Colorimetric Assay for Lecithin Using Two Co-immobilized Enzymes and an Indicator Dye Conjugate

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According to the new assay for lecithin, phospholipase D (PL), choline oxidase (ChOX), and the indicator conjugate bromothymol blue−glutathione (BTB−GSH) are co-immobilized on glutaraldehyde-activated polyacrylamide transparent gel. When a sample containing lecithin is added to the biocatalytic gel, lecithin is hydrolyzed by PL to choline, which is oxidized to betaine by ChOX. The organic acid betaine changes the microenvironmental pH of the enzymes, thus leading the optical absorption of the biocatalytic gel to a hypsochromic shift because of the co-immobilized indicator conjugate, BTB−GSH. The change of the optical absorption at λ_{max} (622 nm) is related linearly to lecithin concentration in the range 20−120 μM. When the optical effect is quantified in a spectrophotometer, the analyte concentration can be determined. The biocatalytic gel is used successfully for lecithin determinations in bovine serum and soybean granules. The principle of the method is applicable to the construction of optical biosensors.

Keywords: *Bromothymol blue; choline; choline assay; choline oxidase; enzyme assay; immobilized enzymes; lecithin; lecithin assay; optical biosensor; phosphatidylcholine; phospholipase D*

INTRODUCTION

Phosphatidylcholine (PC) or lecithin is the class of the most abundant phospholipids in animal tissues, and it is found also in higher plants, yeasts, and other microorganisms but generally is absent among the bacteria, with some notable exceptions (Schiefer and Beutler, 1985). Lecithin is an important source of methyl groups, an essential component of certain lipids and in the nervous tissues of most organisms, and it is a precursor of acetylcholine, which is a major neurotransmitter (Rauch et al., 1997). Determination of lecithin is useful not only for biochemistry but also for food analysis, because lecithin itself is used as a food additive. The classic methods of lecithin determination comprise extraction and fractionation procedures by means of organic solvents or column chromatography, followed by more or less detection methods (Ansell et al., 1973). The most frequent detection methods are based on the determination of inorganic phosphate using ammonium molybdate (Betzing, 1965) or the colorimetric determination of choline-specific Reinecke salt (Glick, 1944). However, these methods are very cumbersome and their precision is unsatisfactory, so they are rarely used nowadays. Although high-performance liquid chromatography (HPLC) (Jungalwala et al., 1976) offers an effective alternative for lecithin analysis, all nonenzymatic methods require extraction with organic solvents prior to further analysis, a step that undoubtedly leads to relatively poor precision. Resolution of molecular species of natural phospholipids, for example, phosphatidylcholine and phosphatidylethanolamine, was done by RP-HPLC (Abidi and Mounts, 1992). Improvement of the resolution and reduction of the run time in glycerophospholipid analysis by RP-HPLC can be effected with gradient elution (McHowat et al., 1997). On the other hand, quantitative analysis of natural phospholipids by HPLC with evaporative light scattering detection is unsuitable because of the incompatibility of the detection systems with the mobile phase electrolytes. A fast and convenient phospholipid analysis method has been developed using thermospray LC/MS; however, it is not sensitive enough for many metabolic studies that require the detection of phospholipid molecular species at low or sub-picomole levels (Kim et al., 1994; Ma and Kim, 1995). A universally applicable analytical method for membrane lipids based on electrospray ionization ESI-MS, is characterized by high sensitivity and moderate experimental complexity and provides reproducible results (Brugger et al., 1997).

Application of enzyme-based methods for lecithin determination avoids the preliminary extraction step and, thus, simplifies the assay and improve its precision. Noncolorimetric, enzyme-based assays for lecithin have been based on the amperometric principle; however, they are cumbersome (Matsumoto et al., 1980; Karube et al., 1979; Masoom et al., 1990; for more information see Results and Discussion). Enzymatic photometric determination of lecithin is generally based on the hydrolysis of lecithin by the action of phospholipase C, yielding phosphorylcholine and diacylglycerol, or by the action of phospholipase D (designated here PL), yielding choline and phospatidic acid (Schiefer and Beutler, 1985). According to a commercial method for lecithin determination (Boehringer Mannheim, 1984), lecithin is hydrolyzed by phospholipase C to diacylglycerol and phosphorylcholine, which are then hydrolyzed by alkaline phosphatase to choline and inorganic phosphate. After inactivation of alkaline phosphatase at >95 °C, choline is phosphorylated to form again phosphorylcholine from ATP, in the presence of choline kinase. The

