Sol−Gel-Entrapped Cholinesterases: A Microtiter Plate Method for Monitoring Anti-cholinesterase Compounds†

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Herein is reported the successful entrapment of three esterase enzymes within silica, by means of the sol−gel process. The enzymes were acetylcholinesterase (AChE) from electric eel (ee), AChE from bovine erythrocytes (er), and butyrylcholinesterase (BChE) from horse serum. Enzyme entrapment was carried out in a novel configuration by casting the doped sol−gel material in 96-well microtiter plates. The study determined the apparent kinetics of the activity and inhibition of the three entrapped enzymes and elucidated the optimal sol−gel matrix composition and preparation procedure. The enzyme mode of action within the sol−gel matrix was compared with that obtained in solution, under various experimental conditions. The activity of entrapped ee-AChE was found to depend on the concentration of the entrapped enzyme and on the sol−gel preparation procedure and composition. It was found that whereas the activity of ee-AChE was 3−4-times lower than that in solution, one gained significantly higher stability. The entrapped enzymes were sensitive to various organophosphates and to the carbamate carbaryl, with inhibition patterns similar to those obtained in aqueous reactions.

Keywords: *Cholinesterases; residue analysis; sol−gel; organophosphates; carbamates*

INTRODUCTION

The direct entrapment of enzymes in inorganic porous oxides via the sol−gel route has been the fastest growing among methodologies for the immobilization of proteins (Avnir and Braun, 1996). The sol−gel process (Brinker and Scherer, 1990) enables one to prepare inorganic oxide matrices of metals and semimetals by direct hydrolysis and polycondensation of active monomeric precursors. For silicon, the most studied and used element in this context, the reaction usually starts with alkoxides of silicon, such as tetramethoxysilane (TMOS) used in the present study. Upon addition of water, this monomer polymerizes into high-surface-area, porous silica. The reactions are performed at room temperature, thus enabling the entrapment of organic and bioorganic molecules within the forming silica network by their simple addition to the polymerizing mixture.

Examples of enzymes successfully immobilized within sol−gel matrices [for recent reviews, see Avnir and Braun (1996), Livage (1996), Avnir et al. (1994), and Dave et al. (1994)] include glucose oxidase, trypsin, acid and alkaline phosphatase, Cu−Zn superoxide dismutase, catalase, urease, nitrate reductase, parathion hydrolase, lipase, G6PDH, and, of relevance to the present paper, cholinesterase (Diaz and Peinado, 1997; Akbarian et al., 1997) (see Discussion). The reason for this intense activity lies in the special features that the sol−gel entrapment provides over currently used immobilization methods. These include the simplicity of the entrapment, which does not involve the need for covalent bonding between the matrix and the protein; the enhanced stability of the entrapped enzyme; the practically zero leaching of the entrapped protein; the optical transparency of the matrix; its action as a high-surface-area adsorbent; its chemical and photochemical inertness; and the ability to obtain this novel material in any desired shape and form (monoliths, thin films, powders, etc.). Proven applications include a variety of optical sensing reactions (Shtelzer and Braun, 1994), electroenzymatic reactions (Sampath and Lev, 1996), the design of photomaterials (Chen et al., 1996), detoxification reactions (Dozorets et al., 1996), the performance of reactions of biotechnological importance (Campostrini et al., 1996), etc. Another important recent objective of these studies has been the entrapment of antibodies (Wang et al., 1993; Aharonson et al., 1994; Collino et al., 1994; Zuhlke et al., 1995; Turniansky et al., 1996; Jordan et al., 1996; Cichna et al., 1997a,b; Bronshtein et al., 1997) and of catalytic antibodies (Shabat et al., 1997). Current problems of the sol−gel methodology are the lower reaction rates for some of the entrapped enzymes compared to those obtained with other techniques and the pressure exered on the quaternary structure of some proteins (Edmiston et al., 1994). These problems are not inherent but rather reflect the immaturity of this young field.

The present paper is devoted to the sol−gel entrapment of a family of enzymes of major importance: the cholinesterases (Massoulie et al., 1993; Taylor and Radie, 1994). These enzymes are inhibited by a variety of organophosphorus compounds (OPs) and carbamates

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(CBs) used as agrochemicals and chemical warfare agents (Fukato, 1990). The analysis of the inhibition of enzymes by these compounds could serve as a basis for their detection, which could be carried out simply by following the activity of the noninhibited enzyme and by comparing it with the activity of the inhibited one. One of the colorimetric methods for the analysis of cholinesterase activity is Ellman's classical procedure (Ellman et al., 1961), which is used in the present study.

In this study, we describe the successful sol−gel entrapment of several cholinesterases, namely, acetylcholinesterase (AChE) from electric eel (ee), AChE from bovine erythrocytes (er), and butyrylcholinesterase (BChE) from horse serum, and their inhibition by several of anti-cholinesterases. A practical advance we made here was to entrap the enzymes in sol−gel matrices cast in standard microtiter plates. This development enables one to perform simultaneous and systematic analyses of many samples and allows one the repetitive use of the same enzyme batch by means of convenient washings, which are not practicable in the solution tests. This development is essential for the effective use of these immobilized enzymes as the basis for the development of OP and CB sensors.

