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Enzyme-based high-performance liquid chromatography stationary phases as metabolic reactors

Immobilization of non-solubilized rat liver microsomes on an immobilized artificial membrane high-performance liquid chromatography support

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ABSTRACT

Non-solubilized rat liver microsomes have been non-covalently immobilized on an immobilized artificial membrane (IAM) HPLC stationary phase. The microsomes were immobilized on the IAM interphase of both loose IAM support and in a 1 cm × 3 mm I.D. HPLC column filled with IAM stationary phase. The activity of the immobilized microsomes was assessed by following the formation of 7-hydroxycoumarin (7-OHC) from the O-deethylation of 7-ethoxycoumarin. The 7-OHC was quantified by either HPLC analysis of an aliquot from an incubation mixture containing the loose microsome–IAM support or directly using the chromatogram from the microsome–IAM HPLC column. Both forms of the IAM-immobilized microsomes were active. The result of this study indicate that the IAM stationary phase can be used to produce immobilized microsomal reactors which can be used in HPLC systems for direct on-line determination of biosynthetic and metabolic processes.

INTRODUCTION

Immobilized enzyme reactors (IMERs) have been developed by a number of groups and applied to synthetic and pharmacological studies. IMERs are of interest because they have a number of advantages relative to soluble enzymes including: the immobilized enzymes can be reused; isolation of the products from the reaction mixture is easier; processes can be run continuously; immobilization can stabilize the enzymes.

The ability to follow biosynthetic and metabolic processes using an IMER has been demonstrated by a number of research groups. Fenselau *et al.* [1] covalently immobilized solubilized microsomal enzymes on cyanogen bromide-activated Sepharose beads and used the resulting IMER to synthesize glucuronides. Lehman *et al.* [2] used the same system in the N-demethylation of ethylmorphine and the O-demethylation of *p*-nitroanisole. The Sepharose-based IMERs were not only active, but also showed excellent stability with respect to both time and temperature [2].

Solubilized microsomes have also been immobilized by entrapment into alginate beads in

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the presence of polyethyleneimine [3]. The resulting IMER showed a substantial increase in UDP-glucuronyltransferase activity compared to free microsomes or microsomes immobilized by other methods. The alginate bead-entrapped microsomes were used to synthesize 3′-azido-3′-deoxythymidine (AZT), ether- and acyl(ester)-glucuronides.

Another approach to the non-covalent immobilization of enzymes involves their entrapment in the interphase of an immobilized artificial membrane (IAM) HPLC stationary phase. IAM phases are produced through the covalent immobilization of 1-myristoyl-2-[(13-carboxyl)-tridecanoyl]-*sn*-3-glycerophosphocholine on an aminopropyl silica through an ω-carboxyl group on the C-2 fatty acid chain [4]. In the resulting support, the phosphatidylcholine head-groups form the surface of the support and the hydrocarbon side chains produce hydrophobic cavities which extend from the charged head-group to the surface of the aminopropyl silica.

IAM stationary phases have been used to non-covalently immobilize the enzymes α-chymotrypsin (ACHT) and trypsin [5]. The resulting IAM-IMERs retained the hydrolytic activity of the non-immobilized enzymes and their sensitivity to enzyme inhibitors, pH and temperature. When a substrate was chromatographed on the IAM–ACHT, the activity of the immobilized enzyme could be determined directly from the chromatogram by calculating the substrate/product ratios. IAM-immobilized ACHT and trypsin can be used as chromatographic probes for qualitative determinations of enzyme/substrate and enzyme/inhibitor interactions [5].

The IAM support has also been used to purify several P-450 isozymes in functional conformations [6]. This has led us to investigate the possibility of producing a new category of biosynthetic IMERs through the entrapment of non-solubilized microsomes on the IAM support. One of the objectives of this work is the development of a microsomal IMER which can be directly linked to an analytical HPLC column for on-line monitoring of metabolic conversions. This note reports the initial results of this project.

EXPERIMENTAL

Materials

7-Ethoxycoumarin (7-EtOC), 7-hydroxycoumarin (7-OHC; umbelliferone), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) and NADPH were obtained from Sigma (St. Louis, MO, USA). IAM HPLC columns and cartridges, as well as loose IAM packing material were from Regis (Morton Grove, IL, USA).