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ADP formed during this reaction is reconverted to ATP from phosphoenolpyruvate with the formation of pyruvate, in the presence of pyruvate kinase. Finally, lactate dehydrogenase is the last catalyst used, converting pyruvate to lactate by NADH which, therefore, is used to quantify lecithin. Obviously this method is laborious and complicated, as it employs five enzymes. Takayama et al. (1977) proposed a much simpler method, suitable for routine determinations, employing only three enzymes [phospholipase D (PL), choline oxidase (ChOX), and peroxidase (POD)], which in the presence of the appropriate chromogenic system leads to color formation:

$$\text{lecithin} + 2H_2O \xrightarrow{\text{PL}} \text{phosphatidic acid} + \text{choline} \quad (1)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChOX}} \text{betaine} + 2H_2O_2 \quad (2)$$

$$2H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{POD}} \text{red dye}_{(500\ \text{nm})} + 4H_2O \quad (3)$$

The above method employs enzymes in free form; therefore, they have to be discarded at the end of each assay. Furthermore, the method is relatively slow, as it requires 30 min before optical readings can be taken. In the present work we propose a new method applicable both to lecithin and to choline determination. According to this method, two enzymes, PL and ChOX, and a purpose-synthesized indicator dye analogue, bromothymol blue−glutathione (BTB−GSH) conjugate, are co-immobilized on transparent polyacrylamide gel. Lecithin hydrolysis by PL yields choline, which is then oxidized to betaine by ChOX. Betaine is an organic acid liberating protons, able to change the pH of the microenvironment of the immobilized biocatalysts, thus bringing about a hypsochromic shift toward shorter wavelength of the optical absorption of the co-immobilized indicator dye conjugate, BTB−GSH. The change of the optical absorption of the biocatalytic gel at λ_{max} is proportional to lecithin concentration in the sample; therefore, when the optical effect is quantified in a spectrophotometer, the analyte concentration can be determined (616 nm for the free enzyme system, 622 nm for the immobilized enzyme system, 438 nm constant for both systems):

$$\text{lecithin} + 2H_2O \xrightarrow{\text{PL}} \text{phosphatidic acid} + \text{choline} \quad (4)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChOX}} \text{betaine} + 2H_2O_2 \quad (5)$$

$$\text{BTB−GSH}_{(\mathbf{616}\ \text{or}\ \mathbf{622},\ \text{and}\ 438\ \text{nm})} \rightarrow \text{BTB−GSH}_{(\mathbf{438},\ \text{and}\ 616\ \text{or}\ 622\ \text{nm})} \quad (6)$$

This new method was evaluated by using samples of pure lecithin as well as real biological samples (bovine serum and commercial lecithin granules from soybean).

EXPERIMENTAL PROCEDURES

Materials. Bromocresol green, bromocresol purple, bromothymol blue, cresol red, bromophenol blue, and 4-aminoantipyrine were obtained from Aldrich, Gillingham, England. Reduced glutathione (crystalline), *Streptomyces chromofuscus* phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4; specific activity = 20 units/mg of lyophilisate), *Arthrobacter globiformis* choline oxidase:oxygen 1-oxidoreductase *E* (EC 1.1.3.17; specific activity = 10 units/mg), and horseradish peroxidase donor:hydrogen peroxide oxidoreductase (EC 1.11.1.7; grade I, specific activity = 250 units/mg) were from Boehringer Mannhein, Germany. Glutaraldehyde solution (25%) was purchased from BDH Chemicals Ltd., Poole, England. The thin-layer chromatography plastic sheets with Kieselgel 60 were from Merck, Darmstadt, Germany. Phoshatidylcholine (lecithin), choline, acrylamide, *N*,*N*-methylenebis(acrylamide) and Triton X-100 were obtained from Sigma, St. Louis, MO. Soybean lecithin (granules) was from Lanes Health Products Ltd., Gloucester, U.K., and from Larkhall Natural Health, London, U.K.