Our main findings are that the activity of sol−gel-entrapped ee-AChE is dependent on the concentration of the entrapped enzyme as well as on the sol−gel preparation protocol and on the composition. Optimal immobilization conditions were identified in which the ee-AChE was active and stable at room temperature for at least 13 days and was sensitive to various OPs and to the carbamate carbaryl, with inhibition patterns similar to those obtained in aqueous reactions.

EXPERIMENTAL PROCEDURES

Chemicals. TMOS (99%) used in all gel preparations was from ABCR (ABCR GmbH & Co. Karlsruhe, Germany). Poly(ethylene glycol) (PEG; Merck analytical grade, average molecular mass of 400 g/mol, which corresponds to approximately 7 ethylene units in a chain) was purchased from Merck. *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), gelatin (type B from bovine skin), tris(hydroxymethyl)aminomethane (Trizma base), 2-(*N*-morpholino)ethanesulfonic acid (MES), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine chloride (ATC), and *S*-butyrylthiocholine chloride (BTC) were all from Sigma Chemicals, of analytical grade. Water was triple-distilled. The anti-cholinesterases omethoate (Folimat), methamidophos (Tamaron), malathion, methidathion (Supracid), chlorpyrifos (Dursban), diazinon, and carbaryl were all from Dr. Ehrenstorfer (Augsburg, Germany). All inhibitors were dissolved in absolute methanol (analytical grade) at a concentration of 100 μg/mL. Stock solutions were kept at 4 °C.

Enzymes. ee-AChE (EC 3.1.1.7, type VI-S), er-AChE type XII-S, and BChE (EC 3.1.1.8 from horse serum) were all purchased from Sigma Chemical Co. The enzymes were dissolved in double-distilled water (DDW) in the presence or absence of 1% (w/v) gelatin in DDW, at a concentration of 1000 units/mL. The solution was aliquoted and stored at −80 °C.

Enzyme Entrapment in the Sol−Gel Matrices. Gels were made by a two-step method (Brinker et al., 1982; Dave et al., 1994). The first step was an acidic hydrolysis of TMOS to create a silica sol; the second step included the addition of a buffered solution of the enzyme to be encapsulated. The quick rise in pH at this stage caused an increase in the condensation rate of the sol, resulting in quick gelation. For the first step, an acidic silica sol solution was obtained by mixing TMOS with 2.5 mM HCl in a 4, 6, 8, or 10 molar ratio, in triple-distilled water. PEG, when used, was added in 10% volume equivalent with respect to TMOS. The mixture was stirred for 1 min until a clear solution was obtained; it was then sonicated for 30 min with a Branson model R-3, 55-W, 0.5-L sonicator. This sol was usually used immediately for the second step. The compositions of the sol−gel preparations described in this study are referred to as r = 1:4, 1:6, 1:8, or 1:10 ± PEG, indicating the molar ratio of TMOS/HCl in the acidic sol prepared in the first step and the presence or absence of PEG, respectively. The enzymes to be encapsulated were diluted to the desired concentration in 50 mM Hepes, pH 7.4 (Hepes buffer), unless otherwise indicated, and 25 or 50 μL aliquots were placed in wells of an Immuno Module StarWell MaxiSorb 96-well microtiter plate (Nunc, Roskilde, Denmark). In some experiments, microtiter plates from other companies were used, and their specifications are listed in the respective figure captions or table footnotes. An equivolume amount of the acidic sol was added to the buffer containing the enzyme placed in the wells, and the solution was immediately mixed for 5 s. Gelation occurred within 1−2 min. After 15 min, the gels were covered with 200 μL of Hepes buffer and kept covered at room temperature until used. Tests with Merck Neutralit papers, carried out immediately after mixing, showed that the Hepes buffer retained a solution pH of 7.4. The Hepes solution was removed prior to performance of the enzymatic reaction.

Leaching Tests. Gels were cast into microtiter plates using the general procedure described above. From the 200 μL of supernatant buffer (used to cover the sol−gels), 50-μL amounts were taken for analysis. The rest was discarded, and the gels were covered with a new 200-μL aliquot of Hepes buffer. Each cycle is referred to in the text as a "wash" cycle. During each such wash the gels were kept under the buffer for a period of 60 min. The samples taken for analysis were placed in microtiter plates and tested for enzymatic activity according to the regular procedure, as described below. Calibration was performed by reacting known amounts of the ee-AChE in solution, with the same standard reagents in the same plate. Due to low enzyme concentration the reaction was carried out for up to 100 min. Calibration curves were made with low enzyme concentrations (0.002−0.01 unit/mL) to give reaction times similar to those needed for the low amounts of enzyme present in the supernatants.