Rat liver microsomes

Rat liver microsomes were prepared by ultracentrifugation from male rats pretreated with phenobarbital. A solution of phenobarbital in saline was administered intraperitoneally for three days in a dose of 80 mg/kg per day and the animals were decapitated 24 h after the last dose. The microsomes (36.2 mg microsomal protein/ml) were frozen as pellet at −80°C until use. The P-450 content of the microsomal pellet after thawing was 0.45 nmol P-450/mg protein.

Non-covalent immobilization of microsomes

To 25 mg of loose IAM material in a 1.5-ml Eppendorf test tube, prewashed with 3 × 1 ml sodium phosphate buffer (0.1 *M*, pH 7.47), 100 μl of microsomal pellet (equal to 3.62 mg microsomal protein) were added. The slurry was vortexed and left to equilibrate for 10 min. It was then washed with 3 × 1 ml of the same phosphate buffer and the washings collected for protein determination. The immobilization was performed twice using the same microsomal preparation and 3.43 (±0.01) mg microsomal protein were bound to 25 mg IAM material.

The non-covalent immobilization of microsomes on the HPLC column was performed by injecting 5 × 20 μl of microsomal pellet (equal to 3.62 mg of microsomal protein) onto the 1 cm × 3.0 mm I.D. HPLC cartridge filled with IAM stationary phase. The mobile phase flow-rate was 0.1 ml/min. The cartridge was perfused with the suspension containing microsomes in reversed position in order to bypass the cartridge frit and enable non-solubilized microsomes to penetrate into the IAM interphase. The eluate (2 ml) was collected and analyzed for unretained protein; 0.44 mg were detected. The cartridge was re-

versed and the eluate collected in 2-ml fractions to check for the possible leak of microsomes. These fractions did not contain any protein and the total amount of microsomal protein retained non-covalently on the IAM interphase was 3.18 mg.

Analytical methods

Protein was determined according to the method of Lowry *et al.* [7]. P-450 content of microsomes was quantitated by the method of Omura and Sato [8] from the reduced carbon monoxide difference spectrum utilizing an extinction coefficient of 91 mM^{-1} cm^{-1} for the 490–450-nm wavelength pair.

7-Ethoxycoumarin-O-deethylase activity

7-Ethoxycoumarin-O-deethylase activity of both non-immobilized and non-covalently immobilized rat liver microsomes on loose IAM material, as well as of the microsomes non-covalently immobilized on the IAM interphase in a HPLC cartridge, was measured by the fluorimetric method of Ullrich and Weber [9] adapted for HPLC.

With non-immobilized microsomes and microsomes non-covalently immobilized on loose IAM support. A 1-ml volume of 0.1 m*M* 7-EtOC in sodium phosphate buffer (0.1 *M*, pH 7.47) was pipetted into two Eppendorf 1.5-ml test tubes (each containing 3.43 mg of non-covalently immobilized microsomal protein on 25 mg of loose IAM chromatographic support), and into two test tubes each containing 50 μl of microsomal pellet (corresponding to 1.81 mg microsomal protein). After short vortexing and equilibration for 5 min at 37°C in a water bath, the enzymatic reaction was started by addition of 5 μl of 10 m*M* NADPH. The test tubes were incubated unstoppered in a water bath at 37°C and vortexed every 5 min to facilitate the oxygenation of the incubation mixture. After 20 min, the test tubes were spun for 2 min at 15 000 *g* and a 100-μl aliquot of the supernatant removed and injected into the HPLC system. Blanks without microsomes and without NADPH were run together with the samples. Before the start of each new incubation with a fresh substrate, the microsomes–IAM slurry was washed with 3 × 1 ml of sodium phosphate buffer (0.1 *M*, pH 7.47).

The productivity of the non-covalently immobilized microsomes, measured as nmoles of 7-OHC/mg microsomal protein, was measured using the same approach. In this experiment, aliquots of the incubation mixtures were removed for HPLC analysis of 7-OHC concentration at 0, 20, 40, 60, 80 and 100 min total incubation time. At each sampling time from 20 to 80 min, 5 μl of 10 m*M* NADPH were added to the incubation mixture.

With microsomes non-covalently immobilized on IAM chromatographic column. A 20-μl volume of mixture A or B were injected directly into the chromatographic system, where: A = 5 ml of 0.1 m*M* 7-EtOC in sodium phosphate buffer (0.1 *M*, pH 7.47) with 5 μl of 10 m*M* NADPH/ml; B = 5 ml of 1 m*M* 7-EtOC in sodium phosphate buffer (0.1 *M*, pH 7.47) with 20 μl of 10 m*M* NADPH/ml. Controls contained the 7-EtOC solution but no NADPH.