Synthesis of the Bromothymol Blue−Glutathione Conjugate (BTB−GSH). The method for synthesis of the BTB−GSH conjugate is a modification of that of Goldfinch and Lowe (1980). BTB (1 g, 1.6 mmol) and reduced glutathione (GSH) (2.5 g, 8.1 mmol) were dissolved in distilled H_2O (100 mL), and the pH was adjusted to 7.0 with 0.8 M NH_4OH. Nitrogen gas was introduced, before the reaction mixture was left at 50 °C for 4 days, with daily adjustment of the pH to 7.0. The progress of the reaction was followed by thin-layer chromatography (TLC; silica gel plates; developing system: *n*-butanol/acetic acid/water 4:1:5 v/v), analyzing daily sample spots and controlling the change of colored bands. The TLC plates were dried by blowing warm air, and the colored bands were evaluated without prior processing of the plates. After completion, the reaction mixture was evaporated (50 °C) and lyophilized, the solids were redissolved in 53 mL of methanol/H_2O (50:50 v/v), and the solution was applied to a Sephadex LH-20 column (60 × 240 mm), which was previously equilibrated with methanol/H_2O (50:50 v/v). The column was run isocratically, and the following four main bands were separated (in order of elution): a yellowish band corresponding to GSH (identified by TLC as above; the dimensionless parameter describing the ratio of the distance migrated by the compound of interest over that of the solvent front, termed R_f, equals 0.17), the band corresponding to BTB−GSH conjugate (identified by TLC as above, R_f 0.33), a mauve band (identified by TLC as above, R_f 0.53), and the band corresponding to BTB (identified by TLC as above, R_f 0.8). The major product BB−GSH was concentrated in a rotary evaporator (60 °C) and lyophilized (overall yield approximately 20%), displaying two absorptions λ_{max} (438 and 616 nm) in distilled water.

Polyacrylamide Activation and Co-immobilization of the BTB−GSH Conjugate and the Enzymes PL and ChOX. *Polyacylamide Activation with Glutaraldehyde.* Polyacrylamide gel (5% v/v, 2.5 g wet weight, 1.5 mm thickness) was cut in two pieces (1 × 4 cm), washed with distilled water, and equilibrated in 20 mL of 0.1 M phosphate buffer, pH 6.9, for 10 min. The two minigels were transferred in a fresh solution of glutaraldehyde (24 mL, 25% v/v) in the same buffer (18 mL) and left to react at 37 °C for 20 h under gentle shaking. After completion, the minigels were washed with 0.1 M phosphate buffer, pH 6.9, followed with water, and incubated in 0.1 M phosphate buffer, pH 7.7, for 4 h.

Immobilization of BTB−GSH Conjugate on Activated Polyacrylamide. BTB−GSH conjugate (25 mg) was dissolved in 2 mL of 0.1 M phosphate buffer, pH 7.7, and into this solution were added the two activated minigels. The system reacted for 17 h at 25−28 °C under gentle shaking. The gels were washed exhaustively with water, followed with 0.1 M phosphate buffer, pH 7.7.

Immobilization of PL and ChOX on BTB−GSH−Polyacrylamide. Enzyme immobilization on one of the minigels was performed as follows. A minigel from the previous stage was transferred in 2 mL of 0.1 M phosphate buffer, pH 7.7, containing 200 units of PL and 40 units of ChOX. The system was incubated at 4 °C for 2.5 days under gentle shaking. Upon completion of the reaction, the biocatalytic polyacrylamide gel was exhaustively washed with water, followed with 0.1 M Tris-HCl, pH 8.5, and stored in the same buffer.

Effect of pH on the Optical Absorption of the Free and Immobilized Systems of PL, ChOX, and BTB−GSH Conjugate. *Free System*. BTB−GSH conjugate solution (25 μL, 2 mg/mL) was added in 2 mL of 50 mM buffer, pH 5.0−9.5, and the optical absorption was measured at two wavelengths, 438 and 616 nm.

Immobilized System. The biocatalytic polyacrylamide minigel was equilibrated in buffer for 10 min and added in 3 mL of the same buffer (50 mM, pH 5.0−9.5). The optical absorption was measured at 622 nm. The buffers used were acetic acid/NaOH, pH 5−5.5, Mes/NaOH, pH 6−6.5, Tris-HCl, pH 7−9, and borate/NaOH, pH 9.5.

Preparation of Lecithin Solution. Pure lecithin from eggs or soybean lecithin (granules) (0.5 g) was suspended in 25 mL of water and stirred at approximately 40 °C until a homogeneous emulsion was formed. The emulsion was cooled at 4 °C and ultrasonicated for approximately 2 min, taking care for the temperature not to exceed 12 °C. The almost clear emulsion was mixed with Triton X-100 solution (1:1 v/v) to form a clear solution (Keesey, 1987a).

Preparation of Bovine Serum. Bovine fresh blood (30 mL) was centrifuged (4 °C) for 10 min at 976*g* (3000 rpm) to remove blood cells. The supernatant (serum) was diluted (1:1) with Triton ×-100 solution (10% v/v) and used for lecithin assays.