Enzymatic Reactions in Sol−Gel Matrices. Hepes buffer was removed from the gels, and three to five consecutive washes (carried out at 30−60-min intervals) were performed, each using 200 μL of Hepes buffer; 200 μL of substrate solution containing 0.5 mM ATC (for ee-AChE and er-AChE) or 0.5 mM BTC (for BChE) in Ellman reagent (1 mM DTNB in 10 mM Tris-HCl containing 0.01% gelatin, pH 7.6) (Ellman et al., 1961) was then applied on top of the gels. Reaction mixtures were incubated at room temperature for 5−45 min, and the developing color was monitored at 405 nm with a Labsystems Multiskan ELISA reader. Each plate included duplicates of substrate blanks (wells with sol−gel prepared as above but without enzymes) and five or six replicates of enzymatic reactions under each set of test conditions. Enzymatic reactions in the sol−gel matrix were highly reproducible, and the coefficient of variance did not exceed 10−15%.

Stability of Sol−Gel Entrapped Enzyme. ee-AChE (2.5 units/mL) was entrapped in a 1:4 sol−gel matrix and the activity monitored on the day of entrapment, 6 h after gelation as described above. After absorbance had been monitored, the supernatant (containing the reaction buffer, unhydrolyzed substrate, and product molecules) was washed five times with Hepes buffer. Fresh Hepes buffer was then applied on top of the gels, and the microtiter plates were kept at room temperature for an additional period of 24 h. The reaction was performed once again, followed by the same washing procedure, and the plates were kept for another period of 13 days.

Enzyme Inhibition in Sol−Gel Matrices. Hepes buffer was removed from the gels, and three to five consecutive washes were carried out with 200 μL of Hepes buffer. Inhibition of esterases was performed by application of 50 μL of the appropriate OP (obtained by standard oxidation of the thio analogues) or of the CB, diluted in Hepes buffer, on the gels for 2 or 20 h at room temperature. The OP inhibitors that were used in this study included methidathion (Supracid), malathion, chlorpyrifos (Dursban), methamidophos, diazinon,

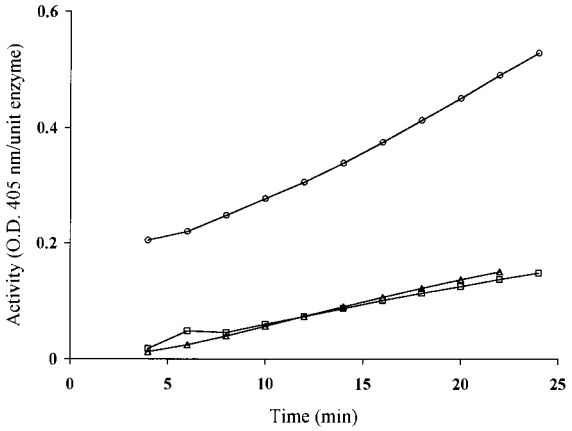

Figure 1. Activity of ee-AChE (□), BChE (△), and er-AChE (○) entrapped in silica sol−gel matrices. ee-AChE and BChE were tested at concentrations of 10 units/mL, and er-AChE was tested at 1 unit/mL. For comparison purposes activities are normalized per unit of enzyme. Enzymatic activity was determined in 1-day-old, 1:4 sol−gel matrix (prepared in Nunc microtiter plates). The 1:4 composition indicates the molar ratio of TMOS/water (containing HCl catalyst) in the first-step acidic sol. Values for each enzyme represent the mean OD of five or six repetitions minus the OD obtained in the substrate blank wells. Background values ranged from 0.17 to 0.18 OD unit, at 4 and 24 min, respectively. Absorbency of er-AChE at $T = 0$ was 0.185 OD unit.

omethoate, and the CB carbaryl [for structural formulas, see Worthing (1979)].

Inhibitor concentration was 0.1−100 ng/50 μL. At the end of the incubation period, the inhibitors were removed from the gels by excessive washings (four or five wash cycles with Hepes buffer) and the reaction was carried out as described above. Each plate included duplicates of substrate blanks (wells with sol−gel prepared as above but without enzymes), five or six replicates of control wells (enzyme without inhibitor), and a similar number of replicates for each concentration of the tested OP or CB.

Enzymatic Reactions in Solution. Solution reactions for comparative and leaching tests, described above, were carried out according to the micro assay described by Doctor et al. (1993). Reactions were performed in 96-well microtiter plates (Costar, Cambridge, MA) and contained 50 μL of enzyme (at concentrations ranging from 0.3 to 10 units/mL), diluted in 50 mM Hepes buffer containing 0.01% (w/v) gelatin, pH 7.6, and 200 μL of substrate solution containing 0.5 mM ATC in Ellman reagent (1 mM DTNB in 10 mM Tris-HCl containing 0.01% gelatin, pH 7.6). Reaction mixtures were incubated at room temperature for 5−45 min, and the developing color was monitored at 405 nm with a Labsystems Multiskan ELISA reader.

Statistical Analysis. Statistical analysis was performed by ANOVA. Differences among means were tested for significance by the Newman−Keuls test at $P < 0.05$. Means with a common letter do not differ significantly.

