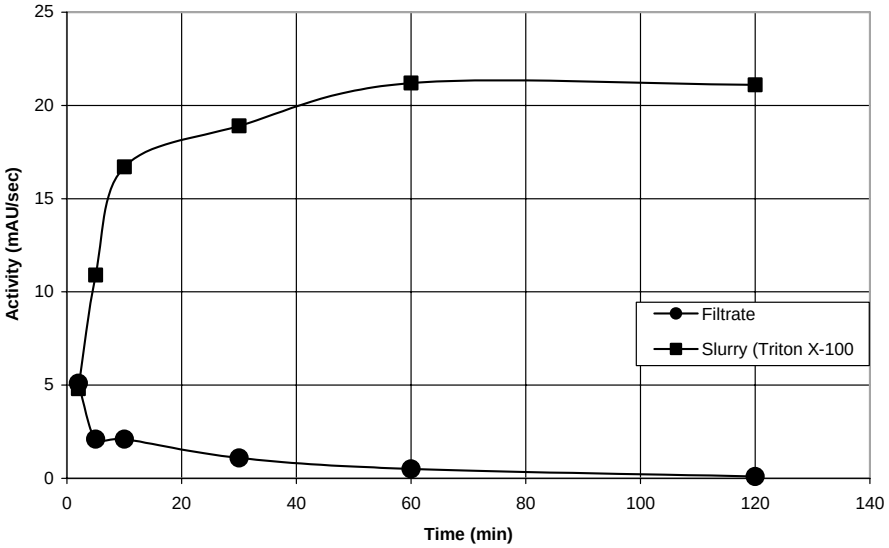

Fig. 4. Hydrophobic vs. covalent binding of PLE onto HEMA-30.

involved in the transfer of very small quantities (20 μl) of slurries with the *p*-nitrophenyl acetate assay provided the impetus to examine the more conventional assay in assessing the specific activity of the immobilized PLE material. The assay was conducted first using free PLE (0.4 mg) and a value of 133 units/mg was recorded. The HEMA-30 support (containing 0.4 mg) of PLE gave a value of 91 units/mg, providing a specific activity of 68%. This value is identical to the activity level reported for PLE bound to an oxirane-functional support [22].

3.2.5. Effects of temperature and pH

The stabilizing effects of immobilization with regard to these two parameters were minimal. PLE immobilized on HEMA-30 showed only slightly enhanced stabilization compared to free PLE; essentially no loss in activity was observed with immobilized samples at temperatures up to 50 °C, while activity with the free PLE declined above 45 °C. Activities of free and immobilized PLE (on HEMA-30) were virtually identical over the pH range of 6.0–9.0.