Lecithin Determination with the Routine Method, Using Three Free Enzymes (PL, ChOX, and POD). The assay is based on the change of optical absorbance (Takayama et al., 1977) and performed in a Perkin-Elmer Lamda 16 double-monochromator UV−vis spectrophotometer. The cell holder, suitable to accept 10 mm path length cells, was thermostated electronically (37 °C) by a PTP-1 peltier temperature programmer. In this method, phospholipids (e.g., lecithin) were hydrolyzed by PL and the liberated choline is subsequently oxidized by ChOX to betaine with the simultaneous production of hydrogen peroxide, which, by oxidation in the presence of POD, couples 4-aminoantipyrine and phenol to yield a chromogen with a maximum absorption at 500 nm (Takayama et al., 1977). A sample of 100 μL from lecithin solution (or 20 μL of bovine serum) was mixed with 200 μL of 0.1 M Tris-HCl buffer, pH 8.0 (or 280 μL of buffer when assaying bovine serum), 50 μL of 0.1 M $CaCl_2$ and 50 μL of PL (0.25 unit in final reaction mixture). The reaction was incubated for 10 min at 37 °C before 2.5 mL of 0.1 M Tris-HCl buffer, pH 8.0, which contained 1.5 μmol of 4-aminoantipyrine, 0.21 μmol of phenol, 3 μmol of EDTA, 3.8 units of POD, and 0.25 unit of ChOX, was added The assay mixture was left for 20 min before its optical absorption at 500 nm was taken against a control cuvette in which lecithin was omitted. The absorption coefficient of the produced dye equals 12 L/mmol/cm (Keesey, 1987b). The lecithin concentration of the unknown was determined from a standard curve that made using pure lecithin in the range 10−90 μM.

Lecithin Determination Using Two Enzymes (PL and ChOX) and BTB−GSH Conjugate, Free in Solution (Free System). In 2.5 mL of 50 mM Tris-HCl, pH 8.0 (or 2.58 mL when bovine serum was assayed) buffer were added 100 μL of lecithin solution (or 20 μL of bovine serum), 50 μL of 0.1 M $CaCl_2$, and 50 μL of PL solution (0.25 unit in final assay mixture). The mixture was incubated at 37 °C for 5 min; 0.25 unit of ChOX was then added, and the mixture was left for 10 min (37 °C). Finally, 25 μL of BTB−GSH solution (2 mg/mL) was added to the mixture, which was left at 37 °C for 10 min before the change of its optical absorption was measured at 616 and 438 nm against a control in which lecithin was omitted. The lecithin concentration of the unknown was determined from a standard curve that made using pure lecithin in the range 40−160 μM.

Lecithin Determination Using Two Co-immobilized Enzymes (PL and ChOX) and BTB−GSH Conjugate (Immobilized System). The solid phase consists of polyacrylamide gel (5% v/v, 10 × 40 × 1.5 mm) bearing immobilized BTB−GSH conjugate, ChOX, and PL. The gel is placed in a spectrophotometer cuvette (1 × 1 × 4 cm, L × W × H) containing 2.65 mL of 0.1 M Tris-HCl buffer, pH 8.5 (or 2.83 mL of buffer when bovine serum was assayed) and 50 μL of 0.1 M $CaCl_2$. After the system was equilibrated for 5 min at 37 °C, 200 μL of lecithin solution (or 20 μL of bovine serum) was added for lecithin assay (*note*: when enzyme activity of the system was assayed, a saturating concentration of lecithin was used, 0.45 mM). The reaction proceeded for 15 min before the change of the gel's optical absorption was measured at 622 nm against a control gel, which was treated as above except that the lecithin sample had been substituted for an equal volume of buffer. A standard curve was made using pure lecithin, in the range 20−120 μM, and from its slope the lecithin concentration of the unknown was determined.

Assay of ChOX for the Immobilized System. The biocatalytic gel (5% v/v, 10 × 40 × 1.5 mm) is placed in a plastic cuvette (1 × 1 × 4 cm) along with 3 mL of 0.1 M Tris-HCl buffer, pH 8.5, containing 0.1 μmol of EDTA and 0.9 μmol of KCl. After the system was equilibrated for 5 min at 37 °C, a saturating concentration of choline (50 μL, 0.15 mM) was added and the reaction was left to proceed for 15 min before the change of its optical absorption at 622 nm was read against a control, which was treated as above except that choline had been substituted for an equal volume of buffer.

Stability of the Immobilized System (PL, ChOX, and BTB−GSH Conjugate). Assays were performed daily for establishing the activity of (a) the whole immobilized enzyme system (PL and ChOX) and (b) immobilized ChOX alone. The procedures employed were the last two described just above, using saturated substrate concentrations, 0.45 mM lecithin and 0.15 mM choline, respectively.