RESULTS

The first part of the study involved immobilization of various esterases in sol−gel to determine whether the enzymes were active in their entrapped form. Three different esterases, ee-AChE, er-AChE, and BChE, were tested at various concentrations (ranging from 0.5 to 10 units/mL for ee-AChE and BChE and from 0.5 to 1 unit/mL for er-AChE). The data presented in Figure 1 [representing the activity monitored with only one of all the tested concentrations (10 units/mL for ee-AChE and BChE and 1 unit/mL for er-AChE)] show that all three enzymes were active, although they differed in their reaction rates. The reaction rate (amount of substrate formed per unit of entrapped enzyme per minute) of ee-AChE was similar to that of BChE (0.16 and 0.20 nmol/min, respectively) and 2.3−2.9-times lower than that of er-AChE (0.46 nmol/min) (Table 1). Similar differences in reaction rates were detected at all tested concentrations (0.5, 1, 5, and 10 units/mL for ee-AChE and BChE and 0.5 and 1 unit/mL for er-AChE) and with enzymes tested 1 and 2 days after entrapment (data not shown).

Table 1. Comparison of Reaction Rates of ee-AChE, er-AChE, and BChE Entrapped in Silica Sol−Gel Matrices and in Solution

reaction conditions	reaction rate[a] (nmol/min)
sol−gel	
ee-AChE	0.16
er-AChE	0.46
BChE	0.20
solution	
ee-AChE	7.68
er-AChE	8.27
BChE	1.32

[a] Reaction rates (R) were calculated according to the following formula: absorbance/min = eRl (Ellman et al., 1961), where e is the extinction coefficient of the product (1.36×10^4), R is the reaction rate in moles per liter per minute, and l is the optical distance (0.72 cm for a reaction volume of 250 μL in a microtiter plate). Values of absorbance/minute normalized for 1.0 enzyme unit were determined from the slopes of Figure 1 (for reactions in gels) and from data on reactions in solution (unpublished results). Sol−gel and solution reactions were carried out as described under Experimental Procedures.

Examination of the reaction rates of these enzymes in solution (under equivalent or similar experimental conditions) revealed that ee-AChE and er-AChE exhibited similar reaction rates (7.68 and 8.27 nmol/min, respectively) that were 5.8−6.3 times higher than that exhibited by BChE (1.32 nmol/min) (Table 1). Comparison of the reaction rate of each enzyme in sol−gel with that in solution revealed lower activities in the sol−gel for all three enzymes. The decrease in activity, however, was different for each enzyme, being highest for ee-AChE (48-fold) and lower for er-AChE and BChE (18- and 7-fold, respectively). Attempts to improve ee-AChE activity by the addition of 0.01% gelatin to the enzyme-diluting buffer during the gelation process were ineffective.

The next set of experiments involved examination of enzymatic stability as a function of storage at room temperature and checking the repeatability of the enzymatic reaction. Experiments were performed with ee-AChE, and activity was determined at various post-entrapment times (using the same entrapped enzymes). As shown in Figure 2 the esterase was found to be active for up to 13 days, and the activity after that time did not differ from that obtained with the same enzyme tested 1 day after entrapment or from that of an enzyme tested shortly after gelation. No changes in the physical properties of the sol−gel (such as volume or texture) were observed after 13 days of storage.

The surface area and morphology, the average pore size, the pore geometry, and pore size distributions are all known to be affected in a nontrivial way by the various parameters of the composition and of the preparation procedure (Brinker and Scherer, 1990). Optimization of performance requires, therefore, a multiple-parameter scan. A preliminary optimization study was carried out here by changing the water/silane ratio (the r parameter) and by adding PEG, as described under Experimental Procedures. Thus, we entrapped ee-AChE in sol−gel compositions of $r = 1{:}4$, 1:6, 1:8, and 1:10 and analyzed its enzymatic activity. At each ratio, 10% (v/v) of the TMOS in half of the sols was replaced by PEG. The addition of PEG, which is generally

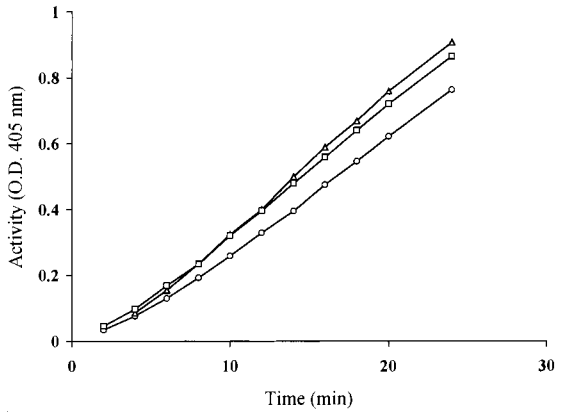

Figure 2. Stability of sol−gel-entrapped ee-AChE. Activity was tested on the day of entrapment (○) and after 1 (△) and 13 (□) days of entrapment. Microtiter plates (Greiner) with the sol−gel-entrapped enzyme were kept at room temperature during the entire experiment. The concentration of the enzyme was 2.5 units/mL, and the reaction was carried out in the 1:4 sol−gel composition. Values, on each day, represent the mean OD of five to six repetitions minus the OD obtained in the substrate blank wells. Background values ranged from 0.13 to 0.16 OD unit, at 2 and 24 min, respectively, in all tested days.