Inhibition of 7-ethoxycoumarin-O-deethylase activity with metyrapone

After the first 20-min incubation (carried out as described above), 10 μl of metyrapone (0.01 *M*) (final concentration in incubation mixture 0.1 m*M*) were added to one test tube with non-covalently immobilized microsomes on IAM, and 10 μl of buffer to the control. After addition of another 5 μl of 10 m*M* NADPH to both test tubes, the incubation at 37°C was continued for another 20 min. The 7-OHC generated was determined by direct injection of the supernatant into the HPLC system as described above.

Stability (time vs. activity profile) of non-covalently immobilized microsomes

This was studied by testing the O-deethylase activity of the immobilized microsomes.

(a) On loose IAM material, the same microsomes were kept at 37°C while incubating and at 25°C for all other manipulations during the days 1 and 2 (*ca.* 10 h/day) and days 7 and 10 (*ca.* 4 h/day). The rest of time microsomes were kept refrigerated at 4°C.

(b) On the microsomes–IAM chromatographic

column, all the experiments were done at 25°C in one day.

Chromatographic system

The chromatographic system consisted of an Spectroflow 400 pump (ABI Kratos, Ramsey, NJ, USA), 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) with a 20-μl sample loop, 470 Waters scanning fluorescence detector (Millipore Waters, Milford, MA, USA) and a Data Jet integrator (Spectra-Physics, San Jose, CA, USA). The chromatographic columns used were an 1 cm × 3.0 mm I.D. cartridge (used as an IMER while containing microsomes) and an 3 cm × 4.6 mm I.D. column, in series, both filled with the IAM chromatographic support (Regis). The mobile phase used was sodium phosphate buffer (0.1 *M*, pH 7.47). The flow was kept at 0.5 ml/min except where otherwise stated. The excitation and emission wavelengths for fluorescence detection were set at 360 and 460 nm, respectively.

RESULTS AND DISCUSSION

The catalytic activity of the microsomal monooxygenatic system of mammalian liver is determined by interactions between the three essential components: P-450, NADPH–P-450 reductase, and phosphatidylcholine. Due to the membrane-bound character, protein–phospholipid interactions are considered of special importance for the functional activity. Although IAM.PC (acronym of the first commercially available IAM based on the most prevalent membrane lipid, phosphatidylcholine, PC) contains monolayers of amphiphilic membrane lipid molecules bonded to silica, this chromatographic support has the hydrophobicity of only a C_3 reversed-phase chromatographic surface. Therefore quite mild mobile phases, like buffers, may be used, which makes IAM.PC columns suitable for protein immobilization. Pidgeon *et al.* [6] have found that solubilized P-450 isozymes bind to IAM in the presence of 0.6% cholate. (The presence of detergent was necessary to maintain membrane proteins in solution and prevent protein aggregation.) They have also found, however, that most other microsomal proteins, including NADPH–P-450 reductase, do not bind to IAM under the same conditions. Thus, the lack of possibility to immobilize all three essential components for microsomal monooxygenation prompted us to try to immobilize non-solubilized microsomes into the IAM.PC interphase. Consequently, we were able to perform P-450-catalysed reactions on-line in the HPLC system without undergoing the lengthy procedure of solubilization of microsomes.

The monooxygenase activity of the non-immobilized and immobilized microsomes was monitored by following the formation of 7-OHC from the O-deethylation of 7-EtOC [9]. This assay is a sensitive indicator of monooxygenase activity but the ability of this approach to detect low levels of 7-ethoxycoumarin-O-deethylase activity using a spectrofluorometer cuvette is limited by the presence in the reaction mixture of other fluorescent compounds, NADPH and 7-EtOC. Several authors have overcome this problem by extracting 7-OHC from the reaction mixture [10,11]. In this study, extractions were avoided by direct HPLC analysis of the incubation mixture. The chromatography was able to directly separate and quantify the product 7-OHC in the presence of the substrate (7-EtOC) and cofactor (NADPH). Representative chromatograms from this study are presented in Fig. 1.

The non-solubilized microsomes were initially immobilized on loose IAM chromatographic support and the monooxygenase activity assessed using Eppendorf test tubes as reaction vessels. Parallel studies were performed using aliquots of the same microsomal pellet suspension from which the immobilized microsomes were obtained. The results of this comparison indicate that the immobilized enzymes retained only 14% of the initial activity of the non-immobilized microsomes (Table I).