RESULTS AND DISCUSSION

The existing routine method for lecithin determination (Takayama et al., 1977) is colorimetric and based on three free-in-solution enzymes, PL, ChOX, and POD, which at the end of the assay are discarded. The method proposed here is based on a co-immobilized system of two enzymes, PL and ChOX, and a pH indicator dye conjugate, BTB−GSH, thus offering many reuses of the enzyme system. The principle of the method is described in the Introduction. The end result is the change of the microenvironmental pH of the immobilized system and, consequently, the shift of the optical absorbance maximum of the immobilized indicator BTB−GSH conjugate, a phenomenon that is then quantified for lecithin determination. Appropriately modified indicator dyes have been exploited by Goldfinch and Lowe (1980, 1984) for the determination of albumin, penicillin, urea, and glucose.

Choice of the Indicator Dye. To identify the most suitable indicator dye, the following five dyes were tested for their ability to shift their λ_{max} during the conversion of lecithin to betaine by free PL and ChOX: BPB, working pH 3.0−4.6; BCG, pH 3.8−5.4; BCP, pH 5.2−6.8; BTB, pH 6.0−7.6; CR, pH 7.1−8.8. Of these indicator dyes, only BTB and CR have shown the desirable property, and of the two candidates, BTB was finally chosen for further studies because it possesses suitable electrophile groups (bromine) which can be substituted by the nucleophilic group of a spacer molecule (the sulfhydryl group of GSH) so that the derived BTB−GSH conjugate can be immobilized, via its terminal free animo group of GSH, on glutaraldehyde-activated polyacrylamide gel.

Figures 1 and 2 show the absorption spectrum of free BTB and free BTB−GSH conjugate, respectively, in 50 mM Tris-HCl buffer, pH 8.0, before (a) and after (b) catalytic breakdown of lecithin in the presence of PL (acting on lecithin) and ChOX (acting on choline, product of the previous reaction). The absorption spectrum shows two maxima, at 438 and 616 nm, the

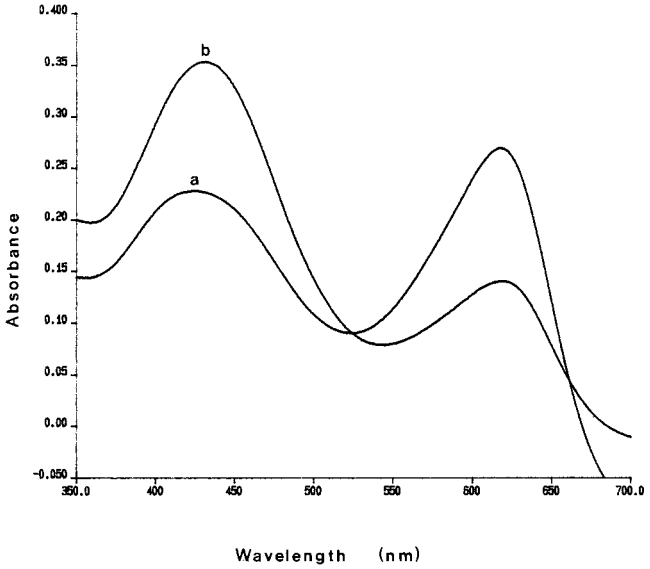

Figure 1. Absorption spectra of the indicator dye BTB in 50 mM Tris-HCl, pH 8.0, before (a) and after (b) the enzyme reaction with lecithin where a hypsochromic shift (from 616 to 438 nm) is observed.

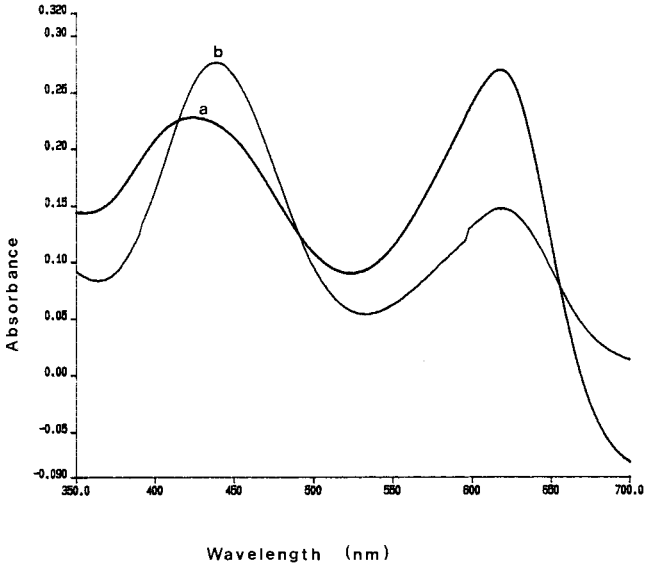

Figure 2. Absorption spectra of the indicator dye conjugate BTB−GSH in 50 mM Tris-HCl, pH 8.0, before (a) and after (b) the enzyme reaction with lecithin where a hypsochromic shift (from 616 to 438 nm) is observed.