Table 2. Reaction Rates of ee-AChE in Various Sol−Gel Compositions

sol−gel format	reaction rate[a] (nmol/min)				
	0.5 unit/mL	1.0 unit/mL	2.5 units/mL	5.0 units/mL	10.0 units/mL
1:4	0.41	0.35	0.43	0.43	0.26
1:4 + PEG	0.49	0.42	0.74	0.35	0.23
1:6	1.51	1.34	0.90	0.54	0.30
1:6 + PEG	2.14	1.62	0.85	0.49	0.26
1:8	1.53	1.68	0.97	0.58	0.30
1:8 + PEG	2.63	1.82	0.88	0.48	0.25
1:10	2.42	2.06	0.98	0.52	0.29
1:10 + PEG	2.71	1.86	0.88	0.51	0.24

[a] Reaction rates were calculated as explained in the footnote to Table 1. Values of absorbance/minute were determined from slopes of experiments performed with ee-AChE entrapped in various sol−gel formats. Activity was tested in 1-day-old sol−gel samples prepared in microtiter plates (Greiner). Activity was monitored for 28 min at 2−4 min intervals.

"friendly" to proteins, creates a composite matrix which is somewhat more flexible than ordinary silicate matrices and, may, therefore, protect the enzyme and increase enzymatic activity. Activity was tested at several enzyme concentrations (0.5, 1.0, 2.5, 5.0, and 10 units/mL) and at two different times (1 and 13 days) after enzyme entrapment. The data in Table 2 reveal that low enzyme concentrations are sensitive to the *r* and PEG parameters, whereas high enzyme concentrations are not. Intermediate concentrations of 1.0, 2.5, and 5.0 units/mL exhibited a gradually decreasing dependence on these parameters. At low enzyme concentrations (0.5 unit/mL) PEG had a more pronounced effect on activity than the *r* value, and the activity obtained in a 1:6 sol−gel with PEG was significantly higher than that obtained in a 1:8 sol−gel with no PEG. Higher enzyme concentrations (10 units/mL) did not show significant differences in activity in sol−gels with different *r* values, and the presence of PEG did not significantly improve the enzymatic reaction rate. The same trend was observed in doped sol−gel matrices tested 13 days post-entrapment.

Analysis of the reaction rates at the various sol−gel compositions revealed that the concentration of the entrapped enzyme also has a profound effect on the enzymatic activity. An increase in the concentration of entrapped enzymes resulted in a marked decrease in activity, in a manner that depended on the sol−gel composition. At a composition of 1:4 ± PEG, at which the enzymatic activity was generally low, there was a 1.6−2.1-fold decrease in activity when the enzyme concentration was elevated from 0.5 to 10 units/mL. At lower *r* values, either in the presence or in the absence of PEG, the activity at 10 units/mL was 10−11 times lower than that at 0.5 unit/mL (Table 2). It is important to note that the combination of low *r* values, PEG, and low enzyme concentration elevated the reaction rate 5.2−6.6 times, compared with that at 1:4 compositions, to a level only 2.8−3.6 times lower than those obtained in solution.

Since dopant molecules may leach out of the highly porous gels, we determined the exact amount of the entrapped enzyme leaching from the sol−gel matrices. Two sets of experiments were performed toward this goal. In the first set we determined leaching by successive washes (these were collected from the sol−gel matrix surface at 60-min intervals). In the second set, we compared differences in the amounts leached out from different sol−gel compositions prepared with and without PEG. Experiments were performed using two sol−gel compositions (1:4 and 1:8) at four enzyme concentrations (0.5, 1.0, 5.0, and 10.0 units/mL). The data that were obtained indicated that leaching is insignificant and in most cases was below the detection limit (namely, below the activity obtained with 0.002 unit/mL ee-AChE after 100 min). The activity detected in the supernatant of the first wash reached only 0.037 and 0.023% of the entrapped enzyme (at the highest enzyme concentration, 10 units/mL) at the 1:4 and 1:8 ratios, respectively. The amounts of enzyme found in the supernatant at lower enzyme concentrations (0.5−5.0 units/mL) were even smaller (<0.001%). Enzymatic activity could hardly be detected in the second, third, and fourth washes at all tested enzyme concentrations. Similar results were obtained with sol−gel compositions prepared with PEG.

A third optimization parameter that was tested was the pH together with the nature of the buffer used to dilute the enzyme in the second step of the sol−gel preparation process. ee-AChE was diluted in three different buffers (Hepes, sodium phosphate, and MES) at various pH values, and the activity of the enzyme was monitored 1 day after gelation. We found that the pH value as well as the type of entrapment buffer itself affected activity, as summarized in Figure 3.