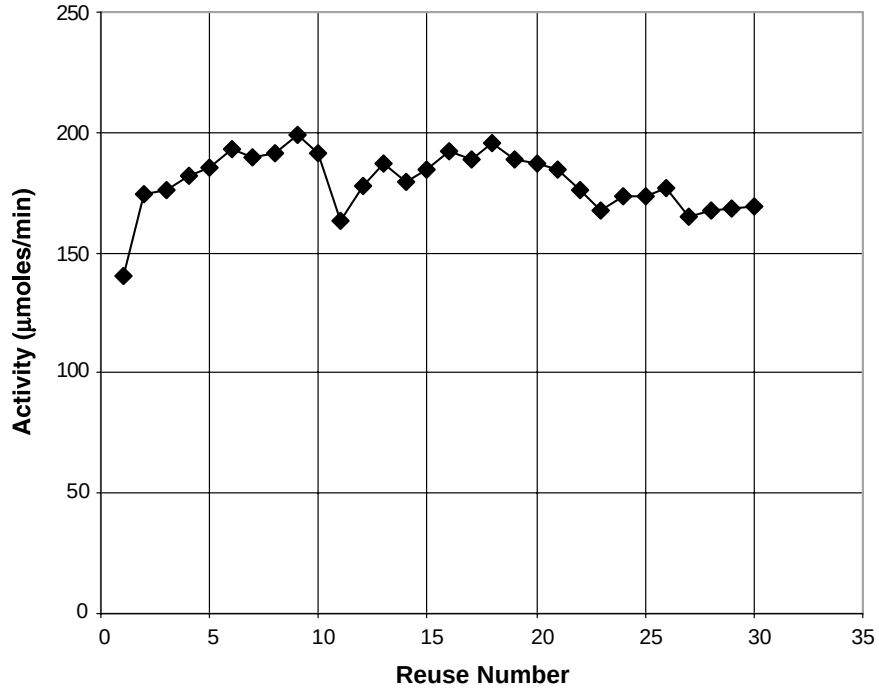

Fig. 5. Experiments examining reuse of HEMA-30/PLE support.

3.2.6. Batch reuse

Perhaps the most important and realistic assessment of the value of an immobilized enzyme is to determine its operational stability over the course of a number of recycles. This can be accomplished by subjecting the immobilized enzyme to repeated conversions of a substrate, including all workup and catalyst recovery operations between reactions, and then monitoring any decrease in reaction rate for subsequent reactions. In this manner, the immobilized biocatalyst is subjected to all possible catalyst deactivation events including enzyme denaturation (during reaction/recovery operations), enzyme leaching, and support fracture (leading to unrecoverable fines).

A batch reuse study with the HEMA-30/PLE(4%) catalyst was conducted using propylene glycol methyl ether acetate as substrate (chosen for its relatively high water solubility, in contrast to ethyl butyrate). Reactions were stirred with an overhead paddle stirrer rather than with magnetic stirring because the latter can be accompanied by significant grinding action that can physically deteriorate support particles. pH control was maintained by autotitration with standard ammonium hydroxide; the quantity of basic titrant added, of course, was a direct measure of the extent of hydrolysis of the acetate substrate. At approximately 25% conversion (50% theoretical conversion of one enantiomer), the catalyst was recovered by filtration, rinsed and redeposited into the reaction flask for the next run. Reaction rates were determined from the slopes of the straight lines obtained by plotting the volume of basic titrant added with time.

The results of 30 reuse experiments are plotted as percentages of initial activity retention in Fig. 5. Reuses 1, 11, and 21 exhibited lower activities primarily because they were the first runs of the day and were obtained with catalyst that was still cold from refrigerated storage. The overall result of the reuse experiments was that approximately 90% of the original catalyst activity was retained after 30 reuses, and a substantial portion of the 10% loss in activity could certainly be attributed to accrued physical losses in transferring the slurry catalyst from reaction vessel to filter and back over the course of the 30 reuses.

4. Conclusions

This report has examined the viability of utilizing the azlactone functional group as a reactive group for covalent attachment of a synthetically useful enzyme, pig liver esterase (PLE). Results indicate that the azlactone group was very useful in this capacity, providing covalent attachment in essentially quantitative yield when a proper binding protocol was utilized that provided for initial hydrophobic binding of the PLE onto the support. Subsequent covalent attachment was observed to be complete within one hour at room temperature. The amide-amide product linkage resulting from nucleophilic addition of a lysine residue on PLE to the azlactone provided stable attachment such that essentially no leaching of the PLE from the support was observed on prolonged storage in buffered media.

Dispersion polymer supports consisting of azlactone-, cross-linking-, and a hydrophilic–hydrophobic third-monomer combinations were easily prepared and adapted for use in immobilizing PLE. Perhaps not surprisingly with an esterase enzyme, it was determined that the more hydrophilic the attaching dispersion polymer support became, the more active was the PLE bound to that support. Operationally, using a 4 wt.% challenge of PLE bound onto a moderately hydrophilic support (HEMA-30), the PLE was immobilized quantitatively and exhibited 68% of the activity of free PLE in solution. Furthermore, the HEMA-30/PLE catalyst could be employed to catalyze a hydrolysis reaction, be recovered by filtration, and re-added to fresh reactant solution at least 30 times with less than 10% loss in catalytic function.

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