second of which offers greater change in optical absorbance. Initially, at alkaline environment, the 616 nm peak (green color) prevails, but during the catalytic reaction the 438 nm peak (green-yellow color) prevails, thus leading to the desirable hypsochromic shift of optical absorption. Study of the effect of pH on the conjugate's optical absorption reveals the effective pH range from which a suitable pH value was chosen for the present method. Figure 3 shows the results from such a study. For the free BTB−GSH conjugate (Figure 3a) the absorption maximum at 616 nm, compared to that at 438 nm, provides a sharper and a greater change in optical absorption for pH values in the range 7.5−8.5. For the immobilized BTB−GSH conjugate (Figure 3b), a sharp and a great change in the optical absorption was observed at λ_{max} 622 nm for pH values in the range 8.5−9.0. These findings justify the choice of pH 8.0 for the free and pH 8.5 for the immobilized enzyme systems.

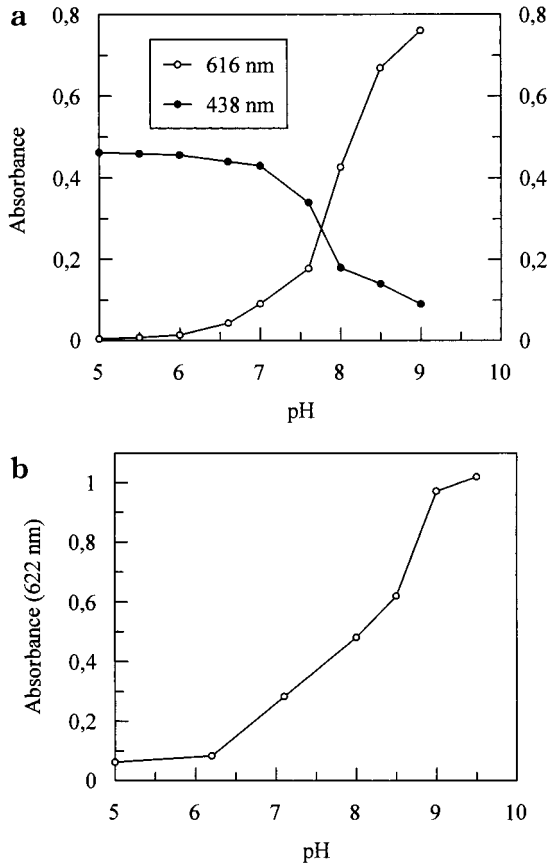

Figure 3. Effect of pH on the optical absorption of the BTB−GSH conjugate: (a) free system at 438 and 616 nm; (b) immobilized system at 622 nm.

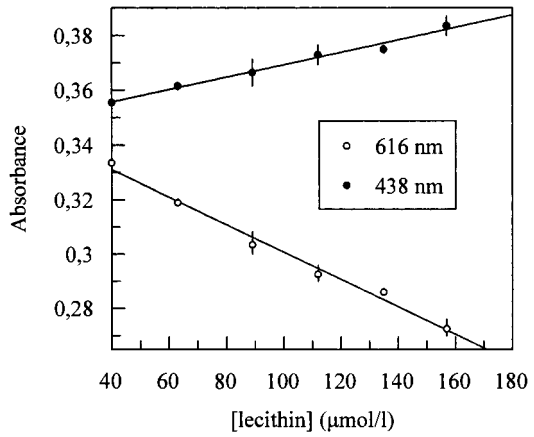

Figure 4. Standard curves for the determination of lecithin at two wavelengths, using free system comprising PL, ChOX, and BTB−GSH conjugate (each point is the mean of four measurements, error < 2.5%).

Notably, these values combine very well with the pH necessary for maximum enzyme activity (8.0).