One of the main characteristics of esterases is their inhibition by compounds belonging to the OP and CB groups. The inhibition pattern of the sol−gel-entrapped ee-AChE was determined with a variety of OP insecticides (malathion, chlorpyrifos, methidathion, diazinon, omethoate, and methamidophos) and the CB carbaryl. Dose−response analysis with the above inhibitors revealed that the entrapped enzyme is effectively inhibited by both OPs and carbaryl (Figures 4 and 5), although different sensitivities were exhibited toward the different compounds. The entrapped enzyme showed a high sensitivity toward methidathion, malathion, and chlorpyrifos and low sensitivities to carbaryl, diazinon, and omethoate (Table 3).

DISCUSSION

In this study we report the successful entrapment of three enzymes belonging to the esterase group: AChE

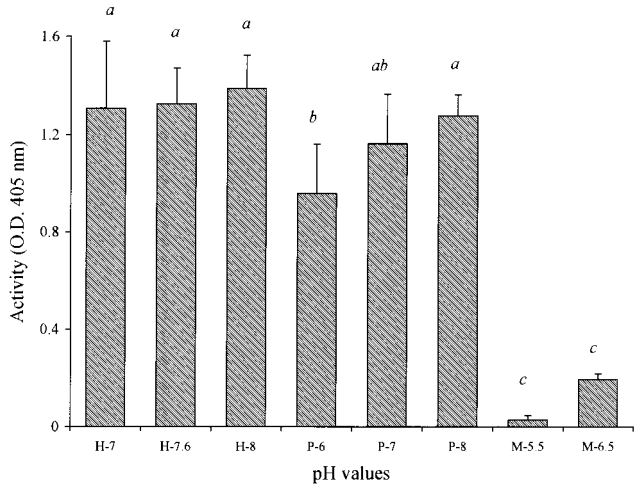

Figure 3. Activity of ee-AChE entrapped in sol−gel matrices prepared in various entrapment buffers (indicated by letters: H, Hepes; P, sodium phosphate; and M, MES) at various pH values (indicated by numbers next to the characters). All buffers were at a concentration of 50 mM and were used as the enzyme dilution buffer in the second step of the sol−gel preparation process (see Experimental Procedures). Activity was tested at an enzyme concentration of 2.5 units/mL in 1-day-old 1:8 sol−gel samples + PEG, prepared in Nunc StarWell microtiter plates. Data represent enzymatic activity after 24 min of reaction. Values, at each pH value, represent the mean OD ± SD of five to six repetitions, minus the OD obtained in the substrate blank wells. Background values ranged from 0.2 to 0.25 OD units, in all tested buffers.

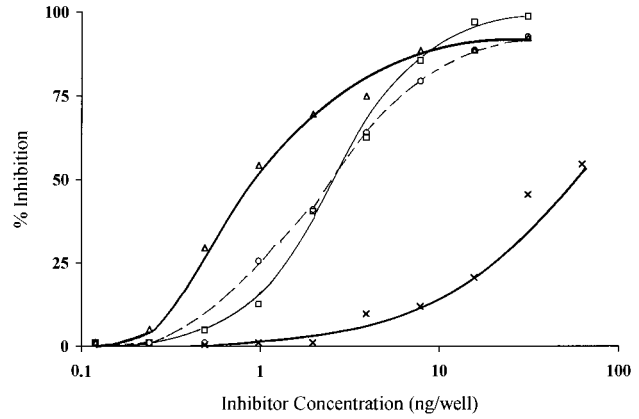

Figure 4. Inhibition of ee-AChE by the OPs malathion (○), chlorpyrifos (□), methidathion (△), and diazinon (×). Activity was tested with 1 unit/mL of ee-AChE in 1-day-old 1:8 sol−gels + PEG, prepared in Nunc StarWell microtiter plates. Inhibition was carried out by incubation of the entrapped enzyme, with 50 μL of 0.12−31.25 ng/well malathion, chlorpyrifos, or methidathion or with 0.49−125 ng/well diazinon. Incubation with the OPs was carried out for 2 h at room temperature. Data represent enzymatic activity after 28 min of reaction. Values represent the ratio (as percent) of the means of enzymatic activities in the presence and absence of inhibitor.

from electric eel, AChE from bovine erythrocytes, and BChE from horse serum. Enzyme entrapment was carried out in 96-well microtiter plates in a manner that enabled performance of many simple, quick, and repeatable assays aimed at characterization of the enzymatic activities and their inhibition patterns. The study determined the apparent kinetics of the activity and inhibition of the entrapped enzymes, and concentrated on the determination of the optimal sol−gel preparation format for maximal activity and minimal leaching of ee-AChE, and on the examination of the inhibition of the enzyme in sol−gel. The enzyme's mode of action in the

