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A Simple Method to Obtain a Covalent Immobilized Phospholipase A_2

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Abstract—In the present work, we obtained an immobilized phospholipase A_2 system through covalent coupling by using an acrylic polymer Eupergit C as support. The immobilized enzyme from cobra venom (*Naja naja naja*) showed good retention activity and excellent stability. Both properties are of great importance for biomedical applications such as hypercholesterolemia treatments.
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Phospholipase A_2 (PLA_2, E.C. 3.1.1.4) catalyzes the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids.[1] In an earlier paper,[2] we obtained an immobilized PLA_2 by using CM-Sephadex as support. This system was characterized for phospholipid hydrolysis and we concluded that the same can be used in a biotechnological process to obtain lysophospholipids, for food and pharmaceutical applications.[3] Nevertheless, such a system could be altered by desorption of the enzyme as a consequence of changes in pH or ionic strength of the medium and therefore was not suitable for biomedical applications. An active and highly stable immobilized PLA_2 system can be applied in hypercholesterolemia treatment. In this sense, PLA_2 was shown to readily hydrolyze phospholipids present in human serum low density lipoproteins (LDL). Moreover, LDL modified by the enzyme was removed from the circulation to the liver more rapidly than unmodified LDL.[4]

Herein, we developed a covalent immobilized PLA_2 system using an acrylic polymer, Eupergit C, as support. To our knowledge, it is the first described Eupergit-PLA_2 system.

PLA_2 from cobra venom (*Naja naja naja*), a gift from Dr. Ismael Bianco, was purified according to Reynolds and Dennis.[5] Its overall amino acid sequence had been determined and the tertiary structure well studied. This PLA_2 has a low apparent molecular mass (13 kDa) and seven disulfide bridges which explain its high stability.[6] Also, the enzyme has an isoelectric point of 5.1. Stock solution of the enzyme was prepared in phosphate buffer saline (PBS) pH 7.4 at a concentration of $0.35\,mg\,mL^{-1}$. Eupergit C, a noncharged support, was generously supplied by Röhm Pharma Polymers, Darmstadt. It is an acrylic polymer, a copolymer of methacrylamide, *N*,*N*′-methylene-bis(methacrylamide) and monomers containing oxirane groups. The oxirane group functions as reactive component and covalently binds sulfhydryl, amino, or hydroxyl compounds in an electroneutral binding mechanism. The content of the oxirane group is higher than 600 μmol per gram (dry). The matrix is electroneutral and predominantly hydrophilic. Eupergit C exhibits a water uptake of 3.0 mL per gram of dry beads. This is a spherical, macroporous, carrier material with a particle size of about 150 microns (Eupergit Basic Information, Röhm). Egg phosphatidylcholine (egg PC), purchased from Sigma Chemical Co., St. Louis, MO, was used as phospholipid substrate. Triton X-100, from Röhm & Haas Co., PBS, potassium barbital and all the other reagents were of analytical grade.

For the covalent immobilization of cobra venom PLA_2 (*Naja naja naja*), the enzyme solution in PBS (2.5 mL) was incubated for 24 h in the presence of the acrylic polymer Eupergit C (200 mg, dry state). After this, the immobilized system was washed several times with buffer and filtered in vacuum to obtain it in wet form. The enzyme fixation level was obtained spectrophotometrically from UV 280 nm absorption of the

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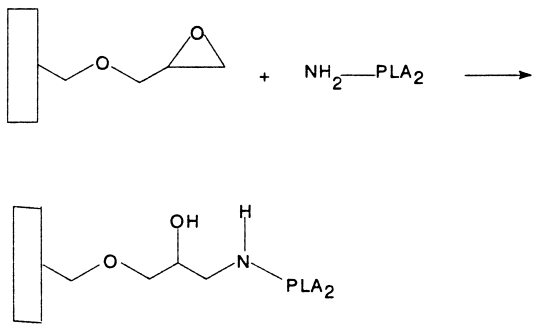

Scheme 1. Schematic representation of the covalent coupling of PLA_2.

Table 1. Kinetic parameters of *Naja naja naja* PLA_2

PLA_2 form	V_{max} ($\mu mol\,mg^{-1}\,min^{-1}$)	K_m (mM)
Soluble	649	12.2
Immobilized	333	11.0

initial enzyme solution and the supernatant at the end of the immobilization process. The enzymatic assays of either unbound enzyme or the immobilized form (20 mg) were performed by using a mixed micelle system of phospholipid/Triton X-100 1:4 molar ratio. The reaction medium (2 mL) was rotated in Büchi RE-111 rotator during 1 min (initial rate) at 60 rpm. Because the support of the immobilized system was Eupergit, it was necesssary to avoid the presence of Tris or similar buffers by virtue of its reactivity toward oxirane groups. Potassium barbital was used as buffer for this purpose. Assays were carried out at 40 °C in 20 mM potassium barbital pH 8.1 and 5 mM $CaCl_2$. Ca^{2+} is an essential activator for secretory PLA_2s[1]. The activity was obtained by liquid–liquid partition followed by titrimetric microdetermination of released fatty acids as described in a previous work.[2]

The enzymatic kinetic parameters V_{max} (maximum velocity) and K_m (Michaelis–Menten constant) were determined by using GraFit software.[7] Data were obtained by nonlinear regression treatment for curve fit in the hyperbolic graphic. The reported values (Table 1) were obtained from averages of duplicate assays of at least two independent experiments.

The covalent immobilization of cobra venom (*Naja naja naja*) on the acrylic polymer Eupergit C was satisfactory since the retention activity (noninitial rate) for this particular system was 48% compared to the original soluble activity. In other work, when the enzyme was a lipase, the immobilized system showed only 3% residual activity.[8] By using Eupergit polymer, the covalent linkage is the result of a nucleophilic attack to oxirane carbon. The spacer unit between matrix and the protein largely minimizes interferences between the support and the enzyme (see Scheme 1). However, the enzymes from other sources such as bee venom (*Apis mellifera*) or pig pancreas lead to practically inactive immobilized adducts. In the case of the *Naja naja naja* enzyme, covalent coupling onto acrylic beads yielded a stable system since the immobilized enzyme maintained almost all the original activity through at least 10 cycles of different batches of substrate. By this reuse assay, the same sample of immobilized system was recycled several times to study its operational stability.

The values of Michaelis–Menten constant (K_m) obtained for soluble and covalent immobilized PLA_2 (Table 1) indicate that the affinity for substrate was hardly modified by the immobilization process. However, the catalytic step was affected since at saturated phospholipid concentration, the initial rate was decreased. The fixation of the enzyme to support would impose severe restrictions on PLA_2 to work in the classical scooting or hopping modes of secretory PLA_2s action.[2] Nevertheless, the immobilized *Naja naja naja* PLA_2 showed good perfomance in considering that generally, immobilized enzymes decay in their catalytic properties.[9]

Further optimization of this covalent immobilization process should lead to an immobilized PLA_2 system of great efficiency and useful for biotechnology processes[3] or biomedical applications such as hypercholesterolemia treatment.[10,11]

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