Assay of Lecithin Using Free and Immobilized Systems. The present assay for lecithin was evaluated using both free and immobilized enzymes (PL and ChOX) and BTB−GSH conjugate, and it was compared with other enzyme-based assays of the literature. Figure 4 shows the influence of lecithin concentration on the optical absorption when assaying with the free system at two wavelengths, 438 and 616 nm. The change of the absorption remains linear in the range 40−160 μM lecithin at both wavelengths, but the wavelength at 616 nm provides a steeper slope (optical

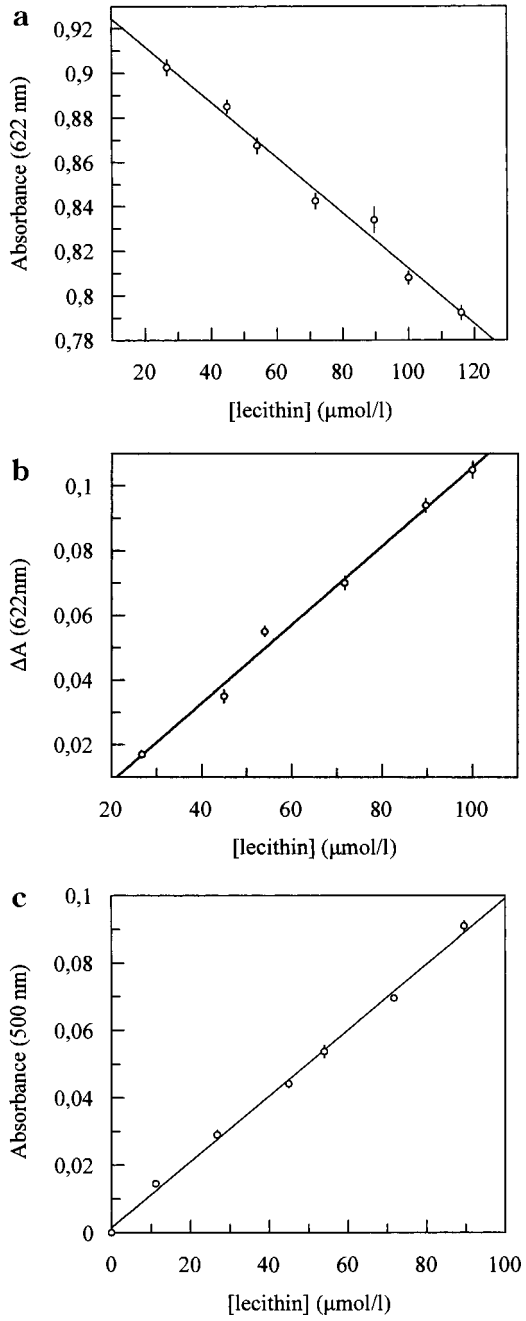

Figure 5. Standard curves for the determination of lecithin using (a and b) the immobilized system comprising PL, ChOX, and BTB−GSH conjugate (each point is the mean of four measurements; error < 3%) and (c) the routine system comprising three free enzymes, PL, ChOX, and POD (each point is the mean of three measurements; error < 1%).

absorption = 0.35249 − 0.0051[lecithin], r = 0.997). Likewise, when using the immobilized system, linearity was observed over a broad range of lecithin concentrations, 20−120 μM (Figure 5a) (optical absorption = 0.94013 − 0.00131[lecithin], r = 0.986, at 622 nm) when taking values of absolute absorbance, and 20−100 μM (Figure 5b) (optical absorption = −0.0146 + 0.012-[lecithin], r = 0.995) when taking values of absorbance difference (ΔA). The present assay method, whether employing free or immobilized system, offers linearity over a substantially broader range of lecithin concentrations compared to the colorimetric routine lecithin assay (Takayama et al., 1977) (Figure 5c), which offers linearity in the range 10−90 μM (optical absorption = 0.00107 − 0.00096[lecithin], r = 0.997). All three methods proposed for lecithin assay were tested with real samples originating from two commercial lecithin products and fresh bovine serum. The results are summarized in Table 1. Unlike the bovine serum (sample C), the commercial lecithin products (samples A and B) contained approximately 20% choline (assays were performed as described in Experimental Procedures except that PL was omitted), for which the lecithin values shown (Table 1) have been corrected. It is evident that the new assay using immobilized enzymes offers, for all samples, standard deviation and error comparable to those of the routine assay, and therefore it should be applicable to lecithin determinations. The same biocatalytic gel could be used for determinations of total choline (free and bound on lecithin).