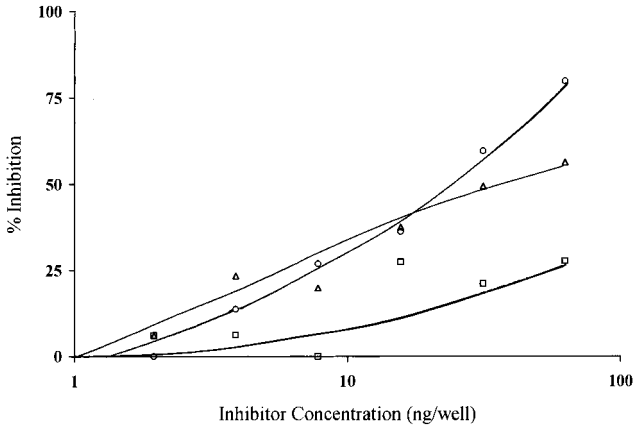

Figure 5. Inhibition of ee-AChE by the OPs omethoate (□) and methamidophos (○) and the CB carbaryl (△). Incubation was carried out for 20 h at room temperature, at concentrations ranging from 1.95 to 125 ng/well (50 μL). Data represent enzymatic activity after 28 min of reaction. Values represent the ratio (as percent) of the means of enzymatic activities in the presence and absence of inhibitor.

Table 3. ee-AChE Sensitivity to Various Organophosphates and the Carbamate Carbaryl[a]

compound	I_{50} (ppb)	I_{20} (ppb)
methidathion (Supracid)	19	8
malathion	44	18
chlorpyrifos (Dursban)	52	24
methamidophos	400	116
carbaryl	680	140
diazinon	840	230
omethoate	2000	440

[a] Inhibition was performed as described in the captions to Figures 4 and 5. I_{50} and I_{20} values were calculated from the curves presented in those figures, representing 50 and 20% inhibition, respectively.

sol−gel matrix, under various experimental conditions, was compared with that obtained in solution.

The first set of experiments was designed to determine whether the three esterases were active in their entrapped form and to compare their activities in the sol−gel matrix with those in solution. We found that all three enzymes (ee-AChE, er-AChE, and BChE) were active in their entrapped form, with the er-AChE exhibiting the highest activity. Comparison with the activities of the three enzymes in solution revealed lower activities in the entrapped form for all three enzymes. The decrease in activity was different for each enzyme, being highest for ee-AChE (48-fold) and lower for er-AChE and BChE (18- and 7-fold, respectively) (Table 1).

Several reasons can account for the lower overall activity of the enzymes in sol−gel than in solution and for the differences in the reaction rates of the enzymes under the two experimental conditions. It is possible that the lower overall activity of the enzymes in the sol−gel matrix reflects the presence of a smaller number of active sites of the enzyme molecules accessible to diffusing substrate molecules or that the enzyme molecules entrapped within the sol−gel matrix exhibit lower affinities toward the substrate due to denaturing processes imposed on the enzymes by the polymerization process around the protein. Another possibility is that enzyme activity is hindered by retarded diffusion of both substrate and product, through the porous matrix as compared with solution. Evaluation of this hypothesis will require detailed kinetic analysis of the enzyme activity, which is at present an ongoing project in our laboratories.

The differences among the activity levels of the three enzymes indicate that the polymerization and the matrix have different effects on them. Thus, it was found that ee-AChE is most susceptible to matrix effects (under the tested conditions, see below) since the decrease in its activity was the highest (48-fold); er-AChE and BChE are less sensitive. The higher stability exhibited by er-AChE may be explained by the fact that this enzyme is hydrophobically attached to the erythrocyte membrane (Low et al., 1986), which may provide it with some resistance against the physical constraints imposed on the protein molecules by the sol−gel matrix. Alternatively, it is possible that the hydrophobic moiety helps to orient the enzyme in a way that exposes its active site to the intrapore liquid and the substrate molecules in it. Differences in orientation may also account for the smaller decrease in the activity of the BChE than that of ee-AChE in sol−gel matrices. Further confirmation for the crucial effect of the composition of the sol−gel matrix on the enzymatic activity is presented below.

Despite its relatively low activity, ee-AChE exhibited high stability in the sol−gel matrix when kept at room temperature, and the enzymatic activity obtained with enzyme molecules entrapped in sol−gel for up to 13 days did not differ from that obtained on the day of encapsulation or 1 day later (Figure 2). The stability was much higher than that obtained in solution, where enzyme molecules that were kept at room temperature in Hepes buffer or adsorbed to microtiter plate wells lost 50% of their activity after 14 days (unpublished data). The higher stability of the entrapped enzyme may be attributed to the protective nature of the matrix, which reduces the freedom of peptide chain refolding causing the denaturation and inactivation of biomolecules. Stability against temperature and pH changes has been demonstrated for sol−gel-entrapped enzymes such as glucose oxidase (Shtelzer and Braun, 1994) and acid phosphatase (Shtelzer et al., 1992).