While the reason for the initial loss of enzymatic activity is not immediately apparent, it may be due to the positioning of the functional membrane proteins within the interstitial cavities of the IAM support. Thus, the access of the substrate to the binding site is inhibited because of the topology of the protein on the immobilized surface. A similar effect has been suggested for the observed 50% decrease in the proteolytic activity of α-chymotrypsin [5]. The positioning,

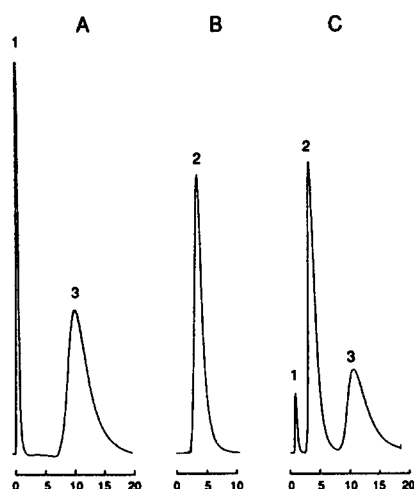

Fig. 1. Chromatograms of (A) the incubation mixture before addition of microsomes, containing only NADPH and the substrate; (B) the 7-OHC standard solution; and (C) the complete incubation mixture containing rat liver microsomal 7-ethoxycoumarin deethylase non-covalently immobilized on loose IAM. Peaks: 1 = NADPH; 2 = 7-OHC; 3 = 7-EtOC. Abscissa = time in min; ordinate = fluorescence.

or shielding, of the microsomes entrapped in the IAM chromatographic support may also explain the fact that immobilization reduced the inhibitory effect of metyrapone; 0.1 m*M* metyrapone produced a 23% decrease in the 7-ethoxycoumarin-O-deethylase activity of the IAM-immobilized microsomes and a 62% decrease in the same activity of the non-immobilized enzymes.

It should also be mentioned that the functional activity of a protein may be diminished by the lack of cofactors at the site of reaction. Taking into account that the enzymatic reaction takes place in the chromatographic column, it is of utmost importance to ensure the simultaneous presence of both substrate and cofactor(s) at the reaction site. If the cofactor(s) and the substrate do not have more or less the same k', they will be separated after the first few mm of the HPLC column and no enzymatic reaction could be expected.

As has been previously demonstrated for microsomes covalently attached to Sepharose beads [2], immobilization enhanced the stability of the enzymes relative to the non-immobilized microsomes (Table I). Both immobilized and non-immobilized microsomes were stored under the same conditions, 4°C, and the 7-ethoxycoumarin-O-deethylase activity determined on days 2, 7 and 14 post immobilization. The non-immobilized enzymes lost 55.5% of their activity by day 2, 92.8% by day 7 and 98.9% by day 14. The IAM-immobilized microsomes lost 32.9% of their activity by day 2, 63.4% by day 7 and 63.8% by day 14. As a result, the relative difference between the activity of the immobilized:non-immobilized microsomes went from 0.13:1 on day 1 to 4.74:1 on day 14.

The productivity of the microsomal IMER as measured by the O-deethylation of 7-EtOC and the production of 7-OHC was studied by sup-

TABLE I

TIME PROFILE OF THE AVERAGE 7-ETHOXYCOUMARIN O-DEETHYLASE ACTIVITY FROM RAT LIVER MICROSOMES NON-COVALENTLY IMMOBILIZED ON LOOSE IAM CHROMATOGRAPHIC SUPPORT IN COMPARISON WITH NON-IMMOBILIZED MICROSOMES

	nmol 7-OHC per mg microsomal protein per 20 min			
	Day 1	Day 2	Day 7	Day 10
Non-immobilized microsomes	1.785	0.794	0.128	0.019
Range	1.464–2.265	0.540–1.000	0.115–0.140	0.016–0.022
n	4	4	2	2
Microsomes on loose IAM	0.249	0.167	0.091	0.090
Range	0.233–0.272	0.105–0.212	0.086–0.096	0.118–0.061
n	6	4	2	2

plying the same incubation mixture with fresh NADPH every 20 min after removing aliquots from the reaction mixture for HPLC determination of 7-OHC concentration. The increase in product formation was linear (Fig. 2).