Table 1. Validation of the Proposed New Assay for Lecithin[a]

sample[b]	routine assay (Figure 5c) (mM)	new assay with free enzymes (Figure 4) (mM)	new assay with immobilized enzymes (Figure 5a) (mM)
A	0.840 ± 0.025 (3.0)	0.819 ± 0.028 (3.4)	0.795 ± 0.023 (2.9)
B	0.690 ± 0.030 (4.3)	0.656 ± 0.030 (4.6)	0.646 ± 0.025 (3.9)
C	3.000 ± 0.066 (2.2)	3.300 ± 0.079 (2.4)	3.200 ± 0.096 (3.0)

[a] Results are average of five determinations ± standard deviation (% error) for each of the samples A−C. [b] A, B, commercial lecithin products; C, bovine serum.

Two nonenzymatic methods for lecithin determination are based on HPLC technology. HPLC is used for the determination of dipalmitoylphospatidylcholine in amniotic fluid, showing an analysis time of 10 min and a detection range 0.13−31.5 μM (Alvarez et al., 1997). Total PC in serum can be detected after extraction with a mixture of chloroform/methanol, followed by HPLC in a solvent system of acetonitrile/methanol/acetic acid where the lecithin eluted is detected colorimetrically in the presence of erythrosin B (Hradec and Dufek, 1997). The method is sensitive to approximately 100 μg but is laborious and not specific enough for lecithin.

Alternative, noncolorimetric enzyme-based assays for lecithin have been based on the amperometric principle; however, they are cumbersome. Matsumoto et al. (1980) combined ChOX immobilized on aminated polyacrylonitrile membrane attached to an oxygen probe and free PL. This assay offers linearity in the range 40−100 μM lecithin, but no information is available on the required assay time. Karube et al. (1979) proposed a system according to which the effluents from an octylagarose column with immobilized PL and ChOX were directed to an amperometric electrode sensing the hydrogen peroxide produced from the oxidase reaction. The method requires the same assay time as the one proposed here (15 min); however, it is less sensitive as it is linear in the range 1−5 mM lecithin. The same amperometric sensing principle was employed later by Masoom et al. (1990) but with a different, more complicated, enzyme system. This system comprised two columns bearing immobilized enzymes, the first phospholipase (C or D) and the second alkaline phosphatase and ChOX. The hydrogen peroxide, produced from the oxidase reaction, present in the column effluents of the system, is being sensed by the amperometric electrode. The method requires the same assay time as before; however, it is less sensitive as it is linear in the range 0.05−2 mM lecithin. Furthermore, both of the above methods, so long they are based on column configurations, require

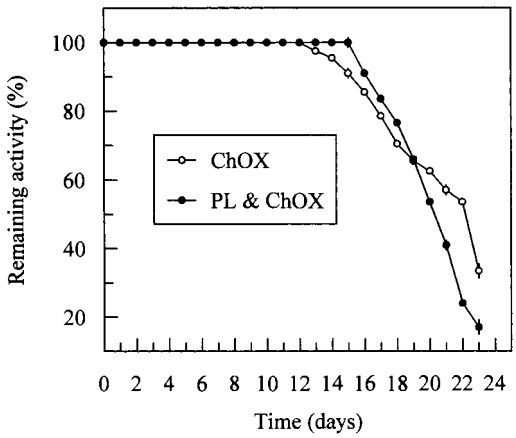

Figure 6. Enzyme stability curves for the immobilized system (PL and ChOX) and of the immobilized ChOX (each point is the mean of three measurements, error < 2.5%).

relatively large samples, are not handy, and limit their wider application.

Stability of the Immobilized System (PL, ChOX, and BTB−GSH Conjugate). Daily assays for determining the activity of the immobilized system (PL and ChOX) and of immobilized ChOX were performed using as saturating substrates lecithin and choline, respectively (Figure 6). Although the decline of ChOX activity is more shallow than the activity of PL (measured as a two-enzyme system), the immobilized system maintained its activity for the determination of lecithin (PL and ChOX are required) 3 days longer than for the determination of choline (ChOX is required). Therefore, when an immobilized system that has maintained > 80% of its original activity was employed, no appreciable effect on the lecithin assay was observed, thus extending the system's effective lifetime to over 2 weeks.

Current work focuses on the development of immobilization techniques for biocatalytic systems, and of solid supports and transducers suitable for use in optoelectronic devices (optical biosensors).

ABBREVIATIONS USED

BCG, bromocresol green; BCP, bromocresol purple; BPB, bromophenol blue; BTB, bromothymol blue; CR, cresol red; BTB−GSH, bromothymol blue−glutathione conjugate; Mes, 2-morpholinoethanesulfonic acid; PL, phospholipase D; ChOX, choline oxidase; GSH, glutathione (reduced); POD, peroxidase; PC, phosphatidylcholine.

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