It has been previously reported that the sol−gel preparation conditions have a marked effect on the activity of the entrapped enzyme (Avnir and Braun, 1996). Because the activity of the sol−gel-entrapped ee-AChE, tested in the first part of the present study, was low, we examined the effects of various sol−gel preparation methods on enzymatic activity, to find a composition that promotes higher activities. Consequently, we used the so-called two-step procedure, which proved to be advantageous in several previous studies (Akbarian et la., 1997), including our own (Turniansky et al., 1996). We chose two parameters as variables: the TMOS/water ratio (which mainly affects surface area and porosity) and the presence of PEG. PEG has previously been shown to improve the activity of encapsulated biomolecules, probably because of the higher flexibility it induces in the silicate matrix and its higher hydration ability. On the basis of data obtained from eight different combinations of sol−gel (containing various concentrations of ee-AChE, ranging from 0.5 to 10 units/mL) at four TMOS/water ratios (1:4, 1:6, 1:8, and 1:10), with and without PEG, we concluded that the most dominant effect on enzymatic activity is that of the concentration of the enzyme trapped within the matrix. Matrix properties (e.g., porosity and presence of PEG) also play an important role, with PEG having a more pronounced effect, but only at low enzyme concentrations (Table 2).

The decrease in activity that was found to be associated with the increase in enzyme concentration results, most likely, from overloading of the matrix with enzyme molecules; this may cause enzyme aggregation, as observed for sol−gel-entrapped trypsin at higher concentrations (Shtelzer et al., 1992). The insensitivity of the enzyme activity at the higher concentration to the nature of changes in the matrix preparation procedure may similarly be attributed to the protective nature of the protein aggregate for the individual active molecule within it.

Another parameter that was found to affect the activity of the entrapped biomolecules was the type and pH of the buffer used for the preparation of the sol−gel matrices (Figure 3). Three explanations may be provided for this behavior. One is the effect the buffer molecules themselves have on the hydrolysis and condensation reactions occurring in the forming matrix. Ionic additives are known to affect these reactions, causing changes in the structure of the forming gel (Brinker and Scherer, 1990). The second effect is the well-documented effect of pH on the structure of the sol−gel materials (Polevaya et al., 1995). The third effect is that related to the buffering capacity and pH of the different buffer types—the lower the buffering capacity and the initial buffer pH, the greater the likelihood that the enzyme will suffer a pH shock when it is mixed, during the second preparation step, with the acidic sol from the first preparation step.

The improved activity that was obtained with the ee-AChE in highly porous gels raised the possibility that the enzyme may leach out of the matrix. The fact that leaching was <0.04% of the entrapped enzyme (after four successive washes that were performed at 60-min intervals) and the fact that the enzyme was active after 13 days in the sol−gel clearly demonstrate that significant leaching of the enzyme does not occur under the tested conditions. Even relatively highly porous gels in the presence of PEG revealed low (<0.25%) leaching.

One of the most powerful tools for enzyme characterization is the use of inhibitors. Esterases have long been known for their high sensitivity to compounds belonging to the OP and CB groups, some of which are among the most common insecticides. In the present study we found that entrapped ee-AChE was effectively inhibited by this group of compounds exhibiting limits of detection (I_{20}) in the parts per billion range (Figures 4 and 5 and Table 3).

Comparison of the I_{50} values of the sol−gel-entrapped enzyme revealed that they were only 3−5 times lower than those obtained with an enzyme in solution (methidathion, 3.8 ppb; malathion, 8.2 ppb; chlorpyrifos, 17 ppb; diazinon, 232 ppb). It should be noted, however, that since OPs are stoichiometric inactivators of the enzyme, the I_{50} values are directly dependent on the amount of active enzyme entrapped in the sol−gel matrix. It is also interesting to note that the potency order of the various inhibitors was the same in both systems, indicating that the sol−gel serves as an inert matrix for these compounds. The lower I_{50} values that were obtained in the sol−gel system may result from apparently lower inhibitor concentrations at the site of inhibition because of the slow diffusion of the inhibitor in the matrix or nonspecific adsorption of the inhibitor molecules to the gel surface lowering the total effective dose.

As indicated above, entrapment of cholinesterases in a sol−gel matrix has been demonstrated in two previous studies (Diaz and Peinado, 1997; Akbarian et al., 1997). These studies showed that sol−gel-entrapped enzymes retain their activity, exhibit high stability during storage, and are inhibited by anti-cholinesterase compounds. It is interesting to note that the sensitivities of the cholinesterases to various OPs found by Diaz and Peinado (1997) were in the parts per million range, that is, 1−3 orders of magnitude lower than those obtained in the present study (see Table 3 and Figures 4 and 5).

In conclusion, our present study demonstrates for the first time the successful entrapment of enzymes in the convenient format of a 96-well microtiter plate. The efficient immobilization of the esterases, their enhanced stability, the lack of leaching, and the patterns of efficient inhibition by a variety of OP compounds and by the CB carbaryl (obtained even after their excessive washing) provide a basis for further development of this system into a sensitive and efficient monitoring assay for pesticide residues belonging to this group of compounds.

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