When the non-solubilized microsomes were immobilized on an IAM support packed in a 1-cm HPLC column, measurable 7-ethoxycoumarin-O-deethylase activity was observed after the injection onto the IMER of 7-EtOC and NADPH by the appearance of 7-OHC in the chromatogram. The chromatographic peak corresponding to 7-OHC was not present when 7-EtOC without NADPH was injected onto the IMER indicating that the appearance of 7-OHC was due to enzymatic activity and not to interactions with the chromatographic phase. The productivity of the IMER (expressed as nmol 7-OHC/mg microsomal protein) was dependent upon the flow-rate of the mobile phase, which, in turn, was a measure of the time that 7-EtOC was in contact with the microsomes (Table II). A decrease in the flow-rate from 0.50 to 0.20 ml/min increased the productivity by 475%. However, this effect appears to be saturable as a decrease in flow-rate from 0.20 to 0.10 ml/min only increased the productivity by 14.4%.

The stability of IMER relative to its 7-ethoxycoumarin-O-deethylase activity was followed during one day (Table III). The observed decrease in enzymatic activity (*ca.* 85%) was greater than the 33% decrease observed for the microsomes immobilized on the loose IAM chromatographic support. This difference may have been caused by the lack of requisite molecular oxygen in the mobile phase or by the pressure exerted on the microsomes during the chromatographic process. Pidgeon *et al.* [6] have reported that with IAM-immobilized microsomes, the P-

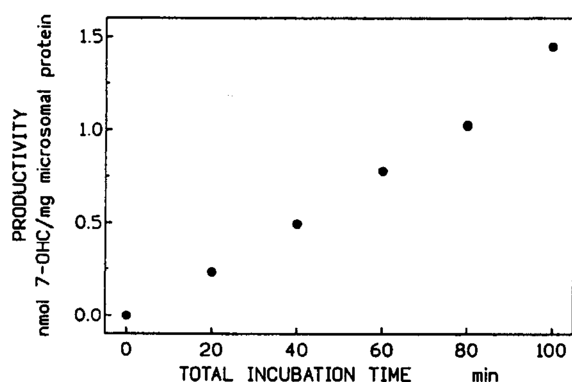

Fig. 2. Production of 7-OHC by rat liver microsomal 7-ethoxycoumarin deethylase non-covalently immobilized on loose IAM chromatographic support. The incubation mixture contained 1 ml of 0.1 m*M* 7-EtOC; 5 μl of 10 m*M* NADPH was added at 0, 20, 40, 60 and 80 min total incubation time. The experiment was performed on day 1, $n = 2$.

TABLE II

7-ETHOXYCOUMARIN O-DEETHYLASE ACTIVITY OF RAT LIVER MICROSOMES NON-COVALENTLY IMMOBILIZED ON IAM CHROMATOGRAPHIC COLUMN IN RELATION TO THE MOBILE PHASE FLOW THROUGH THE HPLC SYSTEM

The experiments were performed within 1 h.

Mobile phase flow (ml/min)	7-EtOC–microsomes contact time (min)	Productivity (nmol 7-OHC/mg microsomal protein)
0.50	2	0.073
0.20	5	0.347
0.10	10	0.397

TABLE III

TIME-YIELD PROFILE OF 7-HYDROXYCOUMARIN GENERATED FROM 7-ETHOXYCOUMARIN INJECTED INTO A HPLC CHROMATOGRAPHIC COLUMN WITH RAT LIVER MICROSOMES NON-COVALENTLY IMMOBILIZED ON IAM CHROMATOGRAPHIC SUPPORT

Elapsed time after immobilization (h)	Substrate and cofactor concentrations (nmol 7-OHC/mg microsomal protein)	
	0.1 m*M* 7-EtOC, 0.05 m*M* NADPH	1 m*M* 7-EtOC, 0.20 m*M* NADPH
1.51	0.059	
1.87	0.069	
2.22		0.384
2.65	0.073	
3.20		0.199
4.55	0.035	
4.87	0.033	
5.65	0.019	
5.97	0.024	
6.35	0.011	

450 tertiary conformation about the haeme catalytic centre appears to be pressure sensitive. This is most likely the result of shearing forces generated at the solid support–mobile phase interface and the best chromatographic results were obtained by maintaining HPLC back pressure below 800–1000 p.s.i. (1 p.s.i. = 6894.76 Pa).

The results of this study indicate that non-solubilized microsomes can be entrapped on an IAM chromatographic support with retention of their enzymatic activity. Further, they indicate that an HPLC system composed of a microsomal IMER can be developed for the direct study of metabolic processes. Additional work in the development of this system is underway in our laboratory.

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